THE NUCLEIC ACIDS

Volume III

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Edited by

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Preface

Since 1955, when the first two volumes of this treatise were published, knowledge relating to the chemistry and the biological importance of the nucleic acids has continued its vigorous growth; a progress which, however, has not been uniform in all directions. After much discussion and deliberation we came to the conclusion that, while an entirely new edition was not yet justified, a way could be found to prevent the premature obsolescence of a compendium of the kind offered by us. It was decided to provide what could be called a "diagonal supplement," i.e., a selection of chapters cutting across, as it were, the systematic arrangement observed in the preceding volumes. Care was taken to choose topics that had not received sufficient emphasis before or that had acquired particular importance since the publication of the earlier volumes. Moreover, a slight shift in perspectives was also made easier by the decision to limit contributions to the present volume to authors who had not collaborated in the preceding ones.

Of the twelve articles presented here, the first six, Chapters 29 to 34, deal with topics related to Volume I of the parent work, whereas Chapters 35 to 40 are thematically near the subject matter treated in Volume II. As regards the question of nomenclature and abbreviations, we are aware of the proposals which have been made by the Nomenclature Commission of the International Union of Pure and Applied Chemistry, Section of Biological Chemistry; but until these have been universally accepted we have felt it best to let each author decide and define the manner best suited to his purpose.

April, 1960

Erwin Chargaff
J. N. Davidson

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Page 145. Paragraph 1, line 4 should read: simultaneous.
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Page 197. Paragraph 2, line 2 should read: leads.
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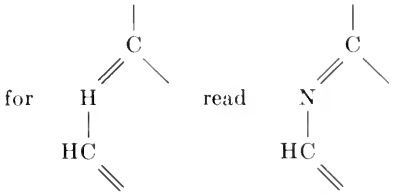

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Chapter 29

Deoxyribonucleic Acids as Macromolecules

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I. Introduction

One knows that it is possible through proper means to extract from the cell nucleus a substance called deoxyribonucleic acid (DNA) which plays a prominent role in the fundamental processes of life. The extraction is performed by means of physicochemical operations that can vary, according to the authors, either in the principles themselves (Hammarsten, Signer, and Schwander, Sevag, Butler, etc.), or in minor details (Vendrely, Pouyet).

The result is a dispersion of the substance in an aqueous solvent of a given pH at a given concentration in an electrolyte such as NaCl. The problem then is to describe the dispersed particles as individuals. We shall see that these particles can be considered macromolecules or sometimes as aggregates of macromolecules which can be separated into their constituents.

In the case of DNA—as it is in general—the complete knowledge of a given macromolecule implies two types of notions.

First, we need to know the chemical composition of the substance as well as the relative position of the atomic groups which it contains.

Second, we have to know the weight as well as the shape, configuration, and dimensions of the macromolecule. For that purpose we have to use

rather special means pertaining to the field of macromolecular physical chemistry. Combining the two types of data one can have in principle a complete and clear description of the macromolecule.

Of course, this distinction of two classes of parameters is somewhat schematic and, in practice, the cut will not be sharp between them. In general, the knowledge of data belonging to one group—the first one for instance—is quite necessary to establish the data of the second one.

The case of DNA will be a very clear example of this fact. According to the situation that we have described, we shall in this paper, admit that a certain number of facts concerning the composition and structure of the DNA macromolecules are well established and we shall devote all our attention to the second group of parameters, that is, weight, shape, and dimensions, that we shall call morphological parameters.

Now it is clear that in a solution as it has been prepared by the biochemist, we have no evidence that the macromolecules are all alike. Heterogeneity may exist for the two groups of parameters. Since we have chosen to treat the second only, we shall have to consider that the solution we have to deal with is in principle polydispersed relatively to the weight, shape, and dimensions of the particles—or macromolecules—that it contains.

We then have to consider not only one molecular weight, one shape, and one dimension, but we have to find out the respective laws of distribution of these three parameters for the assembly of the macromolecules contained in a given sample of DNA. This problem, which we have set forth in its more general terms, is one of the most difficult in macromolecular physical chemistry, and we shall see that we are still far from a complete and satisfactory answer.

II. A Brief Survey of the General Principles of the Determination of Weight, Shape, and Dimensions of Large Molecules in Solution

Before coming to the special case of DNA it may be useful to give, in a preliminary chapter, a short summary of the general methods which are used in the determination of the morphological parameters of large molecules in solution.[1] We shall as far as possible avoid any detail that does not apply here, and we shall keep as close as possible to the information strictly necessary for a through understanding of the results obtained in the case of DNA.

The methods of investigation can be usefully divided into two groups: the so called "hydrodynamical" methods and the "optical" methods.

[1] For more details see, for example, C. Sadron, *Prog. in Biophys.* **3,** 237 (1953).

1. Hydrodynamical Methods

a. Monodispersed Solutions

Let us first assume that we have to deal with a solution containing only one type of macromolecule. It is possible then by proper operations to obtain, among many other data, the following ones: the Brownian diffusion constants of the macromolecules (A for translation, B for rotation); their velocity v of sedimentation in an external field γ (produced, for instance, by ultracentrifugation); the intrinsic viscosity $[\eta]$ of the solution, which is the limiting value of $(\eta - \eta_0)/\eta_0 c$ (where η_0 = viscosity of the solvent, η = viscosity of the solution at the concentration c in grams per milliliter) when c decreases to zero.

These data are related to the macromolecular parameters in the following way:

1. The constants of diffusion obey the general laws

$$A = kT/f \tag{1}$$

$$B = kT/C \tag{2}$$

where k is the Boltzmann constant, T the absolute temperature of the solution, f and C the unitary coefficients of the viscous friction respectively exerted by the solvent on the particle when it moves with a constant velocity of translation or of rotation along a given axis.

The coefficients f and C do not depend on the molecular weight M of the particle, but they are obviously functions of its shape and dimensions.

2. When the permanent state is attained, the velocity of sedimentation in the field of inertia γ is given by the equation

$$v/\gamma = M(1 - V_{sp}\rho_0)/\mathfrak{N}f \tag{3}$$

where V_{sp} is the partial specific volume of the particle, ρ_0 the density of the solvent, and $\mathfrak{N}$ the Avogadro number. The sedimentation constant S is given by $S = v/\gamma$.

3. The relative increase of viscosity (or specific viscosity), $\eta_{sp} = (\eta - \eta_0)/\eta_0$, is proportional at very large dilutions to the number, $c\mathfrak{N}/M$ of particles per unit of volume. It can be shown that $\eta_{sp} = (c\mathfrak{N}/M) \cdot F$ or, according to the definitions of $[\eta]$,

$$[\eta] = \mathfrak{N}F/M \tag{4}$$

In these equations F is a factor which depends only on the shape and dimensions of the macromolecule.

Knowing the experimental values of A, B, S, and $[\eta]$, it is then possible to derive the morphological characters of the macromolecule.

For instance, one obtains directly from Eqs. (1) and (3) the relation

$$M = S\mathfrak{R}kT/A(1 - \rho_0 V_{sp}) \tag{5}$$

Then, having measured V_{sp} [generally by pycnometry, a method which is not above all criticism; see ref. (1)], we see that the value of M can be calculated from the experimental values of S and A.

The derivation of the shape and dimensions from the values of f, C, or F calculated from A, B, or $[\eta]$ is not straightforward. In fact, we have to find out from empiric experience or from theory, or from both, the relationship between f, C, or F and the dimensions of the particle, and that for every possible shape of the latter. This is conceivable only in the case of very simple geometric shapes, for which dimensions can be fixed with very few—one or two—linear parameters.

A method which is widely used is to assume that the particle is a compact ellipsoid of rotation of volume V and elongation p, moving in a continuous medium (the solvent). Explicit equations giving respectively f, C, and F as functions of V and p are then derived from the application of the general theory of the hydrodynamics of continuous fluids. Knowing the values of f, C, and F from experimental determination, it is possible to calculate V and p from these hydrodynamical equations.

It is evident that it remains to determine what could be the relationship between the shape and dimensions of a real particle and the "equivalent ellipsoid." Each case has to be specially discussed in the light of all possible data obtained from the consideration of the chemical structure or of the various physical properties of the given macromolecule.

b. Polydispersed Solutions

In this case, each special category of macromolecules contained in the mixture behaves as if it were alone in the solution (at least at infinite dilutions) and the experimental effect is the sum of the effects which would have been observed for each component.

1. The most general case is that of a mixture of molecules of which weight, shape, and dimensions are simultaneously different and with no correlation between them: for example, a mixture of ellipsoids of revolution of different V and p and of different densities (polydispersity of the third order).

2. Fortunately, experience shows that things are generally simpler. For instance, the particles in the mixture very often have the same density. It means in the case of the ellipsoids of rotation that only two of the three parameters V, p, and M can vary independently (polydispersity of the

second order). However, we cannot in that case make a classification of the molecules as a function of M only, since the value of p can change when V is given.

3. One may understand that even then it is extremely difficult to go through a complete analysis of the mixture and we shall only consider here the case of a polydispersity of the first order. That is to say that we assume that only one parameter is sufficient to characterize each macromolecule. This would happen, for instance, for ellipsoids of rotation of the same density and elongation. We can then classify the molecular species by the molecular weight M (or by V).

This polydispersity of the first order in molecular weight is of great interest, since it is realized in the very important class of linear macromolecules (though, very unfortunately, probably not in the case of DNA) and accordingly we shall very rapidly recall some principles.

The number of macromolecules per milliliter, the weights of which lie between M and $M + dM$, is

$$dn = f(M)dM \tag{6}$$

$f(M)$ is called the number distribution function of M. The concentration of these molecules is

$$dc = g(M)dM \tag{7}$$

$g(M)$ is the weight distribution function of M. Obviously

$$g(M) = Mf(M) \tag{8}$$

In the case of polydispersed solutions these distribution functions are new unknown quantities which are to be determined.

Quite generally if one parameter λ is sufficient to describe the macromolecule, the quantities A, B, S, and F are dependent on λ and we shall have to consider the distribution functions f or g in number or in weight corresponding to each.

1. It is generally possible to determine experimentally the function $g(S)$ by means of ultracentrifugation,[2] and with some difficulties the function $g(A)$ by means of the study of free Brownian diffusion.[3]

If the relationship between M and S (or A) is known, one or the other experiment gives directly $g(M)$. In this respect, an interesting case may arise. If one observes that the experimental curves $g(S)$ and $g(A)$ can be brought to coincide through a simple change in the scale of the abscissa S or A, it means that S and A for each molecule are uniformly proportional.

[2] J. W. Williams, K. E. van Holde, R. L. Baldwin, and H. Fujita, *Chem. Revs.* **58**, 716 (1958).

[3] M. Daune, Thesis, Strasbourg (1958).

Since, for each species we have

$$S = \frac{M(1 - V_{sp}\rho_0)}{\mathfrak{N}kT} A$$

it means that even if the particles have different shapes and dimensions, M is the same for all the molecules, and it is then possible to calculate its value.

2. When we measure some property of the solution in a permanent state (say its viscosity), we no longer obtain a distribution curve, but rather a mean value of the parameters. For instance, it will be easily seen that the specific viscosity of a solution containing a mixture of particles of known distribution function $g(M)$, and for which the morphological coefficient F [see Eq. (4)] is supposed to be a function of M only, is

$$\eta_{sp} = \int_0^\infty \frac{\mathfrak{N}F(M)}{M} g(M)\, dM$$

And we have

$$[\eta] = \int_0^\infty \frac{\mathfrak{N}F(M)}{M} g(M)\, dM \Big/ \int_0^\infty g(M)\, dM$$

Then $[\eta]$ is equal to the mean value of $\mathfrak{N}F(M)/M$.

Let us recall, on this occasion, that if λ is a parameter and $f(\lambda)$ or $g(\lambda)$ its distribution functions, it is possible to calculate the following different averages for λ:

$$\begin{aligned}
&\text{number average} \quad \lambda_n = \int_0^\infty \lambda f(\lambda)\, d\lambda \Big/ \int_0^\infty f(\lambda)\, d\lambda \\
&\text{weight average} \quad \lambda_w = \int_0^\infty \lambda^2 f(\lambda)\, d\lambda \Big/ \int_0^\infty \lambda f(\lambda)\, d\lambda \qquad (9) \\
&z \text{ average} \quad \lambda_z = \int_0^\infty \lambda^3 f(\lambda)\, d\lambda \Big/ \int_0^\infty \lambda^2 f(\lambda)\, d\lambda
\end{aligned}$$

If λ is a function of M, we have $g(\lambda) = Mf(\lambda)$ and we obtain, by replacing in the above equations $f(\lambda)$ by $g(\lambda)/M$, the expressions of the different averages of λ as functions of $g(\lambda)$.

2. Optical Methods[4]

a. Monodispersed Solutions

Let us consider a parallel beam of vertically polarized light passing through a solution of macromolecules whose index of refraction n is differ-

[4] K. A. Stacey, "Light Scattering in Physical Chemistry," Butterworths, London, 1956.

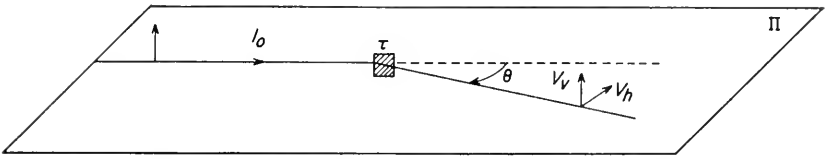


FIG. 1. Definition of the Θ angle in light-scattering experiments.

ent from the index n_0 of the solvent. We shall assume that there is no absorption of the incident light.

If V_v is the difference of the intensity of the light scattered—in direction Θ (Fig. 1)—by the same volume τ of the solution and of the pure solvent, one knows that V_v is given by the relationship:

$$V_v = \alpha \cdot c \cdot MP(\Theta) \tag{10}$$

where α is a factor which can be calculated from the conditions of the experiment. $P(\Theta)$ is a scalar function of Θ and of the dimensions and shape of the scattering particles. Its maximum is equal to unity when $\Theta = 0$; and its value decreases continuously when Θ increases.

Equation (10) is often written:

$$\frac{c}{K} = \frac{1}{MP(\Theta)} \tag{11}$$

where again K—a quantity proportional to V_v—can be measured by a proper device. In principle, Eq. (11) applies only when c = zero. It is an approximation of the more complete expression

$$\frac{c}{K} = \frac{1}{MP(\Theta)} + 2Bc \tag{12}$$

where B is the second virial coefficient of the solutions.

The experiment consists in measuring K/c for different values of Θ, each set of experiments being made for solutions of decreasing concentrations. Then, with proper extrapolations, one obtains K/c for all the Θ values at concentration, $c = 0$.

Plotting $(K/c)_{c=0}$ as a function of Θ, one obtains the curve $MP(\Theta)$, the intercept of which with the axis of ordinates $\Theta = 0$ is equal to M. At the same time the graph gives the function $P(\Theta)$.

The light-scattering technique is then of great interest, since it gives simultaneously the molecular weight of the particles, and a function $P(\Theta)$ which depends only on their shape and dimensions.

When—as is the case for DNA solutions—the whole of the scattered light is vertically polarized, it is possible to calculate the $P(\Theta)$ function for a particle of a given shape.

In fact, we consider a particle as formed by an assembly of punctual

oscillators. Each of these, when excited by the incident light, emits energy in all directions. If the dimensions of the particle are sufficiently small relatively to the wavelength λ of the incident light—say less than about $\lambda/20$—all the oscillators are practically in phase and all of them can be considered as a unit. In that case, the intensity is scattered according to Eq. (10) where $P(\Theta) = 1$ whatever the value of Θ may be. There is no dissymmetry in the distribution of the scattered intensity of light.

When the dimensions of the particle are larger than $\lambda/20$, a detectable difference of phase between the wavelets scattered by each oscillator occurs and interferences take place, hence, a decrease of the intensity of the energy scattered in each direction is taken care of by the function $P(\Theta)$, which can be calculated without any difficulty when the distribution of the oscillators in the space is known.

It is established in general that $P(\Theta)$ is a function of the product hL where L is a length characteristic of the dimensions of the particle, and where

$$h = \frac{4\pi}{\lambda} \sin \frac{\Theta}{2}$$

λ being the wavelength of the incident light in the solution; ($\lambda = \lambda'/n_0$ if λ' is the wavelength in vacuum).

Let us recall some expressions of $P(\Theta)$ for different types of particles.

1. Sphere of radius L

$$P(\Theta) = \left[\frac{3}{h^3L^3}(\sin hL - hL \cos hL)\right]^2 \tag{13}$$

2. Rigid rod of length $2L$

$$P(\Theta) = \frac{1}{hL} S_i(2hL) - \left(\frac{\sin hL}{hL}\right)^2 \tag{14}$$

where

$$S_i(2hL) = \int_0^{2hL} \frac{\sin x}{x}\, dx$$

3. Thin disc of diameter $2L$

$$P(\Theta) = \frac{2}{h^2L^2}\left[1 - \frac{1}{hL} J_1(2hL)\right] \tag{15}$$

where J_1 is the first order Bessel function.

4. Gaussian chain molecule. If we assume that the elementary oscillators succeed each other like the beads of a necklace, the distance between two successive oscillators being able to vary around its mean square value b^2 according to a Gaussian distribution, $P(\Theta)$ is given by

$$P(\Theta) = \frac{2}{(h^2L^2/6)^2}\left[e^{-h^2L^2/6} + \frac{h^2L^2}{6} - 1\right] \tag{16}$$

In this case

$$L^2 = Nb^2 \tag{17}$$

where N is the number of elementary oscillators, and L^2 the mean square value of the distance between the two ends of the chain.

5. Zigzag chain molecule. It is very interesting to examine the case of a thin chain molecule which can be figured as a succession of rods of length b and have all the possible relative orientations with a random distribution.

If each rod has a length which is well under $\lambda/20$ it behaves—as we have said—like a single oscillator and the $P(\Theta)$ function is then the same as for the necklace assembly of beads. But if the elementary length is larger than $\lambda/20$ one has to consider first the interferences produced by the oscillators pertaining to each single rod and then, for each value of Θ, to compose the intensities of the light scattered by all the rods.

In this case, the resulting $P(\Theta)$ function is the sum of two terms: one depending upon the $P_R(\Theta)$ function for a rod [Eq. (14)]; and the second taking into account the correlation of the rods in the whole chain.

Such cases have been recently treated by J. J. and J. Hermans.[5] However, here we shall give the results of the calculation in the form previously established by Benoit and Luzzati[6] who give the expression for $P(\Theta)$

$$P(\Theta) = \frac{1}{N} P_R(\Theta) + \left[\frac{1}{hb} S_i(hb)\right]^2 P_M(\Theta) \tag{18}$$

with

$$P_M(\Theta) = \frac{2}{N^2} \frac{N(1-a) - 1 + a^N}{(1-a)^2} \tag{19}$$

where

$$a = \frac{\sin hb}{hb}$$

These chains will be called zigzag chains. It is clear that they will behave according to Eq. (16) if b is smaller than $\lambda/20$.

In Fig. 2 is shown the shape of $P(\Theta)$ as a function of $\sin(\Theta/2)$ in some simple cases.

b. Limiting Expressions of $P(\Theta)$

(1) *Small Values of hL.* For very small values of hL the expression of $P(\Theta)$ can be replaced by its expansion in increasing powers of hL, which, limited to the first two terms, is:

[5] J. J. Hermans and J. Hermans, Jr., *J. Phys. Chem.* **62**, 1543 (1958).
[6] H. Benoit and V. Luzzati, unpublished (1959).

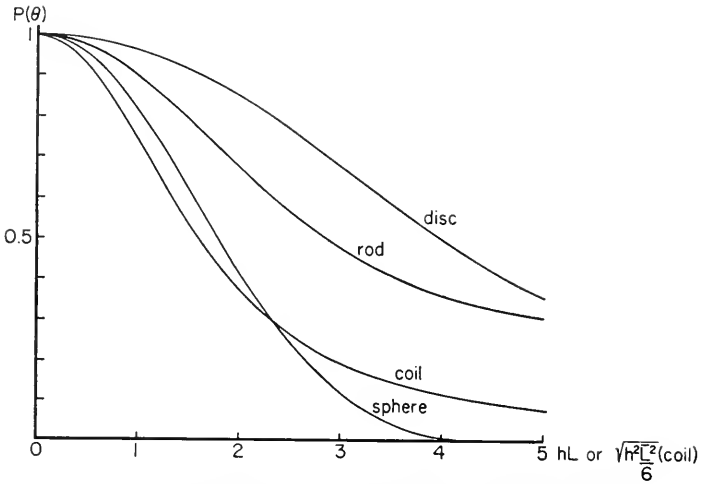


FIG. 2. $P(\Theta)$ functions for a few simple-shaped particles.

$$P(\Theta) = 1 - h^2 \frac{\bar{R}^2}{3} \tag{20}$$

where $\bar{R}^2$ is the mean square value of the radius of gyration of the particle.[6a]

It is very important to note that Eq. (20) is quite general and independent of the model chosen to give a representation of the particle.

As a conclusion, we see from Eq. (12) that

$$\left(\frac{c}{K}\right)_{\Theta=0} = \frac{1}{M} + 2Bc \tag{21}$$

and from Eqs. (12) and (20) that

$$\left(\frac{c}{K}\right)_{c=0} = \frac{1}{M}\left(1 + \frac{h^2\bar{R}^2}{3}\right) \tag{22}$$

With the use of two proper extrapolations giving $(c/K)_{c=0}$ it is possible to measure M (intercept of the curve with the ordinate) and $\bar{R}^2$ (initial slope of the curve) whatever the shape and dimensions of the particle.

Very often these two operations are made according to a process due to Zimm: the values of c/K are taken as ordinates and the values of $\sin^2 \Theta/2 + nc$ as abscissa where n is an arbitrary numeric factor whose magnitude is chosen according to the commodity of the graphic representation.

[6a] The radius of gyration of an assembly of N points A_i with the same weight m is $\bar{R}^2 = \Sigma_i \bar{r}_i^2/N$ where r_i is the distance of A_i to the center of masses of the assembly: for a sphere (radius L), $\bar{R}^2 = 6L^2/10$; for a rod (length $2L$), $\bar{R}^2 = L^2/3$; for a Gaussian chain, $\bar{R}^2 = L^2/6$; for a zigzag chain, $\bar{R}^2 = b^2/6\ (N - 1 + 1/2N)$.

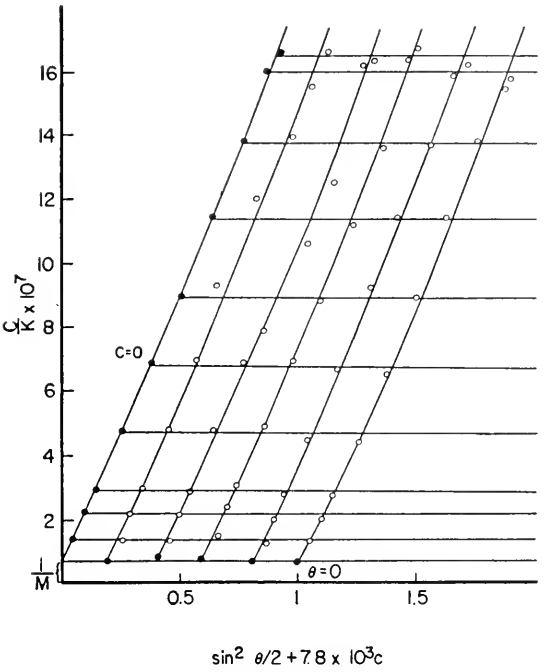


FIG. 3. An example of a diagram according to Zimm's representation.

Thus a diagram is obtained such as is given in Fig. 3.

The two extrapolated external curves *1* and *2* have the same intercept with the axis of ordinates which corresponds to the value of $1/M$, and initial slopes which respectively give the values of B and of $\bar{R}^2$.

(2) *Large Values of hL.* In this case which is realized for $\Theta \to \pi$ and $L/\lambda \to \infty$, $P(\Theta)$ or c/K can be replaced by its asymptotic expression.

One obtains in the different cases:

sphere
$$\frac{c}{K} \simeq \frac{2(hL)^4}{9M} \tag{23}$$

rod
$$\frac{c}{K} = \frac{2}{M\pi^2} + \frac{2hL}{\pi M} \tag{24}$$

disc
$$\frac{c}{K} \simeq h^2 \frac{1}{2} \frac{L^2}{M} \tag{25}$$

Gaussian chain
$$\frac{c}{K} = \frac{1}{2M} (1 + h^2\bar{R}^2) \tag{26}$$

zigzag chain (large values of b)

$$\frac{c}{K} = \frac{N}{\pi^2 M}\left[2 - \frac{\pi^2}{2} \cdot \frac{N-1}{N}\right] + \frac{h}{\pi}\frac{Nb}{M} \qquad (27)$$

where $Nb = L$

It follows from these equations that if the experimental values of c/K for very large values of hL tend to be proportional to sin $\Theta/2$, the particle is a rod or a zigzag chain with large elements, and if they are proportional to $\sin^2 \Theta/2$, the particle is a disc or a Gaussian chain or a zigzag chain with small elements. At the same time the value of the proportionality factor gives the weight per unit length for a rod or for a large zigzag or per unit surface for a disc.

However an important feature is to be noted. From Eq. (24) we see that for a rod the intercept of the linear asymptote with the ordinate is $2/(M\pi^2)$, a positive value. On the contrary, for a large zigzag chain the value of $N/(\pi^2 M)\{2 - (\pi^2/2)[(N-1)/N]\}$ is always negative for all values of N but unity.

Unfortunately, the observation of a negative ordinate is not unambiguously connected to the existence of a large zigzag chain. Benoit and Luzzati[6] have shown that the same effect can be produced by rod-shaped particles crossing each other, or with branching. For instance, the asymptote for a particle consisting of two long rods of length b crosssing each other in their midst with relative orientations distributed at random is given by

$$\left(\frac{c}{K}\right)_{b\to\infty} = \frac{1}{M}\left[\frac{hb}{\pi M} + \frac{4 - 2\pi^2}{\pi}\right]$$

It is then highly probable that the asymptote to the c/K function still has an expression such as

$$\frac{c}{K} = A + \frac{h}{\pi}\frac{Nb}{M}$$

where A has a negative value.

We can sum up this discussion by giving in Fig. 4 the three different elementary configurations leading to an asymptote with negative value of the ordinate.

Finally, let us remark that—since the smaller the λ, the larger the h—it will be of interest, in order to obtain the asymptotic limit of c/K, to use an incident beam of X-rays, for which the wavelength is about four thousand times less than for visible light.

Conversely, K will be very small, except for very small values of Θ, and the intensity of the scattered light will be measurable only at the vicinity of the direction of the incident beam. Hence, the name of "central diffusion" for this type of X-ray scattering.

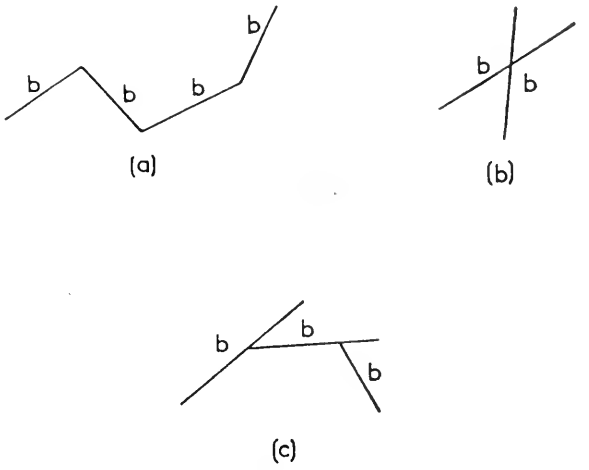


FIG. 4. Configurations giving a negative ordinate of the asymptote $(c/K)_{HL\to\infty}$ when $b > \lambda/20$.

As a conclusion, it is seen that the use of visible light, with λ as large as possible, is to be chosen when one wants to measure the values of M and $\bar{R}^2$, whereas central diffusion of X-rays is to be used for the determination of the shape and specific density of the particles.

c. Influence of Polydispersity

In this case the light-scattering method leads to mean values of the molecular weights or of the morphological parameters.

It can be easily shown that:

1. Whatever the heterogeneity of shapes of the molecules in the mixture, the intercept of the extrapolated curve $(K/c)_{c=0}$ with the axis of ordinates is equal to the molecular weight average

$$M_W = \int_0^\infty M^2 f(M)\, dM \Big/ \int_0^\infty M f(M)\, dM$$

The fact that this result is quite general, even if there is no correlation between the weight and the dimensions of the macromolecules, gives a great advantage to the use of light scattering.

2. The slope of the initial curve gives a mean value of $\bar{R}^2$.

If there is a biunivocal relationship between R and M this mean value is of the z type for all models of particles.

$$R_z^2 = \frac{1}{M_W} \int_0^\infty R_M^2 \cdot M \cdot f(M)\, dM \tag{28}$$

For instance, in the case of a mixture of Gaussian coils with different

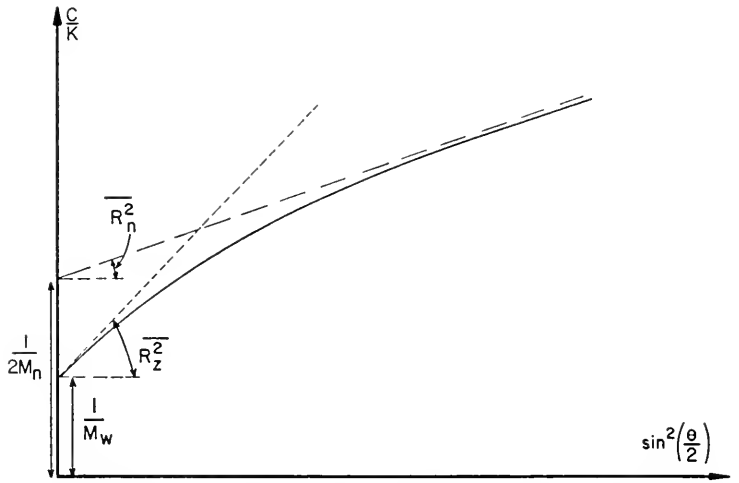


FIG. 5. c/K as a function of $\sin^2 \Theta/2$ in the case of a mixture of Gaussian coils.

values of N but the same value of b^2 (then N is proportional to M) the result of the calculation[7] is:

$$\left(\frac{c}{K}\right)_{\Theta=0} = \frac{1}{M_w}\left[1 + h^2 b^2 \frac{N_z}{18}\right] \tag{29}$$

where N_z is the z average of N.

3. It is evident that for a mixture of particles of uniform type, but of different dimensions, the asymptote of (c/K) for large values of hL will be of the same form as for a monodispersed solution of particles of the same type.

For instance, in the case of Gaussian coils,

$$\left(\frac{c}{K}\right)_{hL\to\infty} = \frac{1}{2M_n}\left[1 + h^2 b^2 \frac{N_n}{6}\right] \tag{30}$$

where N_n is the number average value of N.

From Eqs. (29) and (30) one may draw the graph in Fig. 5, which illustrates all the conclusions that one can obtain from the complete knowledge of the light-scattering curve. Other calculations of the same kind have been made for mixtures of rods or mixtures of discs.[8, 9] For instance, for a mixture of rods of different length L and the same specific weight M/L, one obtains

$$\left(\frac{c}{K}\right)_{hL\to\infty} = \frac{2}{\pi^2 M_n} + \frac{h}{\pi}\left(\frac{L}{M}\right) \tag{31}$$

[7] H. Benoit, *J. Polymer. Sci.* **11**, 507 (1953).
[8] A. Holtzer, *J. Polymer. Sci.* **17**, 432 (1955).
[9] O. Kratky and G. Porod, *J. Coll. Sci.* **4**, 35 (1949).

Then knowing the asymptote to the c/K curve, it is possible to calculate the values of M_n and of L/M.

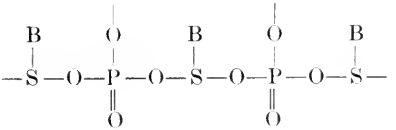
In the case of a large zigzag chain the slope of the asymptote is still $(h/\pi)(Nb/M)$ and it is evident that its intercept with the axis of ordinates is still negative.

If it has been possible for the same solution to measure M_n and M_w, respectively, from c/K at large and small values of Θ, then the comparison between M_n and M_w can give an estimation of the degree of polydispersity of the solution. If it is found that $M_w = M_n$, there is a great probability—though no absolute certitude—that all the particles are identical in weight, shape, and dimensions.

III. DNA Particles in Dilute Solutions

As we have already mentioned, the discussion and the interpretation of the data collected from optical or hydrodynamical investigations on macromolecular solutions are helped to a great extent by all kinds of information that one can obtain from other sources. As far as we are concerned, we shall largely use the simplest notions that chemical analysis as well as X-ray diffraction have made familiar.

It is well known that DNA macromolecules are formed by chains of the type



where S is the deoxyribose molecule and B one of the four bases adenine, guanine, cytosine, and thymine.

X-ray diffraction shows that fibers in the B form—that is to say prepared with the highest degree of moisture—contain bundles of long and thin particles, each of them being formed by the association of two simple chain molecules—or strands—each corresponding to the schematic formula that we have given above.

These two strands are stuck one along the other by means of H bonds between the opposite bases in such a way that there exist only two kinds of pairs: A-T or C-G. The two strands are wound around each other in the familiar shape of the Watson-Crick double helix which once more will be represented in Fig. 6.

The cylinder on which the two helices are wound has a diameter of 20 Å., the distance between the A-T and C-G couples is 3.4 Å. This means that the molar weight of the double helix is about 20,400 gm. for a length of 100 Å.

Owing to this structure, the DNA particle should be rather stiff. Though as everybody knows, one of the most urgent problems is to determine the

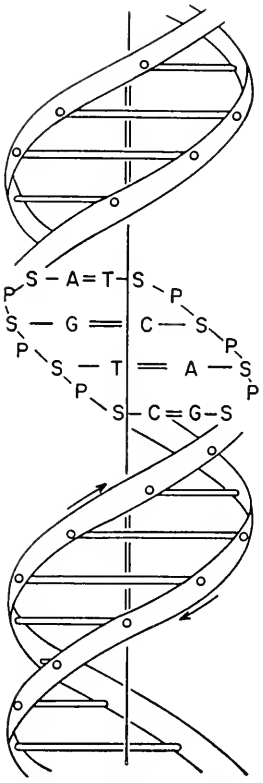


FIG. 6. DNA double helix.

law of sequence of the A-T and C-G groups along a particle of DNA of a given chemical composition, here we shall limit ourselves to the description of its morphological characters, such as weight and configurations.[10]

1. The Effect of Inter- and Intramolecular Interactions: Rigidity of DNA Particles

a. Intermolecular Interactions

First, let us recall that the general methods of analysis of the intrinsic characters of macromolecules in solution are based upon the assumption

[10] Reviews on the same subject have already been given by different authors, for instance,

(a) P. Doty, *Proc. 3rd Intern. Congr. Biochem., Brussels, 1955* p. 135 (1956).

(b) C. Sadron, *Proc. 3rd Intern. Congr. Biochem., Brussels, 1955* p. 125 (1956).

(c) C. Sadron and J. Pouyet, *Proc. 4th Intern. Congr. Biochem., Vienna 1958* p. 5 (1959).

Special mention is to be made of more extensive papers, such as,

(d) K. V. Shooter, *Progr. in Biophys.* **8,** 310 (1957).

(e) J. Hermans, Jr., Thesis, Leiden (1958).

that there are no interactions between them when moving in the surrounding medium; hence, the necessity of extrapolating the experimental data to zero concentration. This means that the actual concentration used in the experiments should be low enough to allow extrapolation with good precision.

In the case of DNA particles, the concentration of solutions must be very low, and this for two reasons:

1. The DNA filament being very long—as we shall see—we have to consider very high dilutions so that every given molecule should be far enough away from the other molecules and, in this way, out of the reach of hydrodynamical perturbation.

2. Aside from these hydrodynamical interactions, we have to consider the effect of electrostatic forces. All the Na phosphates in the molecule being ionized, each particle of DNA carries a great number of negative charges, and moves in an electrostatic field produced by all the neighboring molecules. Hence, when the solvent is pure water, we observe a very large effect of the DNA concentration on the measurements and it is necessary, if some precision in the extrapolation is required, to use such high dilutions that the technique becomes impracticable.

The situation is considerably improved if we introduce a strong electrolyte such as NaCl. In this case the electric charges carried by the DNA particles are practically saturated by the small ions, and the coulombian interactions disappear.

It is easy to illustrate this fact in considering the value of η_{sp}/c in DNA solutions. From Eq. (4) this quantity should be a constant when c changes. One can see in Fig. 7A[11] that this is not the case: η_{sp}/c increases as a function of c, but this effect becomes gradually smaller when the concentration of NaCl increases.

The same situation is met when we consider the sedimentation constant S, and it is established by experience that correct values of S can be obtained only if c is smaller than 1×10^{-4} gm. per milliliter. In fact such a dilution is an obstacle to the use of a refractive optical method (such as Philpot's) to detect the changes of concentration in the solution; and this makes necessary the use of more sensitive devices based, for instance, on the ultraviolet absorption of DNA in the 2.600 Å. band.[12]

b. Intramolecular Interactions: Rigidity of DNA

Since, in consequence, it is necessary to use salt-containing solutions of DNA, the question arises whether the intrinsic character of DNA particles and especially its configurations depend upon the presence of NaCl.

It is well known that in the case of a chain of polyelectrolytes the electro-

[11] J. Pouyet, results on CV 9, unpublished (1955).

[12] K. V. Shooter and J. A. V. Butler, *Trans. Faraday Soc.*, **52**, 734 (1956).

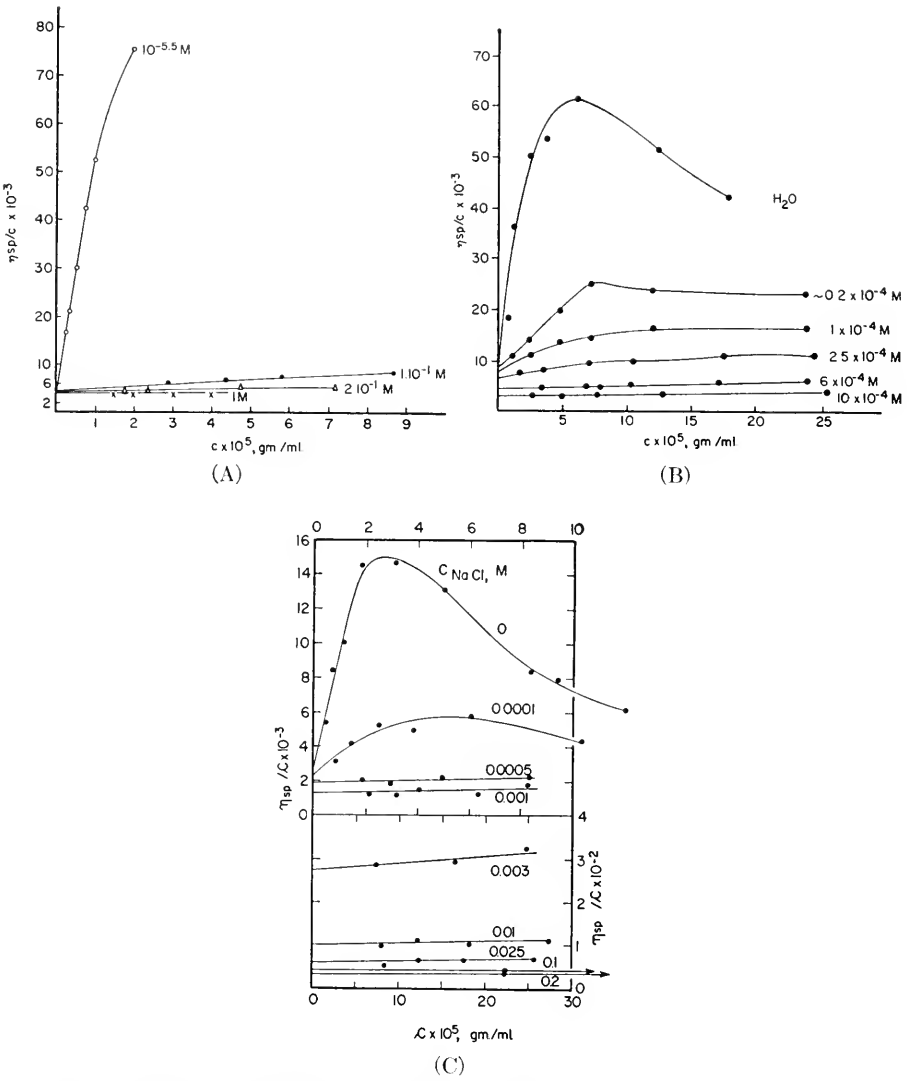


FIG. 7. Viscosimetric behavior of solutions of DNA (A) compared with polyvinylbutylpyridinium bromide solution (B) and RNA solutions (C); T = 25°C., G = 0.25 sec.$^{-1}$. [From U. Z. Littauer and H. Eisenberg, *Biochim. et Biophys. Acta* **32,** 320 (1959).]

static repulsions (or attractions) of the charges located on the different sites of the flexible molecular filament act against the thermal agitation of the elements of the chain and provoke an extension (or contraction) of the coiled configurations. Such parameters as A, $[\eta]$, S, which depend on the

shape of the molecule, will then be a function of the intensity of the electrostatic interaction; that is to say, of the concentration of the added electrolyte. This can be easily observed in Fig. 7B which relates to a solution of flexible chains of polyvinylbutylpyridinium bromide (PVBPB) in water or in salt solutions. Very important differences between the curves in Fig. 7A and 7B may be observed.[13, 14]

First, there is a peak for η_{sp}/c as a function of c in the case of PVBPB, which does not appear in DNA solutions.

Second—and this is even more important—the extrapolated values $[\eta]$ of η_{sp}/c are, in the first case, decreasing as the concentration of the salt increases while they remain constant in the second case. This means that the configuration of the DNA particles in solution does not change whether or not their different parts undergo mutual repulsions.

In conclusion, these particles have no internal mobility. They are stiff, or rigid, bodies. This is the first result that we obtain from the application of hydrodynamical methods.

Other phenomena observed on DNA solutions, such as stream double refraction[15] or structural viscosity,[16] which are connected to the orientation of the particles in solution confirm this statement.

However, in the case of stream double refraction, DNA seems to behave as an intermediary between a quite rigid particle, such as tobacco mosaic virus (TMV), and a quite flexible molecule, such as polystyrene (Fig. 8). This could be interpreted along the lines of Cerf's theory[17] as a slight flexibility of the molecule. But a careful examination of the ultraviolet absorption of DNA in glycerol solutions shows that, at higher concentrations of glycerol, there appears to be a slight denaturation of the molecule which could be responsible for the observed flexibility.

We must maintain the conclusion that DNA particles very probably possess rigid configurations.

Perhaps it is of interest to note here that RNA, in contrast to DNA, behaves as a quite flexible polyelectrolyte[18] as appears clearly from the comparison of Fig. 7A and 7C.

2. Light Scattering in DNA Solutions

The study of light scattering in DNA solutions has been widely developed in the last few years, and many of its conclusions have been discussed in numerous papers. Only recently, however, has the validity of the interpre-

[13] J. Pouyet, *Compt. rend. acad. sci.* **234**, 152 (1952).
[14] H. Eisenberg and J. Pouyet, *J. Polymer. Sci.* **13**, 85 (1954).
[15] H. Schwander and R. Cerf, *Helv. Chim. Acta* **32**, 2356 (1949).
[16] J. Pouyet, *J. chim. phys.* **48**, 161 (1951).
[17] R. Cerf, *Fortschr. Hochpolym. Forsch.* **1**, 382 (1959).
[18] U. Z. Littauer and H. Eisenberg, *Biochim. et Biophys. Acta* **32**, 320 (1959).

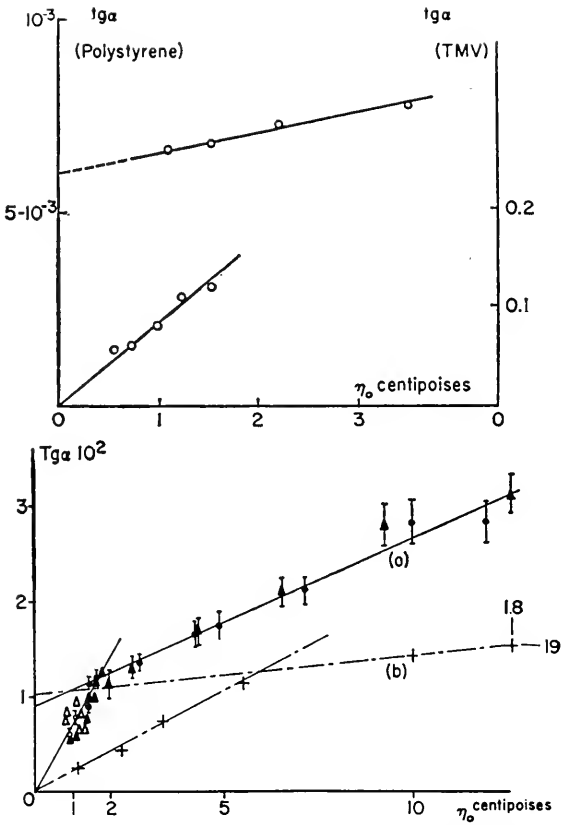


FIG. 8. Rigidity test by stream double refractions: tg α is the slope, at zero shear stress, of the isocline curve at different values of solvent viscosity η_0. *Above*: behavior of a (lower curve) rigid particle (TMV) and of a (upper curve) flexible coil (polystyrene in cyclohexane, chain molecule). *Below*: behavior of DNA particles (in water plus increasing percentage of glycerol). At low concentration of glycerol these particles behave as rigid bodies.

tations of the many, very confused data on the weight and shape of the dispersed DNA particles been subjected to clear criticism.[10c]

It seems that we are now in a much better situation for obtaining significant results, and it is beyond any doubt that in the near future we shall be able to benefit from data that will deserve careful consideration. At the present time, however, we are still constrained to adopt a rather cautious position. The main objections which have been put forward are related to two very different points.

The first is connected with the validity of the light-scattering measurements, and the second with the reproducibility of the DNA preparations.

Though the latter question seems logically to be the first to be answered,

it will be better to begin with the first; after all, the nonreproducibility of physical properties can be judged from a comparison of numerical data.

a. Light Scattering at Small Angles

We have seen [Eqs. (11) and (20)] that the values of M_w and $\bar{R}^2$ were, respectively, calculated from the intercept of the c/K function of Θ with the axis of ordinates, and from its initial slope. It is then necessary to obtain from the experimental determination a set of values of c/K which really can be extrapolated to $\Theta = 0$. This, as we shall see, requires discussion. In fact, in all experimental devices now in use, it is quite impossible to make practically any measurement under a certain limiting value Θ_l of Θ. In general, $\sin(\Theta_l/2)$ is of the order of $\frac{1}{4}$.

There exists, then, a practical limiting value h_l of h, and a minimum value h_lL of the parameter hL which, as we have seen, is the true variable in the c/K function. But the larger L is, the larger h_lL is; and it may happen that for very large particles this value is so large that we are far away from the region where it is possible to represent the experimental curve $P(\Theta)$—that is $P(hL)$—by its limited development as it has been given in Eq. (20). If this is so, it is to be feared that the extrapolation to $\Theta = 0$ of the experimental curve $P(\Theta)$ leads to erroneous results.

For instance, let us consider a rod-shaped particle. At the limit, when it is very long, K/c depends only on M/L (Eq. 31) and not on M (or on L). Then no extrapolation of the experimental curve, when it is practically identical to its asymptote, can be used for the determination of M (or of L).

Unfortunately, this situation seems—to a certain extent—to apply to the particles of DNA as illustrated in Fig. 9A, where we have represented as a function of $\sin\Theta/2$ (full points) the values of $(K/c)_{c=0}$ as they have been measured in a solution of sample CV71 in 1 molar NaCl.

It is obvious that the experimental points lie on the "tail" of the K/c curve and that no safe extrapolation is possible, as shown by continuous curves *1* to *5* which have been calculated from theoretical equations for rods of different radii of gyration (or lengths) (Fig. 9A).

However, in the representation of c/K as a function of $\sin^2(\Theta/2)$ according to Zimm's procedure, the extrapolation seems straightforward (Fig. 9B). Unfortunately, this is a mere appearance due to the use of $\sin^2(\Theta/2)$ and c/K as abscissas and ordinates. For large values of M—as in the case of the DNA particles—a slight deviation in the extrapolation produces a large error in the determination of $1/M$, and the use of the square of $\sin^2(\Theta/2)$ contracting the scale of the abscissa in the region of the small values of Θ, gives still a much greater uncertainty to the extrapolation.

It seems that we cannot avoid the conclusion that the values of M_w, which have been determined from light-scattering data, must be suspected

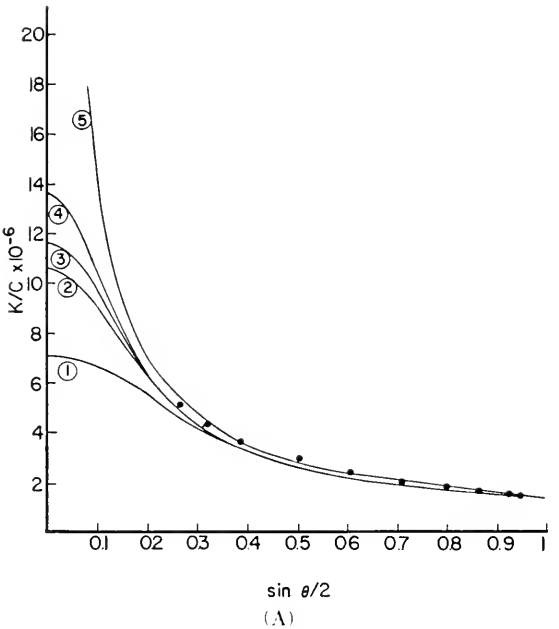


FIG. 9. A: K/c as a function of sin $\Theta/2$ (sample CV71 in 1 molar NaCl). The full line curves are calculated for rods of different radii of gyration; $\sqrt{\bar{R}^2}$ = 1500 Å. (1), 2200 Å. (2), 2400 Å. (3), 2800 Å. (4); and $L = \infty$ (5).

B: c/K values, for the same experiments, in Zimm's representation; $M_w = 7.1 \times 10^6$, $\sqrt{\bar{R}^2}$ = 1550 Å.

of being very uncertain. They are perhaps of the right order of magnitude, but it is difficult to evaluate the extent of the error.

As to the radius of gyration $\bar{R}^2$, since it is obtained from the evaluation of the initial slope of the extrapolated curve $(c/K)_{c=0}$ as a function of Θ, it is clear that the uncertainty is still much greater. We think that it is wise, in the present state of affairs, not to pay too much attention to the numerical values of $\bar{R}^2$ as they have been published so far.

It is beyond any doubt necessary to find new devices in which Θ_l will be much smaller than is now possible. However, one should keep in mind that—since large particles scatter the maximum intensity at $\Theta = 0$—there will still remain the experimental difficulty of getting rid of the error introduced by the eventual presence of dust in the solutions.

In Tables I and II there is listed a large collection of values of M_w as reported by several authors for samples of different origin and different types of preparation.

At first sight, the striking feature is the large spread of the experimental values of M_w, which range from one million to more than thirty million; a difference larger than the experimental error, even in its more pessimistic evaluation.

A more accurate examination reveals that the values are not scattered at random.[19] More than 40% of the numbers lie between 6×10^6 and 8×10^6 (Table I) and between 6×10^6 and 10×10^6 (Table II); more than 30% are between 10×10^6 and 16×10^6; very few values are found to be less than 6×10^6 or larger than 16×10^6. Even if, keeping in mind our suspicion as to the significance of the numerical values, we consider that M_w is nothing more than a parameter more or less specific of a DNA sample with no clear relation to the true mean molecular weight, the fact remains that these parameters roughly belong to two groups: the 6×10^6 to 8×10^6 group and the 12×10^6 to 16×10^6 group.

It is evident from Tables I and II that the origin of the samples cannot be responsible for this grouping: we find that in each group there are DNA samples coming from bacteria as well as from higher organisms. It is, therefore, necessary to question the procedure of extraction and ask whether, using the same material, a given technique gives reproducible results.

This is the reason why—before going on—we shall, in the following section, examine this very important question.

b. Conditions of Reproducibility of the Dispersion of DNA Particles in Solution

A great number of observations have been made in this field. Here we shall sum up the main ones.

[19] C. Sadron, J. Pouyet, and R. Vendrely, *Nature* **179**, 263 (1957).

TABLE I

MOLECULAR WEIGHTS (M_w) AND INTRINSIC VISCOSITIES ($[\eta]$) OF DNA FROM CALF THYMUS

Designation of sample	$M_w \times 10^{-6}$	$[\eta] \times 10^{-3}$	Methods of preparation	Authors
JJA	11.0	6.00	1 and 2	*a*
CV 91	7.9	2.98	1	*b*
RH	5.0	3.90	1	*c*
HB 1	7.0	4.49	4	*d*
AH 1a	18.0	3.60	4	*d*
AH 1b	36.0	3.17	4	*d*
B 1	6.3	3.42	4	*d*
DH 1	24.0	1.90	3	*c*
DH 2	8.0	—	3	*c*
DH 3	14.0	1.20	2	*c*
JC 1	11.6	2.70	4	*d*
JC 2	8.8	3.65	4	*d*
JCK 1	16.0	4.00	5	*d*
JCK 2	13.0	5.60	5	*d*
J 40	5.9	6.80	3	*d*
J 35	5.9	3.30	3	*e*
T 1	9.2	6.80	3	*e*
J 31	8.2	5.00	2	*e*
BB	11.5	2.30	3	*e*

Methods of Preparation

[1] R. Signer and H. Schwander, *Helv. Chim. Acta* **32**, 853 (1949).
[2] M. G. Sevag, D. B. Lackman, and J. Smolens, *J. Biol. Chem.* **124**, 425 (1938).
[3] A. M. Marko and J. A. V. Butler, *J. Biol. Chem.* **190**, 165 (1951). E. R. M. Kay, N. S. Simmons, and A. L. Dounce, *J. Am. Chem. Soc.* **74**, 1724 (1952).
[4] J. A. V. Butler, D. W. F. James, and B. E. Conway, *Trans. Faraday Soc.* **50**, 612 (1955).
[5] K. S. Kirby, *Biochem. J.* **66**, 495 (1957).

Authors

[a] J. Hermans and J. Pouyet, published in Thesis of J. Hermans, Jr., Leiden (1958).
[b] R. Vendrely, C. R. M., Strasbourg.
[c] J. Hermans, Laboratory of Physical Chemistry, University of Leiden.
[d] J. P. Coelingh, Laboratory of Biochemistry, University of Leiden.
[e] J. A. V. Butler, Chester Beatty Research Institute, London.

1. With a given material (calf thymus, hen erythrocytes, etc.) a given procedure of extraction gives a sample belonging to group I or to group II, without any apparent reason.[20]

2. At least with samples of group II, the values of M_w depend on the concentration of NaCl in the solution, and—if precipitation of DNA by ethanol has taken place—of the NaCl concentration of the initial solution. Curves I and II of Fig. 10 illustrate the results obtained by Pouyet on a sample

[20] J. Pouyet, unpublished (1957).

TABLE II

MOLECULAR WEIGHTS (M_w) AND INTRINSIC VISCOSITIES ($[\eta]$) OF DNA FROM DIFFERENT SOURCES

Designation of samples	$M_w \times 10^{-6}$	$[\eta] \times 10^{-3}$	Organ*	Ref.
B.G.	3.5	3.07	1	*a*
SX	3.8	—	1	*b*
CV 42	4.0	1.36	1	*c*
CV 49	4.0	4.30	1	*c*
Simmons	5.8	5.34	1	*d*
S VII	5.9	5.10	1	*d–f*
—	6.0	—	1	*e*
S V	6.0	1.50	1	*f*
I 69	6.0	1.50	1	*c*
CV 74a	6.0	3.40	2	*c*
CV 69	6.0	4.60	1	*c*
CV 71	6.0	4.80	1	*c*
S VIII	6.0	5.70	1	*c*
CV 74b	6.0	5.90	2	*c*
Varin	6.87	4.80	1	*g*
—	7.0	—	3	*h*
Varin	7.7	—	1	*d–f*
Doty-Rice	7.7	—	1	*d*
Brown	9.1	—	3	*c*
S XII	11.6	—	1	*b*
CV 51	11.6	4.00	4	*c*
CV 62	13.0	3.45	1	*c*
CV 64	13.0	5.90	1	*c*
I 62	14.0	2.60	1	*c*
CV 9	15.0	4.00	1	*c*
CV 78	15.0	3.40	4	*c*
CV 5	16.5	3.60	1	*c*
Brown	14.0 13.5	—	5	*e*

* Key: 1 = calf thymus, 2 = troutsperm, 3 = *E. coli*, 4 = chicken erythrocytes, 5 = avian tubercle bacilli

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[a] P. Doty and B. H. Bunce, *J. Am. Chem. Soc.* **74**, 5029 (1952).

[b] R. Bütler, Thesis, Berne (1953).

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[d] M. E. Reichman, S. A. Rice, C. Thomas, and P. Doty, *J. Am. Chem. Soc.*, **76**, 3047 (1954).

[e] G. L. Brown, M. B. Mewan, and M. J. Pratt, *Nature* **176**, 161 (1955).

[f] R. Signer and H. Schwander, *Helv. Chim. Acta* **32**, 853 (1949). R. Signer and H. Schwander, *Helv. Chim. Acta* **33**, 1521 (1950).

[g] M. E. Reichman, R. Varin, and P. Doty, *J. Am. Chem. Soc.* **74**, 3203 (1952).

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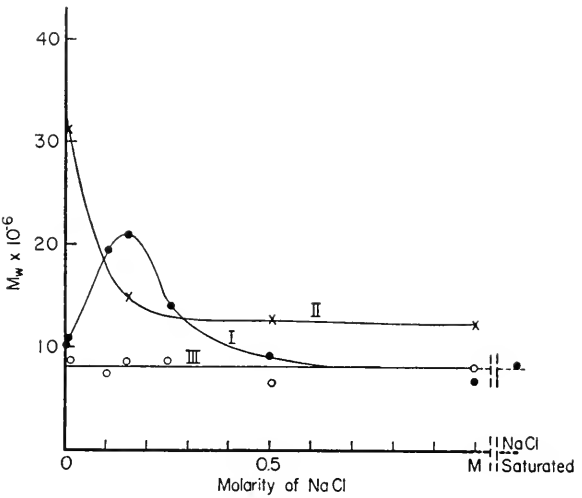

FIG. 10. Values of M_w from light scattering (AMJ_1, hen erythrocytes) as a function of the concentration of NaCl. Curves I and II are relative to solutions not treated with chymotrypsin; they correspond to different treatments (precipitation at different concentrations of NaCl) before being brought again into solution at the desired NaCl concentration. Curve III is relative to the sample treated by the enzyme: M_w is then independent of the concentration of NaCl.

of DNA (AMJ_1, hen erythrocytes) isolated from the nucleoprotein without precipitation, by use of a modified Sevag's method[20]. The result of the extraction is a solution of DNA in saturated NaCl. The value of M_w measured on this solution before any treatment, is found to be 14×10^6.

3. It is possible to shift the value of M_w from group II to group I by different means: (*a*) By moderate heating at 75°C. of a salt solution of DNA.[10e, 21] A rapid drop of M_w to one-half is observed and, afterward, the ordinary process of degradation begins to appear (Fig. 11).

(*b*) By the action of a proteolytic enzyme such as chymotrypsin, as was first shown by J. A. V. Butler and collaborators.[22]

Table III contains the values of M_w for different samples of DNA before and after treatment by chymotrypsin taken from the work of J. Hermans'[10e] and from our own experiments (samples AMJ_1 and MAV).

4. These experiments strongly suggest that particles in group II are aggregates of stable particles of group I held together by a polypeptide or protein residue.[22]

[21] A. M. Freund, J. Pouyet, and C. Sadron, *Compt. rend. acad. sci.* **246,** 1306 (1957).

[22] J. A. V. Butler, D. H. Philipps, and K. V. Shooter, *Arch. Biochem. Biophys.* **71,** 423 (1957).

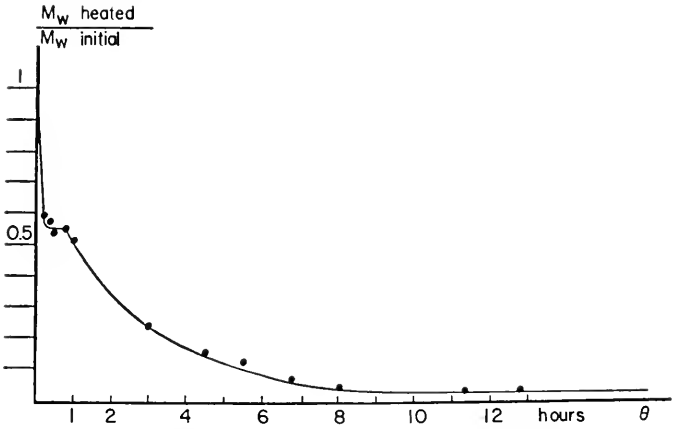

FIG. 11. Effect of heat on a solution of DNA not treated with chymotrypsin. M_w drops from 14×10^6 to 7×10^5 after 20 minutes of heating at 75°C. M_w remains constant ($[\eta]$ decreasing) for times of heating less than 1 hour. Afterward, the normal process of degradation occurs (sample CV51 in 1×10^{-2} molar NaCl).

TABLE III[a]

MOLECULAR WEIGHTS OF SOME DNA SAMPLES BEFORE AND AFTER TREATMENT WITH CHYMOTRYPSIN

Designation of samples	Original $M_w \times 10^{-6}$	Chymotrypsin-treated M_w
JJA	11.0	6.0
—	—	4.3
JC 1	11.6	6.5
JC 2	8.8	6.7
JCK 1	16	5.2
JCK 1	16	5.7
JCK 1	16	5.3
JCK 2	13	6.8
JCK 2	13	5.8
JCK 2	13	5.4
J 40	5.9	7.7
J 35	8.8	6.9
T 1	9.2	6.1
T 31	8.2	6.2
BB	11.5	2.9
AH 1a	18	14
AH 1b	36	20

[a] J. Hermans, Jr., Thesis, Leiden (1958); *Biochim. et Biophys. Acta* **32**, 304 (1959).

If, by chance, the measured value of M_w has a close connection to the actual mean molecular weight of the particles, the conclusion would be that the group II particles are dimers of particles of group I.[19]

5. At any rate, with particles treated with chymotrypsin, the complicated effects that we have mentioned in point 2, disappear completely. For instance, curve III in Fig. 10 shows that the value of M_w for sample AMJ_1 after treatment is quite independent of the concentration of NaCl and remains at 7×10^6, a value characteristic of group I.

For this reason, we shall, in the following, devote our principal attention to samples which were preliminarily treated with the enzyme.

c. Light Scattering at Large Angles

Since the values of h_1L seem to be so large that the admissibility of the extrapolation to zero values of Θ must be questioned, it is quite logical to investigate if—on the contrary—we can use the experimental data to ascertain the asymptotic values of c/K at the other end, that is when hL goes to infinity.

For this purpose we shall plot the values of c/K as a function of sin $\Theta/2$,

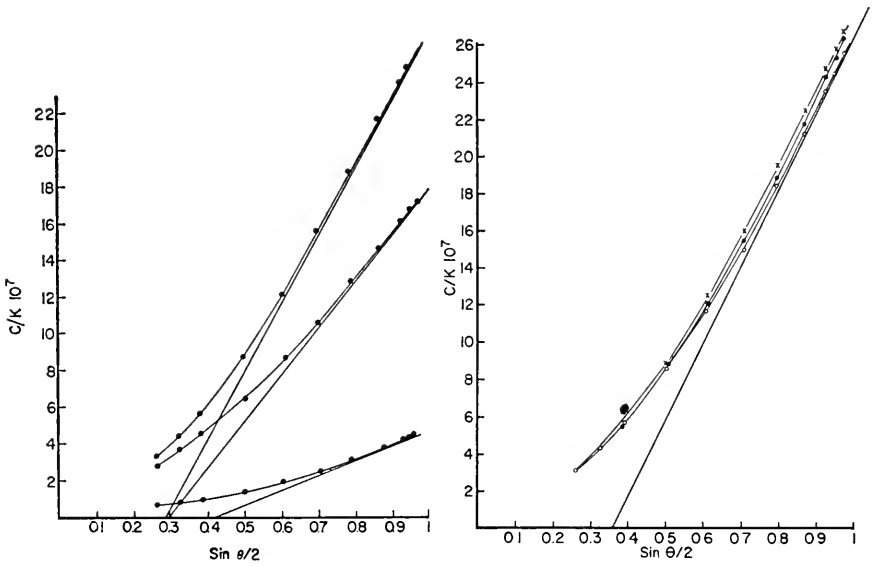

FIG. 12. A: c/K as a function of sin $\Theta/2$ for MAV (not treated). The three curves are respectively relative to solutions of 1, 0.5, and 0.1 molar NaCl (downward). B: c/K as a function of sin $\Theta/2$ for MAV (treated). The effect of the concentration of NaCl has practically disappeared; ● = saturated NaCl, × = 1 molar NaCl, ○ = 0.25 molar NaCl.

and we shall limit our discussion to the case of samples MAV and AMJ_1 which, as we have seen, had been prepared without any preliminary precipitation of the DNA.

1. As we have already mentioned, the c/K curves, for samples not treated with chymotrypsin, depend on the concentration of NaCl in the solution. This effect disappears almost completely after treatment with the enzyme (Fig. 12A and B).

2. Within the limits of the precision of the experiments, it seems that these curves admit a linear asymptote as it appears in Fig. 12A and B and more clearly in Fig. 13 relative to sample CV71.

3. Thus we may assume that the DNA particles behave like rods, or like large zigzag chains, and the slope of the asymptote gives the value of M/L.

For MAV and AMJ_1—without previous treatment with chymotrypsin—the values of M/L depend on the concentration of NaCl in the solution. Figure 14 shows that M/L has a maximum for a concentration of 0.15 molar NaCl just as has been observed for M_w. M/L is the same for the two samples at a concentration of 1 molar NaCl, and its value is 250 (mole per Å.). The effect of the salt concentration disappears if the sample have been treated by chymotrypsin and the constant value of M/L lies between 200 and 220. It is important to note that the M/L values are quite compatible, within the range of experimental errors, with the theoretical value

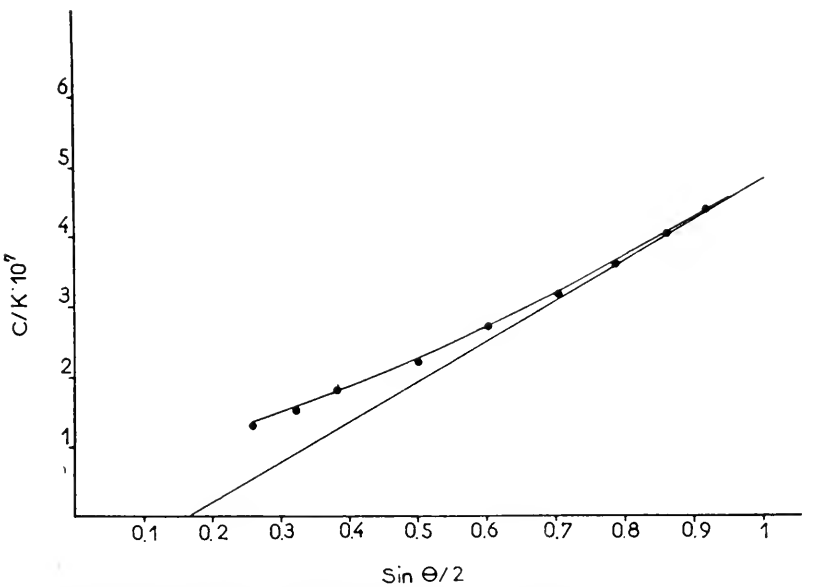

FIG. 13. Another example (sample CV71 in 1 molar NaCl) of linear variation of c/K as a function of sin $\Theta/2$ for large values of Θ.

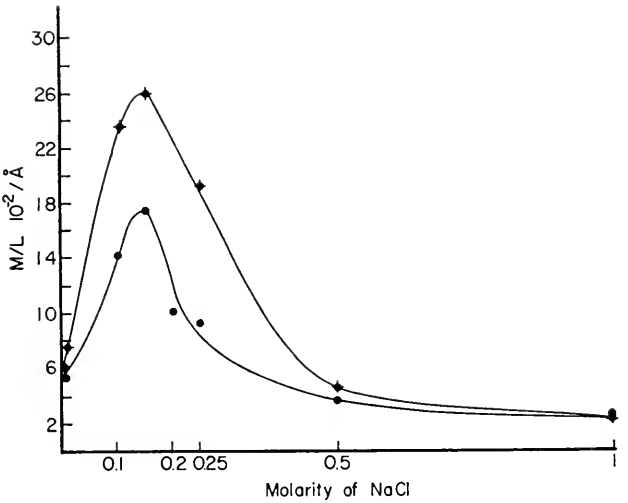

FIG. 14. The value of M/L depends on the concentrations of NaCl for samples not having been treated previously by chymotrypsin. M/L, however, is the same in 1 molar NaCl solutions and is practically equal to the theoretical value (200 per Å); ● = MAV and ⊛ = AMJ_1.

of 204 corresponding to the Watson-Crick model. However, in the case of CV71 (Fig. 13), the slope of the asymptote gives the much larger value $M/L = 1.400$ per Å.

4. It is evident from Figs. 12 and 13 that the ordinate of the intercept of the asymptote with the c/K axis is negative. Hence, the particle of DNA cannot be a single rod, an inference confirmed by the value of the intrinsic viscosity of the solution. On the contrary, its configuration can be the one discussed in Section II,2,*a*,1.

3. Hydrodynamical Methods

Intensive work has been done in the field of the application of hydrodynamical methods to DNA solutions. Till now, only the values of S and $[\eta]$ have been systematically used and discussed. No values of the Brownian diffusion constant A have yet been published owing to the difficulty of experiments at the large dilutions that are required for such extrapolation.

We have already given some of the conclusions that can be drawn from the consideration of $[\eta]$. We shall now briefly discuss the results of the sedimentation experiments and, afterward, give a brief survey of the conclusions that have been drawn from the comparison of S and $[\eta]$ values for different samples.

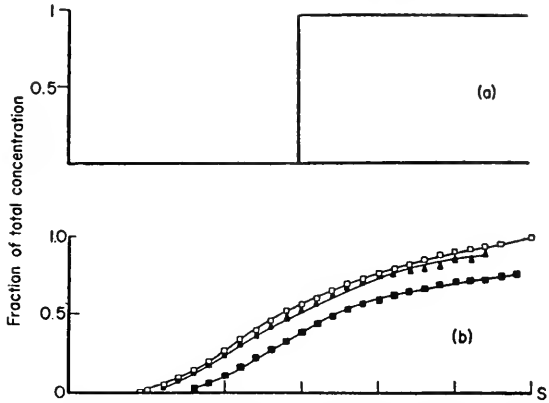

FIG. 15. (*a*) The $G(S)$ function for a monodispersed solution. (*b*) The experimental $G(S)$ function. [From J. A. V. Butler, D. J. R. Laurence, A. B. Robins, and K. V. Shooter, *Proc. Roy. Soc.* **A250**, 1 (1959).]

a. Sedimentation Constant: The Polydispersity in DNA Solutions

At such large dilutions, it is almost necessary to measure the distribution of the concentrations in the ultracentrifuge cell by using an ultraviolet absorption method. Consequently, one does not obtain directly the distribution function $g(S)$ of the sedimentation constant, but the integral function:

$$G(S) = \int_0^S g(S)\, dS$$

$G(S)$ is then the weight fraction of the particles with a sedimentation constant smaller than S. If S were the same for all the DNA particles, $G(S)$ would be represented by Fig. 15*a*. The result of the experiment (Fig. 15*b*) clearly shows that the S values cover a wide range beginning at 10 and up to more than 50 in Svedberg units.[22a] The DNA solutions then are clearly polydispersed.

If we refer to the definition of S (Section II,1,*a*) and if we assume that the partial specific volume V_{sp} is the same for all the particles, we have to conclude that the ratio M/f of the molecular weight to the friction coefficient differs from one particle to another. But we cannot say, from the single sedimentation determination, if the heterogeneity of M/f is only due to a heterogeneity of the molecular weight.

In order to answer this very important question it would be necessary either to measure the distribution of the Brownian diffusion constant A (see Section II,1,*b*)—but we have seen that no data are available as yet—or to determine, by a proper comparison with the viscosity and light-scattering

[22a] The Svedberg unit is equal to 10^{-13} c.g.s. units.

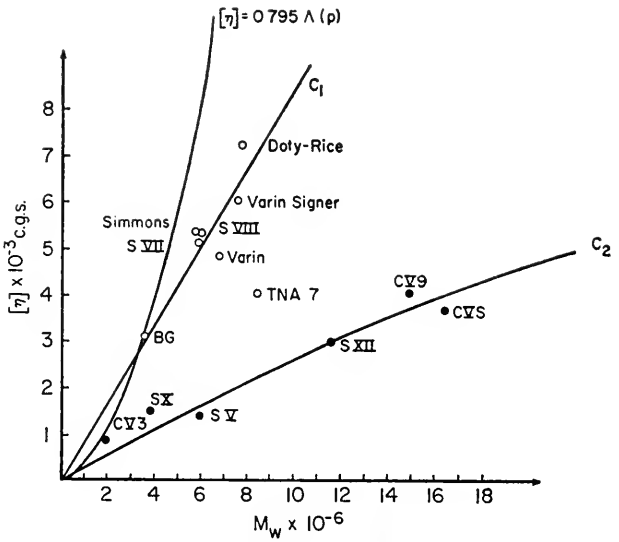

FIG. 16. There is no simple correlation between M_w and $[\eta]$; ○ = category C_1 and ● = category C_2.

values of M, if there is a univocal relationship between M and the morphological parameters on which S is dependent, that is to say between M and S. It is clear that if S is a function of M, the polydispersity of S is due to the polydispersity of M, and that the law of distribution $g(M)$ of M can be derived from the experimental law of distribution of S.

b. Comparison with Other Results

(1) *Light-Scattering and Viscosity.* If there exists such a relationship between M and the shape and dimensions of the particles, the measured values of $[\eta]$ for different samples should be a simple function of the measured weight.

Figure 16 shows that such a correlation apparently does not exist. The points giving $[\eta]$ as a function of M_w are dispersed in the part of space contained between the two curves C_1 and C_2. Such a dispersion appears still more accentuated from the results collected by J. Hermans.[10e] It is difficult to avoid the conclusion that particles of different shape and dimensions may correspond to a given value of M.

(2) *Sedimentation and Viscosity.* This conclusion should lead us to think that the use of hydrodynamical methods is, at the present moment not profitable and that we have to wait for further developments before being able to derive from it more precise conclusions. However, it is worthwhile to mention some interesting attempts made by different authors,[23] espe-

[23] P. Doty, *J. Cellular Comp. Physiol.* **49**, 27 (1957).

cially by Butler and his co-workers,[24] in order to obtain some significant results in the present situation.

The general idea is to get rid of the dispersion of the S values by considering only one value S_0, which corresponds to the maximum of the distribution curve $g(S)$. This S_0 value is the abscissa of the inflexion point of the $g(S)$ curve (cf. Fig. 15*b*). Each sample then is characterized by $[\eta]$ and S_0. It is assumed that all particles of the same molecular weight have the same $[\eta]$ and the same S_0 or, in other words, that there exist such definite function as $S_0(M)$ and $[\eta](M)$. These functions are supposed to be the same as those which have been observed to be valid in the general case of high polymer solutions, for instance Fox and Flory's equations which, by a proper combination[25] lead to the so-called Mandelkern equation

$$\frac{S_0[\eta]^{1/3}\mathfrak{N}\eta_0}{M^{2/3}(1 - V_{sp}\rho_0)} = \beta$$

where β is an empirical constant. In comparing the DNA particle with a high polymer coil, β is chosen to be equal to 2.6×10^6. Then all is ready for the calculation of M from S_0 and $[\eta]$.

Butler[24] has found that, for a series of five different preparations coming from calf thymus, the molecular weights thus calculated lay between 8.5×10^6 and 10.8×10^6. These values are of the same order of magnitude as those which have been found for M_w (group I). However it has to be emphasized that the author has measured the light-scattering molecular weights for the same samples and has found them to be between 3 and 4 million. He concludes that there is no agreement between M_w and $M_{\eta s}$ values. On the contrary, a good agreement of the same quantities is found for X-ray-degraded samples, for which the common value of M_w is somewhat under 4×10^6.

IV. Conclusions

The general conclusion to be drawn from the brief survey attempted here is that the situation still is rather confused. On the one hand, we have shown that light-scattering measurements, at least so far as the molecular weight of DNA particles is concerned, are suspect, and, on the other, that the use of hydrodynamical methods relies on assumptions (Section III,3,*b*) whose validity is very difficult to accept or, at any rate, to estimate.

Despite our necessarily critical attitude the following conclusions may, however, be proposed:

(*a*) The character of the particles in dilute solution depends in general

[24] J. A. V. Butler, D. J. R. Laurence, A. B. Robins, K. V. Shooter, *Proc. Roy. Soc.* **A250**, 1 (1959).

[25] L. Mandelkern, P. J. Flory, W. R. Krigbaum, and H. A. Scheraga, *J. Chem. Phys.* **20**, 1392 (1952).

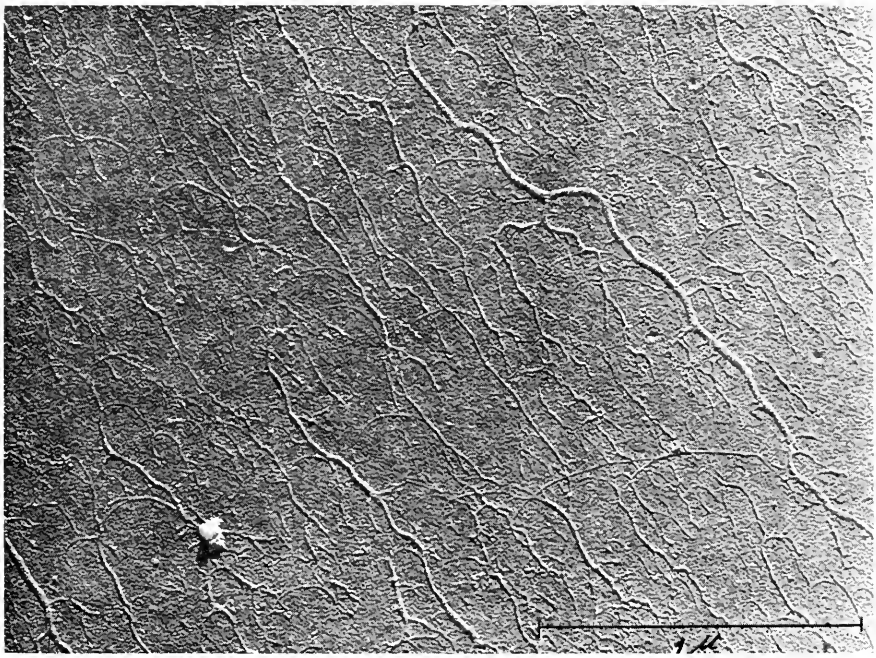

FIG. 17. DNA filament.

on the process of extraction of DNA from the living organisms, and the conditions that have to be realized in order to obtain reproducible results do not yet seem to be clearly established. However, a proteolytic enzyme treatment (chymotrypsin or even trypsin) of samples of different characters removes the discrepancies observed before the treatment. The assumption[12] that the enzyme breaks residual protein bridges between different particles seems to be in agreement with the observed facts. Nothing is known about the eventual existence of such residual bridges inside the particle.

(*b*) The particles in dilute solution are not identical. It is not yet clear if they have different molecular weights and, consequently, different shapes and dimensions, or if there is no correlation between their molecular weight and their shape. Particularly, the question is open whether, in a given sample, the particles possess a uniform molecular weight. Electron micrographs could, perhaps, give an answer. The examination of filament-shaped particles (Fig. 17) does not lead to precise conclusions.[26]

When, on the other hand, the particles occur in globular form (Fig. 18),[27]

[26] C. E. Hall, *J. Biophys. Biochem. Cytol.* **2**, 625 (1955).

[27] R. Vendrely, C. Vendrely, and C. Sadron, *Exptl. Cell Research* **15**, 222 (1958).

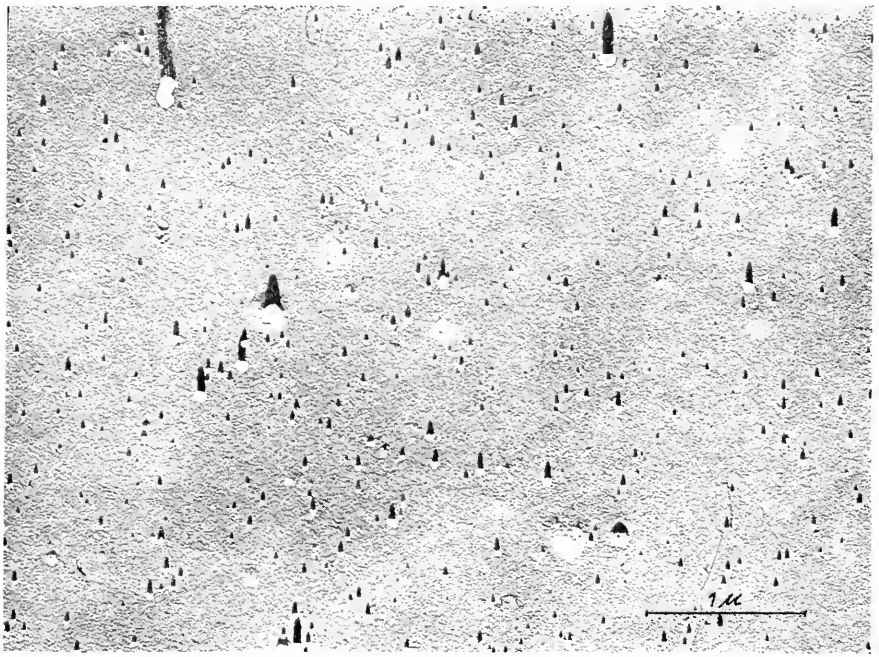

FIG. 18. DNA, globular particles.

it seems that heterogeneity can be observed.[28] It is, however, not impossible that degradation had taken place during the preparation of DNA in globular form.

(*c*) The molecular wheight of DNA particles is very large, certainly of several million. Light-scattering measurements of the mean molecular weight M_w of these particles are open to much criticism on technical grounds and we do not know to what extent the values found for M_w are erroneous. These M_w values can be divided into two main groups. In the first, M_w lies between 6 and 8 million, in the second it ranges from 12 to 16 million. Treatment with chymotrypsin does not change the value of M_w in the first group, but it reduces the M_w values of the second group to those of the first.

The use of hydrodynamical methods can also be criticized, because it relies on assumptions whose adequacy seems difficult to appreciate. The molecular weight obtained by these methods is of the same order as M_w, though somewhat higher.

(*d*) The particles of DNA in dilute solution have a linear configuration and the value found for the weight per unit length is quite compatible within

[28] C. Sadron, *Onzieme Conseil de Chimie Solvay, Brussels, 1959.*

the limits of experimental error, with the value corresponding to the Watson-Crick double helix (2.10^4 per 100 Å.). This linear configuration is not a single straight line (or rod), nor a continuous and flexible coil such as, for instance, in the case of the single-stranded particles of RNA. It could be a large zigzag chain, composed of elements of a length larger than λ 20, that is to say larger than about 300 Å. The molecular weight of each element should then exceed 60,000. If the molecular weight of the total molecule is about 6 to 8 million, this means that each zigzag is formed by less than a hundred rod elements. According to viscosity and stream double refraction this zigzag chain should be practically rigid. Perhaps a rotation of small amplitude—due to thermal agitation—could occur at the bending points.

Finally, there could be branching or, less probably, loose bundle of elementary rods. All these possible configurations have been enumerated in Section II and presented schematically in Fig. 4. In the present state of affairs, neither optical nor hydrodynamical methods permit us to choose between the different figures. If we want to do this, we must rely on other types of considerations. For instance, we may make the following speculations.

It is evident that, if the DNA particles have the double helix structure, and if their molecular weight is of several million, their total extended length should be of several thousand Å., that is to say of the order of half a micron. Such a long filament has to be closely packed in the nucleoprotein particles in the chromosomes. This means that the double helix line has to be pleated abruptly—for instance like an accordion—enclosing the protein

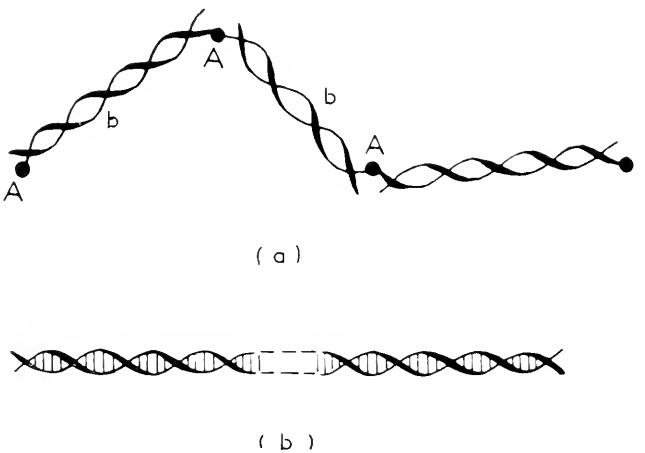

FIG. 19. (*a*) Weak points due to an interruption of one of the streams. (*b*) Weak points due to a local untwisting of the double helix (Ambrose). The proportions have not been respected (the length of each double-helix segment is larger than 300 Å.).

molecules. If one postulates that there is neither branching (Fig. 4*b*) nor formation of bundles (Fig. 4*c*), a zigzag line (Fig. 4*a*) makes it very reasonable to assume that at the bending points, A, there is a kind of dislocation or heterogeneity of the double helix permitting an abrupt bending. It is then necessary to imagine that at such points the local structure is different. It is appealing to assume that at these points the chain is particularly easily broken by physical or chemical agents. In this case, the effect of ultrasonic or X-ray irradiation as observed, respectively, by Doty[29] and Butler,[24] would be to cut the zigzag into rod-shaped AA segments (those segments between two successive points, A) of a length, as we have seen, larger than 300 Å. (molecular weight 60,000).

It could be suggested that these weak points, where the chain is able to bend, correspond to segments of a single strand. Such a structure has already been proposed for other reasons by Dekker and Schachman[30] but with a number of weak points per molecule which is too large to be compatible with the necessary minimum length (300 Å.) of each double-helix segment. Another model, with ladder-shaped zones has been suggested by Ambrose (Fig. 19*b*).

However, it has to be kept in mind that such considerations are strictly speculative, and that so far no direct experiment allows us to say that they are more than assumptions which may be useful in stimulating new investigations.

(*e*) It is evident that owing to the many uncertainties in our conclusions there is no possibility of knowing with precision whether or not the characters of DNA particles depend on the organs or the animal species from which they have been extracted. At present, no significant differences can be seen between different species such as bacteria, fishes, birds, or mammalians. If this point were proved, this could perhaps lead to some interesting conclusions.[19] For this reason, and for many others, it may be believed that, despite the meager results obtained till now regarding the determination of the morphological characters of DNA particles in solution, it is worthwhile not to stop efforts which probably will, in the near future, give valuable results.

ACKNOWLEDGMENT

I want to thank J. Pouyet for having kindly helped me in the redaction of this paper.

[29] P. Doty, B. B. McGill, and S. A. Rice, *Proc. Natl. Acad. Sci. U. S.* **44**, 432 (1958).
[30] C. A. Dekker and H. K. Schachman, *Proc. Natl. Acad. Sci.* **40**, 894 (1954).

CHAPTER 30

Photochemistry of Nucleic Acids and Their Constituents

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I. Introduction

The action of electromagnetic and particle radiations on living cells and their constituent components has developed into an important branch of research in almost every field of biology. With the increasing use of isotopes as scientific tools, and the rapid application of nuclear energy for medical, technical, and industrial purposes, it is not surprising that additional emphasis is being laid on the effects of ionizing radiations. This, however, in no way detracts from the importance of the ultraviolet and visible regions of the spectrum in radiobiology since the process of energy absorption is specific and related to the molecular structure of the substances under investigation, while a good deal more is known about the physics and physical chemistry of the absorption process and the dissipation of the absorbed energy. It is, in fact, becoming common practice to conduct parallel studies on the same systems with ultraviolet and ionizing radiations.

The photochemistry of nucleic acids is of special interest because of their important biological functions; their very high extinction in the ultraviolet region of the spectrum which is most efficient in the production of mutagenic, lethal, and other biological effects; the accumulated evidence from action spectra that they are the immediate receptors of radiation in many photobiological processes; and the recent demonstration, both by physicochemical and biological techniques, that the modifications induced in oligo- and polynucleotides by ultraviolet light are at least partially reversible.

It should be emphasized that, as in the case of ionizing radiations, the photochemistry of nucleic acids is of interest not only from a biological, but also from a physicochemical point of view. A good deal of work has therefore been done in which the primary aim has been to achieve appreciable degradation, consequently involving the use of doses considerably in excess of those required to provoke biological effects. This fact must constantly be borne in mind in any attempted extrapolation of *in vitro* findings to the cellular level.

II. Principles of Photochemistry

1. General

In photochemical reactions we are interested in elucidating the nature of the chemical changes in a system resulting from its exposure to light. Although the obvious fact that absorption of energy must take place for a reaction to occur was formulated many years ago by Grotthus and is known as the first law of photochemistry, its significance has not always been appreciated, e.g., in relation to carbohydrates (Section V, 1) as well as in connection with attempts to photoreactivate simple nucleic acid (NA) derivatives (Section X).

Energy is absorbed by molecules in discrete units or quanta and, according to the Einstein law of photochemical equivalence, each molecule which reacts as a result of exposure to light absorbs one quantum of the radiation provoking the reaction. This does not mean, however, that every quantum absorbed results in a reaction.

If the frequency of the absorbed radiation is $\nu = c/\lambda$ where ν is in vibrations per second, λ is the wavelength in centimeters, and c is the velocity of light, 2.9977×10^{10} cm. per second then the energy of a quantum is $E = h\nu$ where h is Planck's constant, 6.62×10^{-27} erg seconds. The energy per mole of quanta, defined as an einstein, is therefore

$$1 \text{ einstein} = NE = Nh\nu = Nh\frac{c}{\lambda}$$

where N is Avogadro's number, 6.023×10^{23}. Substituting, and expressing λ either in Å. (10^{-8} cm.) or mμ (10^{-7} cm.), we have

$$1 \text{ einstein} = \frac{2.859 \times 10^5}{\lambda(\text{in Å.})} \text{ kcal./mole} = \frac{2.859 \times 10^4}{\lambda(\text{in m}\mu)} \text{ kcal./mole}$$

An einstein of green light, such as the 546.1 mμ mercury arc line, therefore corresponds to an energy of 52 kcal./mole. At 253.7 mμ, the wavelength most commonly used in photobiological work, the corresponding energy is 113 kcal., sufficient to break most chemical bonds. It does not necessarily follow that bond rupture will result from its absorption since the energy may not be localized at the required bond, but distributed over several modes of vibration.

The law of photochemical equivalence is usually expressed quantitatively in terms of the quantum efficiency or quantum yield

$$\phi = \frac{\text{no. of molecules reacting}}{\text{no. of quanta absorbed}} = \frac{\text{no. of moles reacting}}{\text{no. of einsteins absorbed}}$$

which theoretically should equal unity. With a few exceptions[1] this is rarely so, owing to the fact that the photochemical equivalence law applies only to the primary photochemical reaction, that in which the radiation is absorbed. Deviations from quantum yields of unity are due to secondary reactions and it is the products of these secondary "dark" reactions which are normally followed experimentally.

A good illustration of a photochemical reaction for which the quantum yield is practically unity is the dissociation of the carbon monoxymyoglobin complex by either visible or ultraviolet light.[2, 3]

[1] S. Glasstone, "Textbook of Physical Chemistry." Macmillan, London, 1948.

[2] T. Bücher and J. Kaspers, *Biochim. et Biophys. Acta* **1**, 21 (1947).

[3] O. Warburg, "Heavy Metal Prosthetic Groups and Enzyme Action." Oxford Univ. Press, London and New York, 1949.

2. The Primary Photochemical Process

The absorption of a light quantum by a molecule leads to its being raised from its normal, or ground, state to one of a possible number of discrete higher energy levels, or excited states. For absorption in the visible and ultraviolet regions of the spectrum, the excitation energy is mainly electronic. The resulting excited molecule is no longer in thermal equilibrium with its surroundings and must therefore give up its excitation energy in one of several ways.

(*a*) By the reemission of radiation at wavelengths to the red of that absorbed (i.e., of lower energy) in which case we are dealing with fluorescence (if the lifetime of the emission is less than 10^{-4} seconds) or phosphorescence (if the lifetime of emission is greater than 10^{-4} seconds and may even last as long as several seconds).[4]

(*b*) By a process called internal conversion, resulting from the fact that each energy level of a molecule is the resultant of its electronic, vibrational, and rotational energies. It is therefore possible for a transition to occur from a low vibrational level of a high electronic state to a high vibrational level of a lower electronic state, the total energy being the same in both states, so that the molecule does not reemit energy in the form of radiation. Consequently a portion of the electronic excitation energy is "converted" to vibrational energy and it becomes a "hot" molecule. It may then lose this vibrational energy by successive collisions with neighboring unexcited molecules, the excitation energy being thus dissipated as heat.

(*c*) Or it may rupture into two stable molecules or into free radicals, or undergo a rearrangement. If this happens very rapidly the process is referred to as optical dissociation or predissociation.

(*d*) The absorbing molecule may also initiate a chemical reaction in another molecule by loss of its energy to the latter. Reactions of this type are known as sensitized reactions.

3. Excited States of Molecules

At least in the case of phosphorescence, and what is sometimes referred to as long-lived fluorescence, we are dealing with molecules which remain in an excited state for an appreciable length of time by comparison with that required for many chemical reactions. In solution at room temperature, of course, deactivation by collisions competes with, and frequently completely eliminates, phosphorescence. But, by dissolving the substance in a solid medium (such as fused boric acid which sets to a rigid glass, or in a fluid medium which freezes at a low temperature), so that the excited molecules are "trapped" and deactivation by collisions is reduced considerably, both the fluorescence and phosphorescence phenomena may be readily investigated.[4-7]

As a result of such studies, it has been shown that phosphorescence is due to the transition of an excited molecule to a metastable triplet energy level from which it may return to the ground state by the emission of light (phosphorescence). The triplet state is one in which the molecule possesses two electrons with unpaired spins and hence is a diradical which is quite reactive chemically. When to this is added the fact that it is relatively long-lived, the importance of the triplet state in photochemical reactions may be readily understood. Reid[6] objects to the formulation of the triplet state as a diradical and points out that a diradical is not a triplet, while one may also visualize triplet states which are not diradicals.

Considerable impetus has been given to the study of the optically excited states of molecules by the development by Porter[8] and others[9-11] of the flash-photolysis, flash-spectroscopy technique, applicable to molecules in solution at room temperature under conditions identical to those normally used in photochemical studies. The basic principle involved is the very rapid formation of excited molecules followed by the recording of the absorption spectra of these excited molecules. The substance under investigation is submitted to the "flash" from a high-intensity source,[12] lasting several microseconds and intense enough to excite an appreciable portion of the solute molecules. Following a predetermined delay a second short flash is passed through the sample into a spectrograph which records the absorption spectrum of the excited state produced by the first flash. Once the characteristic maxima and extinction coefficients of the excited state have been obtained in this way, the photographic plate of the spectrograph may be replaced by a photoelectric cell by means of which the lifetime and decay kinetics of the excited state may be examined.

An excellent illustration of this technique is its application to the study of the various excited species produced in quinones,[13] which include the triplet state as well as the semiquinone radical and radical ion (Fig. 1).

Figure 2 shows the absorption spectrum of the excited, probably triplet, state of chlorophyll a.[14] While the method has already been applied to studies on the metastable states of proteins and aromatic amino acids,[15] it has not as yet been used on nucleic acids or their derivatives notwithstanding

[4] P. Pringsheim, "Fluorescence and Phosphorescence." Interscience, New York, 1949.

[5] M. Kasha, *Chem. Revs.* **41,** 401 (1947).

[6] C. Reid, "Excited States in Chemistry and Biology." Butterworths, London, 1957.

[7] M. C. R. Symonds and M. G. Townsend, *J. Chem. Soc.* p. 263 (1959).

[8] G. Porter, *Proc. Roy. Soc.* **A200,** 284 (1950); *Radiation Research, Suppl.* **1,** 479 (1959); *Spectrochim. Acta* **14,** 261 (1959).

[9] Q. H. Gibson, *J. Physiol.* **134,** 112 (1956).

[10] S. Claesson and L. Lindqvist, *Arkiv kemi* **11,** 535 (1957).

[11] R. Livingston and E. Fujimori, *J. Am. Chem. Soc.* **80,** 5610 (1958).

[12] R. G. W. Norrish and G. Porter, *Nature* **164,** 658 (1949).

[13] N. K. Bridge and G. Porter, *Proc. Roy. Soc.* **A244,** 276 (1958).

[14] R. Livingston, *J. Am. Chem. Soc.* **77,** 2179 (1955).

[15] L. I. Grossweiner, *J. Chem. Phys.* **24,** 1255 (1956); *Radiation Research* **9,** 124 (1958).

[15a] L. I. Grossweiner and W. A. Mulac, *Radiation Research* **10,** 515 (1959).

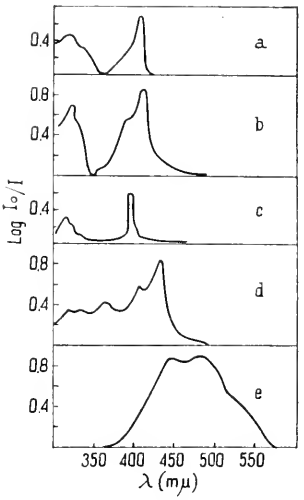

FIG. 1.

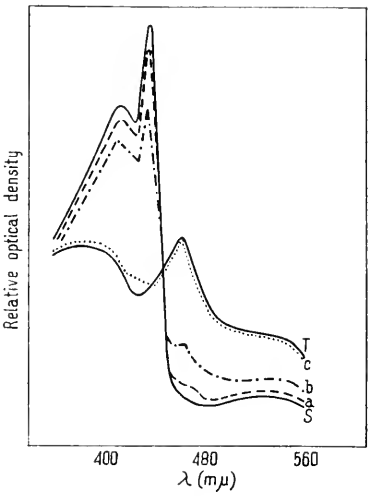

FIG. 2.

FIG. 1. Duroquinone radical and triplet spectra: (*a*), $QH^{\cdot}$ in liquid paraffin; (*b*), $QH^{\cdot}$ in 50% ethanol; (*c*), $QH^{\cdot}$ in hexane; (*d*), $Q^{\cdot-}$ in 50% ethanol; (*e*), triplet state in liquid paraffin [from N. K. Bridge and G. Porter, *Proc. Roy. Soc.*, **A244,** 276 (1958), which should be consulted for methods of identification of various excited states].

FIG. 2. Absorption spectra of 10^{-6} *M* chlorophyll a in cyclohexanol: *S*, ground state; (*c*) as soon as possible after flash excitation; (*b*) 900 μsec. after flash; (*a*) 2600 μsec. after flash; *T*, triplet state, obtained by extrapolation to zero time after flash [R. Livingston, *J. Am. Chem. Soc.* **77,** 2179 (1955)].

its potential importance in examining the excited states of pyrimidine derivatives in connection with the phenomenon of reversible photolysis (Section VII, 1) and the relationship of this latter to photoreactivation (Section X).

4. Free Radicals in Biological Systems

Reid[6] suggests that it would be surprising if triplet states were not found to be intermediates in many biological reactions. Actually a number of observers, e.g., Michaelis,[16] have postulated that electron transport in biological oxidations occurs singly rather than in pairs and consequently proceeds via intermediates containing an unpaired electron, i.e., free radicals. If this were the case, one would expect to find a stationary concentration of such free radical intermediates in actively metabolizing tissues.

The existence of free radicals has now been demonstrated in a variety of lyophilized tissues by the technique of paramagnetic resonance absorption

[16] L. Michaelis, *in* "The Enzymes" (J. B. Sumner and K. Myrbäck, eds.), Vol. II, Part 1. Academic Press, New York, 1951.

or electron spin resonance (ESR) absorption, as it is now referred to, which involves measurements of the absorption of energy in the microwave region of the spectrum as a result of the interaction between an applied variable magnetic field and the magnetic moments of the unpaired electrons in the sample under investigation.[17-22]

Subsequent improvements in technique have made possible the application of this method to wet samples, as a result of which the existence of free radicals has been demonstrated in isolated enzyme systems, viz., various dehydrogenase and xanthine oxidase systems, as well as in a number of actively metabolizing cells including isolated chloroplasts, chlorella cells, bacteria, and mammalian tissues.[23-26] Ehrenberg and Ludwig[25] have, however, emphasized the importance of obtaining adequate kinetic data before concluding that the free radicals observed are real intermediates in the systems studied. Further refinements in technique are obviously desirable in order to make possible the precise identification of the free radicals in living cells and isolated enzyme systems.

It is therefore clear from the above that the study of free radicals far transcends the fields of photochemistry and radiation chemistry and is bound to assume a role of increasing importance in studies on the metabolic activities of living cells. This in turn enhances the value of photochemical studies of biologically important molecules, since optical excitation is probably the most useful and precise method for attaining known excited electronic states.

Finally, mention should be made of the fact that electron spin resonance has been used to study radiation induced damage in proteins[27, 28] and irradiated nucleic acids and their derivatives.[29, 30] It has also been applied to

[17] B. Commoner, J. Townsend and G. E. Pake, *Nature* **174,** 689 (1954).
[18] E. Zavoisky, *J. Phys.* (*U. S. S. R.*) **9,** 211 (1945).
[19] N. N. Semenov, "Some Problems in Chemical Kinetics and Reactivity." Acad. Sci. U.S.S.R., 1954; English translation (M. Boudart), Princeton Univ. Press, 1958.
[20] J. E. Wertz, *Chem. Revs.* **55,** 829 (1955).
[21] P. B. Sogo and B. M. Tolbert, *Advances in Biol. and Med. Phys.* **5,** 1 (1957).
[22] D. J. E. Ingram, "Free Radicals as Studied by Electron Spin Resonance." Butterworths, London, 1958.
[23] B. Commoner, J. J. Heise, and J. Townsend, *Proc. Natl. Acad. Sci. U. S.* **42,** 710 (1956).
[24] B. Commoner, J. J. Heise, B. B. Lippincott, R. E. Norberg, J. V. Passoneau and J. Townsend, *Science* **126,** 57 (1958).
[25] A. Ehrenberg and G. D. Ludwig, *Science* **127,** 1177 (1958).
[26] R. C. Bray, B. G. Malmstrom, and T. Vanngard, *Biochem. J.* **71,** 24P (1959).
[27] W. Gordy and H. Shields, *Radiation Research* **9,** 611 (1958).
[28] L. A. Blumenfeld and E. A. Kalmanson, *Biofizika* **3,** 87 (1958).
[29] H. Shields and W. Gordy, *Proc. Natl. Acad. Sci. U. S.* **45,** 269 (1959).
[30] J. W. Boag and A. Muller, *Nature* **183,** 831 (1959).

an investigation of the fate of free radicals in yeast suspensions in connection with the mechanism of radiation protection.[31] A general outline of the principles involved in such studies has also appeared.[32]

5. Secondary Photochemical Processes

The excited atoms or molecules, or free radicals, produced in the primary photochemical process, may subsequently undergo a wide variety of reactions resulting in the formation of stable products. These secondary steps or "dark" reactions follow the laws of chemical kinetics and in fact, studies of the kinetics of the formation of photoproducts, or the disappearance of reactants, form one of the important tools used for the elucidation of the mechanism of a photochemical reaction.

The various procedures used in the study of the photolytic reactions undergone by nucleic acids and their constituents form the subject of most of this chapter. Standard texts should be consulted for more general information (see General Bibliography at the end of the chapter).

III. Photochemical Techniques

The conditions under which a photochemical experiment is carried out should be reported in sufficient detail so that the results are susceptible of comparison with those of other observers. The minimum of information required for this purpose includes the type of lamp used, whether with or without filter, as well as characteristics of filter, geometrical arrangement of source and irradiated substance, and concentration and layer thickness of irradiated material. In view of the frequent tendency to specify only the incident intensity of the source, one cannot overemphasize the fact that the important quantity is the energy *absorbed* by the system.

1. Sources

The ideal source is obviously a monochromator. Very few laboratories, however, dispose of such instruments suitable for photochemical work; moreover, even a good monochromator will not supply the ultraviolet intensities necessary for many photobiological experiments. In general the application of such instruments has been reserved for investigations of action spectra.

For most photochemical work in the ultraviolet the use of high-pressure mercury lamps is to be definitely discouraged because of the heterogeneity of the emitted radiation and the lack of simple filters for isolation of specific

[31] B. Smaller and E. C. Avery, *Nature* **183,** 539 (1959).

[32] W. Gordy, *in* "Information Theory in Biology" (H. P. Yockey, R. P. Platzman, and H. Quastler, eds.). Pergamon Press, New York, 1958; *Radiation Research, Suppl.* **1,** 491 (1959).

lines. The most convenient type of source is the low-pressure quartz mercury resonance lamp with maximum emission at 253.7 mμ; included in this class are also the familiar germicidal lamps with special glass envelopes. A wide variety of resonance lamps[33] is available from a number of firms who usually supply data regarding emission intensities at different wavelengths, the fractional intensity of which, at 253.7 mμ, may be well over 90%. According to Peel,[34] the British Thermal Syndicate lamp emits 97% of the total intensity at 253.7 mμ, about 2.7% above 300 mμ and a few tenths of 1% at wavelengths below 300 mμ, excluding the 253.7 line.

Many resonance lamps emit traces of radiation at wavelengths to the violet of 253.7 mμ and occasionally as much as 1% or more of the total output at 184.9 mμ. Despite the fact that this latter line is strongly absorbed in air (50% absorption in 2–3 cm. air), its much greater photochemical effectiveness may be troublesome. Santer[35] presents data on the amount of 184.9 mμ radiation emitted by various resonance lamps as well as a method for the measurement of the intensity of this line. By far the simplest procedure, however, is to filter out such radiation with a layer of 25–50% acetic acid. Surprisingly enough, even some germicidal lamps may emit traces of 184.9 radiation;[33, 36] while some lamps (cold-cathode Sterilamps) are made in a number of different glasses so as to transmit desired intensities of the 184.9 line.[33]

For experiments where high doses are necessary, it has become common practice to use batteries of as many as 6–12 germicidal lamps, the irradiated solution being in shallow pans or in a series of quartz tubes. A much simpler and more convenient arrangement, which has been in use in this laboratory for over a year, is that shown in Fig. 3. The solution to be irradiated and introduced at A is contained between the germicidal lamp L and the glass tube T, to the latter of which is sealed a water jacket for temperature control. The diameter of the tube T is from 2–10 mm. larger than that of the lamp, depending on individual requirements; it is tapered at the bottom and cemented to the lamp surface at S by means of a plastic spacer and some plastic cement; it is centered at the top by several plastic spacers P. The stopcock C is sealed to T through a capillary tube by means of which the solution may be stirred during irradiation by passage of a stream of air or nitrogen. During the course of the reaction samples may be drawn off via C or by means of a fine drawn-out glass capillary inserted at A. Should the lamp contain troublesome radiation below 253.7 mμ, it may be necessary to insert an additional (quartz) tube between T and L so as to supply a layer of liquid filter; without this modification, however, we have found this source suitable for preparing sizeable quantities of photo-products of pyrimidine derivatives.

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[33] L. J. Buttolph, *in* "Radiation Biology" (A. Hollaender, ed.), Vol. II. McGraw-Hill, New York, 1955.

[34] G. N. Peel, *Brit. J. Radiol.* **12**, 99 (1939).

[35] E. Santer, *Z. angew. Phys.* **9**, 105 (1957).

[36] A. Canzanelli, R. Guild, and D. Rapport, *Am. J. Physiol.* **167**, 364 (1951).

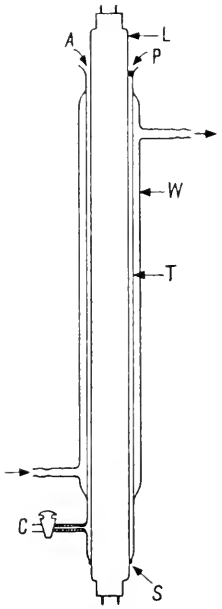


FIG. 3. Simple source of high intensity 253.7 mμ radiation constructed from germicidal lamp; for description see text [Shugar and Wierzchowski, unpublished].

2. Preparative Photochemistry

Photochemical procedures, although limited in scope because of the frequent difficulty of predicting the course of a reaction, have nonetheless been profitably applied to the synthesis of a large number of new compounds, and reference should be made to the excellent and detailed articles of Schenck[37] and Masson *et al.*[38] on the applications of photochemistry in synthetic organic chemistry. It will be shown in Section V that a variety of pyrimidine derivatives, including nucleosides and nucleotides, are transformed into new derivatives instead of undergoing ring rupture.[39] We have found the source described in the previous section suitable for the preparation of 50–60 mg. quantities of such photoproducts in a single run; but this could undoubtedly be stepped up considerably by sealing the tube T to the lamp at the upper end and introducing a continuous flow of the reactant.

3. Light Intensity Measurements

By far the most convenient procedure, as well as the most accurate, for the measurement of light intensities, is by chemical actinometry. The uranyl

[37] G. O. Schenck, *Angew. Chem.* **64**, 12 (1952); **69**, 579 (1957).

[38] C. R. Masson, V. Boekelheide, and W. A. Noyes, *in* "Techniques of Organic Chemistry" (A. Weissberger, ed.), Vol. II. Interscience, New York, 1956.

[39] D. Shugar and K. L. Wierzchowski, *Postepy Biochem.* **4**, Suppl., p. 243 (1958).

oxalate actinometer[40] is still the most widely used and is described in detail by Masson *et al.*[38] For most purposes, however, the simple procedure outlined by Bowen[41] is entirely adequate. If desired, the inconvenient titration of undecomposed oxalate may be replaced by difference spectrophotometry of the amount of oxidant consumed in the control and irradiated samples, using ceric sulphate as the oxidizing agent; this method is also claimed to have the advantage of making possible measurements of tenfold lower intensities.[42]

For much lower intensities (not exceeding 10^{13} quanta/sec.) the malachite green leucocyanide acinometer is perhaps the most suitable, and involves only the measurement of the optical density of dye formation resulting from exposure to radiation;[43] but this has not been widely used.

The potassium ferrioxalate actinometer proposed by Parker[44] has undergone further development and has now been described in very great detail.[45] It is simple to use, since only spectrophotometric measurements are involved, and it is said to be more sensitive than the uranyl oxalate actinometer besides being applicable over a wider range of light intensities.

A thermopile or photocell may be useful for checking the constancy of output of a given source, but the accuracy required in most photobiological experiments is seldom such that this is necessary.

For measuring light intensities of resonance lamps we have found it convenient in our laboratory to follow the rate of decrease in optical density at 262 mμ of a solution of 10^{-4} *M* uridine phosphate (Up) in a 10-mm. cuvette at neutral pH. The rate of decrease of optical density may be calculated from the quantum yield (see Table II) or calibrated against a uranyl oxalate or other actinometer.

It is desirable to carry out actinometry in the irradiation cell so as to eliminate as much as possible errors due to the geometry of the system.

4. Conversion Factors for Light Intensity

Because of the fact that results are frequently reported in the literature with different units for light intensity, the following conversion factors may be found useful:

$$1 \text{ calorie} = 4.185 \text{ joules}$$

$$1 \text{ joule} = 10^7 \text{ ergs}$$

[40] W. G. Leighton and G. S. Forbes, *J. Am. Chem. Soc.* **52**, 3139 (1930).
[41] E. Bowen, "The Chemical Aspects of Light." Oxford Univ. Press, London and New York, 1946.
[42] J. N. Pitts, J. D. Margerum, R. P. Taylor, and W. Brim, *J. Am. Chem. Soc.* **77**, 5499 (1955).
[43] J. G. Calvert and H. J. L. Rechen, *J. Am. Chem. Soc.* **74**, 2102 (1952).
[44] C. A. Parker, *Proc. Roy. Soc.* **A220**, 104 (1953).
[45] C. G. Hatchard and C. A. Parker, *Proc. Roy. Soc.* **A235**, 518 (1956).

$$1 \text{ watt} = 10^6 \,\mu\text{watts} = 1 \text{ joule/sec.} = 10^7 \text{ ergs/sec.}$$

$$1 \text{ quantum} = \frac{47.6}{\lambda(\text{in m}\mu)} \times 10^{-16} \text{ cal.}$$

$$= \frac{1992}{\lambda(\text{in m}\mu)} \times 10^{-12} \text{ ergs}$$

At $\lambda = 253.7$ mμ, 1 quantum $= 7.8 \times 10^{-12}$ ergs.

For a G30T8 germicidal lamp, the effective emitting length of which is 81 cm. and the circumference 8 cm., the total ultraviolet emission is about 7 watts, or 10,801 μwatts/cm.^{2} at the surface, or 108,000 ergs/cm.^{2}/sec. Since most of this is at 253.7 mμ, the emission at the surface is $108{,}000 \times (10^{12}/7.8)$ quanta/cm.^{2}/sec. $= 1.4 \times 10^{16}$ quanta/cm.^{2}/sec. at 253.7 mμ.

IV. Optical Properties and Techniques

A knowledge of the absorption spectrum of a substance is a prerequisite for photochemical studies. In the case of nucleoproteins, nucleic acids, and their constituents this is all the more important in that spectral changes provide a convenient tool for following the effects of irradiation and in many instances for identification of some of the photoproducts. Interpretations of action spectra are also based on absorption spectra under the particular conditions prevailing.

The optical properties of nucleic acids have already been dealt with in Chapter 14 of Volume I and we shall confine ourselves here to some additional material which has appeared since the publication of this review and which is of relevance to the photochemistry of polynucleotides.

1. Far Ultraviolet Spectra

The absorption spectra of deoxyribonucleic acid (DNA) and some nucleic acid derivatives have been extended into the vacuum ultraviolet[46] in connection with photochemical studies on nucleic acids, and necessarily involving the use of dry films. The spectra of DNA and cytosine, extended into the near ultraviolet, were in reasonably good accord with those for the same substances in solution, but marked differences were noted for adenine. Aside from the undesirability of the use of films as compared to aqueous solutions, vacuum spectrophotometers are neither accessible nor applicable readily to routine measurements.

A significant technical advance of the past three years has been the extension of the available wavelength range to almost 186 mμ without the use of vacuum spectrophotometers. The conversion of a Beckman model DU for measurements to 192 mμ has been described by Taylor and Jones[47];

[46] J. W. Preiss and R. Setlow, *J. Chem. Phys.* **25**, 138 (1956).
[47] L. W. Taylor and L. C. Jones, *Anal. Chem.* **28**, 1706 (1956).

Buell and Hansen[48] report details for the construction of an instrument suitable for measurements in the range 230–190 mμ, based on the use of a "solar-blind" photomultiplier.

Of great interest to most laboratories is the development by Beckman of its photomultiplier attachment which increases sensitivity over the entire spectral range and makes possible measurements to 190 mμ. A similar goal has been achieved by Unicam through the use of a fused quartz prism with improved transmission characteristics and which is said to extend the useful working range to 186 mμ, the region of maximum absorption of the peptide bond[49] and most likely of nucleoproteins.

With the use of proper precautions for checking the absence of, or correcting for, stray light, these new instruments should considerably extend the potentialities of spectral techniques to structural studies upon, and the analytical chemistry of, nucleic acids and their derivatives. An analytical procedure for serum proteins has already been described, based on peptide bond absorption at 210 mμ.[50] Many nucleic acid derivatives possess an absorption maximum in the region 190–210 mμ which the new instruments define clearly[51, 52] (Fig. 4) while others, such as some dihydropyrimidines and photoproducts of uracil derivatives, possess a maximum at neutral pH only in this region.

2. Aqueous Solution Infrared Spectroscopy

Most infrared investigations in the past have involved the use of films, mulls or solutions in nonaqueous solvents (Volume I, Chapter 14). The appearance on the market of thin cells using a variety of water-resistant window materials,[53] together with the use of D_2O as solvent in the 3-μ and 6-μ regions of the spectrum where H_2O strongly absorbs, now makes possible routine infrared spectroscopy of nucleic acids and their derivatives in aqueous medium.[53-55] Several laboratories have already begun to exploit this powerful technique in structural studies on pyrimidine nucleosides and nucleotides,[56, 57] as well as on nucleic acids and nucleoproteins.[54, 58] Atten-

[48] M. V. Buell and R. E. Hansen, *Science* **126**, 842 (1957); *Anal. Chem.*, **31**, 878 (1959).

[49] A. R. Goldfarb, L. J. Saidel, and E. Mosowitch, *J. Biol. Chem.* **193**, 397 (1951); J. S. Ham and J. R. Platt, *J. Chem. Phys.* **20**, 335 (1952).

[50] M. P. Tombs, F. Sonter, and N. F. McLagan, *Biochem. J.* **71**, 13P (1959); *ibid.* **73**, 167 (1959).

[51] A. Rörsch, R. Beukers, J. Ijlstra, and W. Berends, *Rec. trav. chim.* **77**, 423 (1958).

[52] M. V. Buell and R. E. Hansen, *J. Biol. Chem.* in press (1960).

[53] H. Sternglanz, *Appl. Spectroscopy* **10**, 77 (1956).

[54] E. R. Blout, *Ann. N. Y. Acad. Sci.* **69**, 84 (1957).

[55] W. J. Potts, Jr., and N. W. Wright, *Anal. Chem.* **28**, 1255 (1956).

[56] H. T. Miles, *Biochim. et Biophys. Acta* **22**, 247 (1956); **27**, 46 (1958); **30**, 324 (1958).

[57] R. L. Sinsheimer, R. L. Nutter, and G. R. Hopkins, *Biochim. et Biophys. Acta* **18**, 13 (1955).

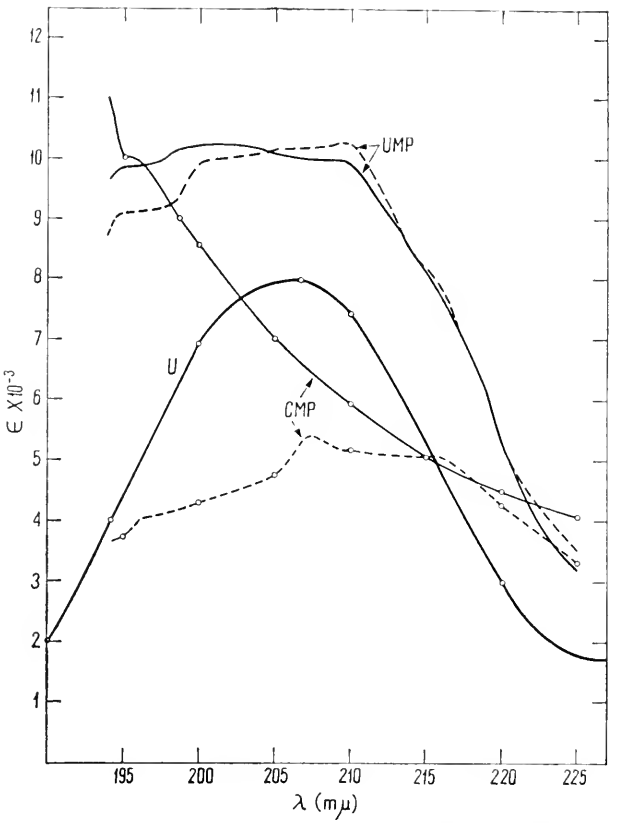


FIG. 4. Absorption spectra of the sodium salts of UMP and CMP in aqueous, unbuffered medium at approximately neutral pH (——) and in 0.2 *N* H_2SO_4 (-----) [from M. V. Buell and R. E. Hansen, *J. Biol. Chem.* in press (1960)] and of uracil (U) at pH 2.4 [from A. Rörsch, R. Beukers, J. Ijlstra, and W. Berends, *Rec. trav. chim.* **77,** 423 (1958)]. For CMP the extinction coefficients should be twice those indicated.

tion is drawn to this relatively new field particularly because of its potential importance in the analysis of stable and reversible photoproducts of pyrimidine derivatives.[59]

3. Dihydropyrimidine Derivatives

Saturation of the 4,5 double bond of pyrimidine derivatives results in a disappearance of the characteristic absorption maxima in the neighborhood of 260–280 mμ,[60] a fact also evident from the spectral characteristics of

[58] E. R. Blout and H. Lenormant, *Biochim. et Biophys. Acta* **17,** 325 (1955).

[59] A. M. Moore and C. H. Thomson, *Science* **122,** 594 (1955).

[60] R. D. Batt, J. K. Martin, J. McT. Ploeser, and J. Murray, *J. Am. Chem. Soc.* **76,** 3663 (1954).

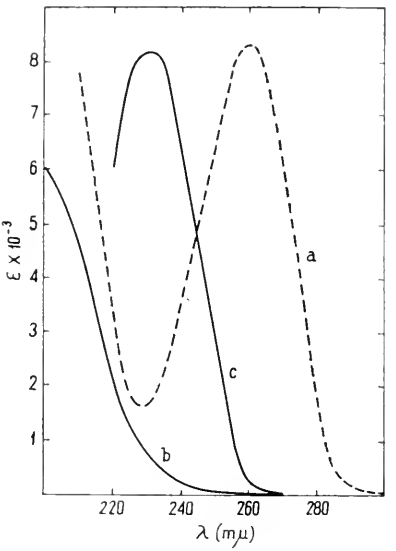


FIG. 5.

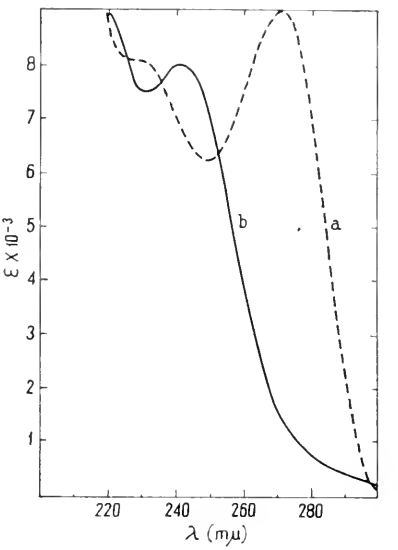


FIG. 6.

FIG. 5. Absorption spectra (*a*) of uracil at neutral pH; (*b*) of dihydrouracil at neutral pH; (*c*) of dihydrouracil at pH 13, corrected for alkaline decomposition [curve *c* from R. D. Batt, J. K. Martin, J. McT. Ploeser, and J. Murray, *J. Am. Chem. Soc.* **76**, 3663 (1954)].

FIG. 6. Absorption spectra at neutral pH of (*a*) deoxycytidine, (*b*) dihydrodeoxycytidine; extinction values for the latter curve are only approximate, see text. Spectrum of dihydrocytosine is similar to curve *b* [Wierzchowski and Shugar, unpublished].

barbital and barbituric acid derivatives at acid pH.[61] The spectra of the dihydropyrimidines and their glycosides are of interest in relationship to the photochemistry of the parent compounds, but the optical properties of these biologically important substances have received very little attention, although some of them are now readily available through hydrogenation with a rhodium catalyst.[62-64] This is due in part to the fact that the absorption spectra are located largely in the somewhat less accessible region below 230 mμ, although newer instruments (Section IV, 1) have now surmounted this obstacle.

Figure 5 shows the spectrum of uracil at neutral pH, as well as that of dihydrouracil at neutral and alkaline pH; the latter curve has been extrapolated to zero time to correct for the drop in extinction due to the alkaline

[61] J. J. Fox and D. Shugar, *Bull. soc. chim. Belges* **61**, 44 (1952).
[62] W. E. Cohn and D. G. Doherty, *J. Am. Chem. Soc.* **78**, 2863 (1956).
[63] M. Green and S. S. Cohen, *J. Biol. Chem.* **225**, 397 (1957).
[64] M. Green and S. S. Cohen, *J. Biol. Chem.* **228**, 601 (1958).

decomposition of dihydropyrimidines to β-ureido acid derivatives.[60] According to Batt *et al.*[60] the spectral changes for dihydrouracil (and the dihydro derivatives of thymine and orotic acid) are consistent with an enol form at alkaline pH. Studies in progress in our laboratory (Janion and Shugar) indicate that the transition from the neutral to the alkaline curve is due to ionization of one of the carbonyl groups. Dihydrodimethyluracil exhibits a similar maximum at 225 mμ in neutral medium, but this is not modified by alkalization prior to decomposition (cf. dimethylbarbituric acid[61]).

The preparation of dihydrocytosine derivatives is more difficult because of the danger of overdehydrogenation and deamination, even at neutral pH.[64] Figure 6 exhibits the absorption spectra at neutral pH of deoxycytidine and a sample of impure dihydrodeoxycytidine, so that extinction coefficients for the latter should not be considered as accurate. The absorption spectrum of dihydrocytosine is similar in shape to that of dihydrodeoxycytidine, but the maximum is at 237.5 mμ. Since position 3 in dihydrodeoxycytidine is blocked, it is clear that the chromophore principally responsible for the peak at about 240 mμ is

It is of interest in this connection to recall that cytosine nucleosides (but not cytosine or 3-methylcytosine) show a point of inflection[65, 66] which, in the case of pyranosylcytosines,[66] cytidine-2′,3′-phosphate,[39, 67] and isopropylidenecytidine (Wierzchowski and Shugar, unpublished), is resolved into a clearly defined maximum at about 236 mμ, due most likely to the above chromophore and resulting from some type of interaction between the pyrimidine and carbohydrate rings (see also Section V, 4, *b*).

4. Hyperchromicity in Oligo- and Polynucleotides

The fractional increase in absorption (hyperchromicity) accompanying the degradation of nucleic acids has already been discussed (Volume I, Chapter 14), particularly with reference to its relationship to nucleic acid structure. Some of the conclusions reached at that time with respect to hyperchromicity in small oligonucleotides require modification in the light of additional evidence which has since become available. Furthermore the development of chemical and enzymic procedures for the synthesis of homo- and hetero-oligonucleotides (see Chapter 31) now make it possible to investigate with more certainty, and considerable simplification, the factors contributing to hyperchromicity. This, in turn, has already proved useful

[65] D. Shugar and J. J. Fox, *Biochim. et Biophys. Acta* **9**, 199 (1952).
[66] J. J. Fox and D. Shugar, *Biochim. et Biophys. Acta* **9**, 369 (1952).
[67] D. Shugar and K. L. Wierzchowski, *Bull. acad. polon. sci., Classe II*, **6**, 283 (1958).

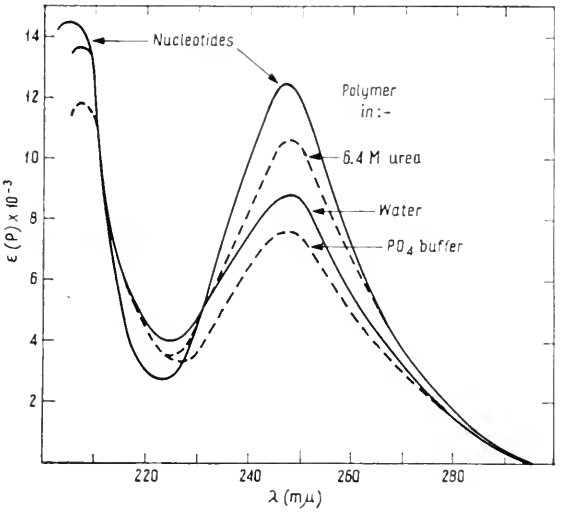


FIG. 7. Absorption spectrum of poly-I, illustrating hyperchromicity in a single polynucleotide chain under various conditions [R. C. Warner, *J. Biol. Chem.* **229**, 711 (1957)].

in attempts to interpret the photochemical behavior of model oligonucleotides.[68, 69]

The magnitude of the hyperchromic effect exhibited by high molecular weight polymers is illustrated by Fig. 7 for polyinosinic acid (poly-I) under various conditions.[70] The hyperchromicity of polyuridylic acid (poly-U) is small (see below) so that the difference between curves *a* and *b* in Fig. 8 is equal to the hyperchromicity of polyadenylic acid (poly-A).

Certain pairs of homopolymers also interact specifically under defined conditions,[71, 72] the resulting complexes exhibiting an additional decrease in extinction (Fig. 8). In fact such interactions were first discovered by spectral methods which are still the most suitable for quantitative studies of polymer interactions.

From Fig. 8 it will be seen that the total hyperchromicity of a twin strand chain is almost 100% (actually 96%), i.e., this is the increase in absorption on quantitative hydrolysis of the complex to mononucleotides. For highly polymerized DNA with an ϵ(P) of 6000, the calculated extinction of mono-

[68] K. L. Wierzchowski and D. Shugar, *4th Intern. Congr. Biochem., Vienna, 1958*, No. 3–35.

[69] K. L. Wierzchowski and D. Shugar, *Acta Biochim. Polon.* **6**, 313 (1959).

[70] R. C. Warner, *J. Biol. Chem.* **229**, 711 (1957).

[71] D. R. Davis and A. Rich, *J. Am. Chem. Soc.* **80**, 1003 (1958); G. Felsenfeld and A. Rich, *Biochim. et Biophys. Acta* **26**, 457 (1957).

[72] R. C. Warner, *4th Intern. Congr. Biochem., Vienna, 1958* Symposium No. 9.

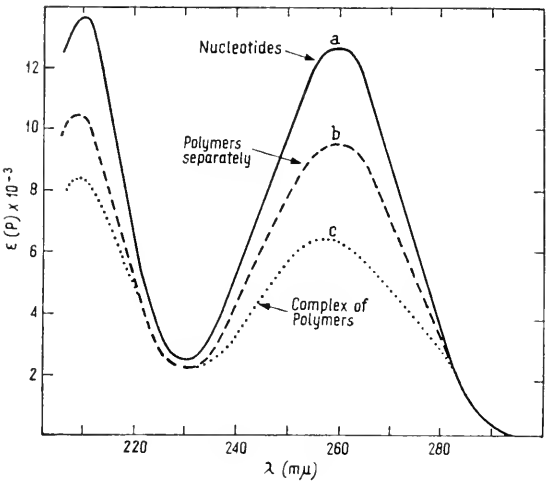


FIG. 8. Hyperchromicity resulting from specific polymer interaction, in this case an equimolar mixture of poly-A and poly-U: (*a*), spectrum of mixture of mononucleotides; (*b*), calculated spectrum for mixture of poly-A and poly-U; (*c*), observed spectrum for mixture of poly-A and poly-U; [R. C. Warner, *J. Biol. Chem.* **229**, 711 (1957)].

nucleotides is 10,100 so that the hyperchromicity should be about 70%[73]; an experimentally observed value[74] based on exhaustive digestion with DNase and phosphodiesterase is 80%; this may be somewhat on the high side because of the fact that following DNase digestion, the pH was raised to 9.2 for diesterase digestion.

It may, however, be concluded from the above that about 40–50% of the hyperchromicity of a DNA chain is due to hydrogen bonding between base pairs in the twin-stranded Watson-Crick structure.

Insofar as single chains are concerned, the hyperchromicity at neutral pH varies from 6% for poly-U to 40% for polycytidylic acid (poly-C), 50–55% for poly-A, and 63% for poly-I.[70] However, Shapiro and Chargaff[75] have shown that the diphosphate of thymidine (pTp) exhibits an extinction coefficient about 8% lower than that of thymidine phosphate (Tp); a similar effect most likely prevails for pUp, so that this alone may fully account for the small hyperchromicity of poly-U. Such an explanation cannot apply to other polymers since, e.g., diphosphates of deoxycytidine possess an extinction coefficient 8% lower than for monophosphates, whereas the hyperchromicity of poly-C is about 40%. The increase in absorption of such polymers under the influence of urea (see Fig. 7) or heat has been taken as evidence for involvement of hydrogen bonding[72] which, in the case of poly-

[73] P. D. Lawley, *Biochim. et Biophys. Acta* **21**, 481 (1956).
[74] R. L. Sinsheimer and J. F. Koerner, *J. Biol. Chem.* **198**, 293 (1952).
[75] H. S. Shapiro and E. Chargaff, *Biochim. et Biophys. Acta* **26**, 596 (1957).

A, may be between amino groups and phosphate oxygens,[76] but this is not the complete explanation.

Additional information is forthcoming from studies on smaller oligonucleotides. A number of observers have confirmed the observations of Sinsheimer[77] and deGarilhe and Laskowski,[78] that even dinucleotides exhibit a hyperchromic effect, the latter authors demonstrating how this varies quantitatively from 2–11% for various pairs of nucleotides (Table I). These findings have been extended in a rather elegant manner by Michelson[79] to a number of small oligonucleotides with chain lengths of from 2 to about 13 residues. The hyperchromicity was found to increase with chain length and attain a limiting value for a chain with 5 to 6 residues (Table I). From a study of the variation of hyperchromicity with pH, as well as the changes in dissociation constants of ionizable groups as a function of chain length, it was concluded that the major cause of hyperchromicity is due to the ordered stacking of the aromatic rings parallel to each other, even in dinucleotides; the resultant interaction between the π-electron orbitals of adjacent rings leads to the formation of electron orbitals extending over several rings with a lower total energy, and, from a geometrical point of view, also a smaller chromophoric area (cf. Lawley[73] and Laland *et al.*[80]).

The behavior of an aromatic ring in a polynucleotide chain, under the influence of absorbed radiation, will consequently depend on its neighbors as well as whether it is near one end of the chain or in the interior. The merging of electron orbitals of adjacent rings would also be expected to lead to a transfer of absorbed energy; such a transfer has actually been demonstrated in DPNH, which is analogous to a dinucleotide and which also exhibits appreciable hyperchromicity (see Section V, 3).

The very low hyperchromicity of poly-U, and the absence of any alteration of its extinction by heat, urea, or ionic strength, has been interpreted by Warner[72] as due to the absence of interaction between the bases of this polymer. Photochemical studies on oligo- and polynucleotides of Up, on the other hand, suggest that such interaction does exist.[69]

Use of $\epsilon(P)$*:* The introduction by Chargaff and Zamenhof[81] of the term ϵ(P) for characterization of extinction coefficients of natural nucleic acids was (and still is) due to the difficulty of characterization of molecular weights; it was based on the fact that there is one atom P per purine or pyrimidine residue. This expression has proved its usefulness, particularly in the characterization of the degree of degradation of a given preparation.

[76] R. F. Beers, Jr., and R. F. Steiner, *Nature* **179,** 1076 (1957); **181,** 30 (1958).
[77] R. L. Sinsheimer, *J. Biol. Chem.* **208,** 445 (1954).
[78] M. P. deGarilhe and M. Laskowski, *J. Biol. Chem.* **223,** 661 (1956).
[79] A. M. Michelson, *Nature* **182,** 1502 (1958); *J. Chem. Soc.* p. 1371 (1959).
[80] S. G. Laland, W. A. Lee, W. G. Overend, and A. R. Peacocke, *Biochim. et Biophys. Acta* **14,** 356 (1954).
[81] E. Chargaff and S. Zamenhof, *J. Biol. Chem.* **173,** 327 (1948).

TABLE I

HYPERCHROMICITY OF SOME NUCLEOSIDE DIPHOSPHATES AND OLIGO- AND POLYNUCLEOTIDES

Substance	Hyperchromicity[a]				References
	pH 2	pH 7[b]	pH 9	pH 13	
(d)pTp	8	8	—	—	*d*
(d)pCp	3	9	—	—	*d*
(d)A5′p3′T5′p	—	18	—	—	*e*
(d)pTpT	—	3	—	—	*e*
(d)pCpC	0	1.5	4		*d*, *f*
(d)pCpT	3	3	2	—	*d*, *f*
(d)pCpA	6	25	7	—	*d*, *f*
(d)pGpA	—	—	11	—	*f*
A2′p5′U	12	16	—	12.8	*g*
A2′(3′)p5′T	—	—	—	12.7	*g*
A3′p5′C3′p	—	—	—	7.2	*g*
Diadenylic acid[c]	0	—	—	15.1	*g*
Triadenylic acid	6.8	—	—	22.8	*g*
Tetraadenylic acid	13.2	—	—	30.7	*g*
Pentaadenylic acid	16.4	—	—	32.9	*g*
Poly-A (13.6)[c]	22.9	36.7	—	36.7	*g*
Poly-A (high)	—	50	—	—	*h*
Poly-U (3.5)[c]	4.8	—	—	4.8	*g*
Poly-U (6.6)[c]	7.5	—	—	6.5	*g*
Poly-U (12)[c]	7.5	7.5	—	8.6	*g*
Poly-U (high)	—	6.0	—	—	*h*
Poly-G (3.2)[c]	9.0	10.3	—	0	*g*
Poly-G (5.4)[c]	14.6	16.5	—	0	*g*
Poly-G (12.5)[c]	18.0	23.0	—	0	*g*
Poly-AG (6.2)[c]	14.7	36.7	—	19.0	*g*

[a] Per cent increase in absorption following degradation to mononucleotides.

[b] Values in this column for oligo- and polynucleotides of Ap, Up, Gp, and ApGp are in unbuffered solution; for influence of ionic strength on highly polymerized polynucleotides see Warner[h].

[c] These refer to polymers containing a mixture of 2′,5′ and 3′,5′ internucleotide linkages (see Michelson[g]); figures in parentheses denote average chain lengths.

[d] H. S. Shapiro and E. Chargaff, *Biochim. et Biophys. Acta* **26**, 596 (1957).

[e] P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.* **80**, 6212 (1958).

[f] M. P. deGarilhe and M. Laskowski, *J. Biol. Chem.* **223**, 661 (1956).

[g] A. M. Michelson, *Nature* **182**, 1502 (1958); *J. Chem. Soc.* p. 1371 (1959).

[h] R. C. Warner, *J. Biol. Chem.* **229**, 711 (1957).

More recently there has been a tendency to use ϵ(P) to express the extinction coefficients of such compounds as nucleoside diphosphates, dinucleotides, and dinucleoside monophosphates.[75, 82] It seems questionable to the writer whether such a procedure retains the simplification it was originally intended to have, when applied to well-defined compounds; and comparisons of absorption spectra on this basis are, if anything, rendered more complicated. Until some uniform procedure is adopted, it

[82] P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.* **80**, 6212 (1958).

is suggested that the above be borne in mind in attempting to present spectra with the greatest possible clarity. Summing up extinctions of the component nucleosides of small oligonucleotides offers some advantages.[82]

In view of the increasing importance and use of hyperchromicity in studies on the structure, photochemistry and analytical chemistry of oligo- and polynucleotides, it is perhaps not out of order to specify the terms used to describe it. When the extinction of a given oligonucleotide is lower than that of its constituent mononucleotides, it is "hypochromic" or exhibits "hypochromicity." If the oligonucleotide is hydrolyzed to mononucleotides, it exhibits "hyperchromicity." If a given oligonucleotide exhibits 75% of the absorption of its constituent mononucleotides, its hypochromicity is 25/100 or 25% whereas its hyperchromicity is 25/75 or 33%.

It should be noted that hyperchromicity is by no means confined to nucleotide polymers and coenzymes. The observations of Katchalski *et al.*[82a] on synthetic polypeptides of aromatic amino acids show the presence of spectral effects which may be ascribed to hypochromicity and attaining values as high as 15%. However, probably few natural proteins are hypochromic since the percentage of aromatic amino acids is small; a notable exception is gramicidin where the high tryptophan content might be expected to result in appreciable hypochromicity.

5. Nucleoproteins and Viruses

Considerable new evidence, based on studies of chemical composition,[83-85] as well as X-ray diffraction data and studies of molecular models,[86] has now accumulated to show that at least some of the nucleoprotamines and nucleohistones extracted from living cells are definite complexes; and Crampto[87] has even demonstrated how nucleohistone may be prepared by methods such that dissociation cannot occur during the extraction procedure (cf. Volume I, Chapter 10). Much remains to be done, however, as regards the nature of the linkages between nucleic acid and protein which, in addition to saltlike bonds, may apparently vary from hydrogen-bonding in a ribonucleoprotein extracted from *Escherichia coli*[88] to very firm alkali-stable bonds in a deoxyribonucleoprotein from liver cell nuclei.[89]

Spectral investigations on nucleoproteins continue to be scanty and the results conflicting, possibly because they have been confined to artificial

[82a] E. Katchalski and M. Sela, *J. Am. Chem. Soc.* **75,** 5284 (1953); A. Patchornik, M. Sela, and E. Katchalski, *ibid.* **76,** 299 (1954); M. Sela and E. Katchalski, *ibid.* **76,** 129 (1954).

[83] P. F. Davison and J. A. V. Butler, *Biochim. et Biophys. Acta* **21,** 568 (1956); *Advances in Enzymol.* **18,** 161 (1957).

[84] C. F. Crampton, W. H. Stein, and S. Moore, *J. Biol. Chem.* **225,** 363 (1957).

[85] R. Vendrely, A. Knobloch, and W. Matsudaira, *Nature* **181,** 343 (1958).

[86] M. Feughelman, R. Langridge, W. E. Seeds, A. R. Stokes, H. R. Wilson, C. W. Hooper, M. H. F. Wilkins, R. K. Barclay, and L. D. Hamilton, *Nature* **175,** 834 (1955).

[87] C. F. Crampton, *J. Biol. Chem.* **227,** 495 (1957).

[88] D. Elson, *Biochim. et Biophys. Acta* **27,** 207 (1958); *ibid.* **36,** 362 (1959).

[89] K. J. Monty and A. L. Dounce, *J. Gen. Physiol.* **41,** 595 (1958).

complexes; e.g., Seibert[90] finds that such complexes exhibit decreases in extinction from additivity, while Nurnberger[91] finds only increases. Klamerth[92] has studied the increase in absorption of DNA-histone complexes at 259 mμ with increasing histone concentrations and showed the existence of two points of inflection, the first at an N/P ratio of 3.8 characteristic of normal DNA histones[83] and a second at an N/P ratio of 9, which is the limit of histone binding of the complex. It must, therefore, be emphasized that spectral investigations of artificial nucleoproteins should be supplemented by additional independent evidence of complex formation, such as by light scattering, sedimentation, diffusion, and electrophoresis.

In general it appears that, in the region 240–300 mμ which is most used for photochemical investigations, the deviations from additivity in a nucleoprotein complex are not important enough to cause apprehension. At shorter wavelengths peptide bond absorption begins to predominate, a fact of considerable significance in studies on action spectra and on the mechanism of ultraviolet inactivation of viruses (Section IX).

The nature of the linkage between nucleic acid and protein can profoundly influence the photochemical behavior of the former, the most striking example being that of the tobacco mosaic virus (TMV), where it has been shown that this is the source of differences in sensitivity of different strains and of previous failures to observe photoreactivation (Section X).

In the case of viruses, quantitative photochemical studies must also surmount the hurdle of scattered light which may be appreciable because of the high molecular weights of these particles. The usual procedure has been to measure spectrophotometrically the scattered light above 320 mμ, where the virus does not specifically absorb, and applying the Rayleigh law of scattering, to calculate graphically the amount of scattering at shorter wavelengths.[93, 94] For tobacco mosaic virus the amount of scattering at 260 mμ has been calculated in this way to be about 16%.[93, 95] A more direct approach has involved the comparison of the optical densities of solutions of x-protein from infected tobacco plants, one at pH 7.3 at which the molecular weight of the protein is low, and the other at pH 5.3 where it is polymerized into rods of the approximate size of TMV; the amount of scattered light calculated in this way is over 20%.[96] Methods based on calculation of

[90] F. B. Seibert, *Discussions Faraday Soc.* **13,** 251 (1953).
[91] J. I. Nurnberger, *in* "Analytical Cytology" (R. C. Mellors, ed.), Chapter 4. McGraw-Hill, New York, 1955.
[92] O. Klamerth, *Z. Naturforsch.* **12b,** 186 (1957).
[93] G. Schramm and H. Dannenberg, *Ber.* **77,** 53 (1944).
[94] R. Dulbecco, *J. Bacteriol.* **59,** 329 (1950).
[95] K. K. Reddi, *Biochim. et Biophys. Acta* **24,** 238 (1957).
[96] A. D. McLaren and W. N. Takahashi, *Biochim. et Biophys. Acta* **32,** 555 (1959).

optical absorption from chemical composition[97, 98] suggest that for TMV about 50% of the apparent absorption at 253.7 mμ is due to scattered light; but the validity of such a procedure is questionable.

From the absorption spectrum of TMV corrected for scattering, Reddi[95] subtracts the protein absorption to obtain an $\epsilon(P)$ for the nucleic acid component which is approximately equal to the $\epsilon(P)$ of the nucleic acid isolated directly from the virus. It is concluded from this that the aromatic rings are not involved in the linkages between protein and nucleic acid in TMV. However, the Rayleigh procedure for correcting for scattering is subject to considerable error in the neighborhood of an absorption band, as well as for particles such as TMV which are of the same order of magnitude as the wavelength of the light,[99] so that the conclusion that the main linkage in TMV is between the ribonucleic acid (RNA) phosphate groups and protein guanidine groups requires independent substantiation; the more so since the protein component of various TMV strains appears to affect appreciably the photochemical behavior of the virus RNA.

V. Photochemistry of Nucleic Acid Constituents

A knowledge of the photochemical behavior of nucleic acid derivatives is an obvious prerequisite to any attempts to interpret the effects of radiation on polynucleotide chains. It should also be borne in mind that living cells contain a considerable pool of free nucleotides as well as nucleotide coenzymes through which radiation effects may likewise be manifested. For instance, a reasonable correspondence has been established between the destruction of adenosine triphosphate (ATP) and the mobility and fertilizing capacity of frog spermatozoa under the influence of irradiation.[100] In view of the demonstrated involvement of uridinediphosphate glucoside (UDPG) in sucrose synthesis in plants, it is perhaps more than a coincidence that the rate of photolysis of UDPG is comparable to the rate at which inhibition of sucrose synthesis in plants occurs under the influence of irradiation.[101] Another example of considerable significance is the observation of Haas and Doudney[102] on mutation enhancement by ultraviolet irradiation

[97] A. Butenandt, M. Friedrich-Freksa, S. Hartwig, and G. Scheide, *Z. physiol. Chem.* **274**, 276 (1942).

[98] G. Oster and A. D. McLaren, *J. Gen. Physiol.* **33**, 215 (1950).

[99] K. A. Stacey, "Light Scattering in Physical Chemistry." Butterworths, London, 1956.

[100] D. Kanazir and M. Errera, *Biochim. et Biophys. Acta* **16**, 198 (1955).

[101] L. P. Zill, *Federation Proc.* **16**, 276 (1957); L. P. Zill and N. E. Tolbert, *Arch. Biochem. Biophys.* **76**, 196 (1958).

[102] F. L. Haas and C. O. Doudney, *Proc. Natl. Acad. Sci. U. S.* **43**, 871 (1957); **44**, 390 (1958).

in the presence of purine and pyrimidine derivatives and their suggestion that this may be due to light activation of nucleotides.

1. Carbohydrates

The saturated carbon chains of carbohydrates would not be expected to exhibit specific absorption in the region normally used for photobiological studies and, in fact, it was shown many years ago that, following careful purification, sugars possess only extremely low end absorption below 220 mμ at neutral pH.[103]

In view of the fact that extensive irradiation of nucleic acids, as well as of some nucleotide coenzymes, leads to the destruction of the sugar moieties (Sections V, 3 and VI, 1), it is pertinent to inquire to what extent this is due to the direct effect of absorption of light. Theoretically, there should be no such effect at wavelengths at least to the red of 220 mμ.

There exist, nonetheless, scattered reports on the destruction of free sugars and polysaccharides by ultraviolet irradiation. According to Holtz,[104] irradiated neutral glucose solutions are decomposed with the formation of an oxidation-reduction system resembling that for ascorbic acid. In a more extensive study Rice[105] found destruction of sugars in irradiated ribose, glucose, cytidylic and adenylic acids as well as RNA and DNA. With the exception of the nucleic acids there is little doubt that such results are due to the use of sources with high emission at short wavelengths. For instance, Hvidberg *et al.*[106] demonstrated that the viscosity of hyaluronate solutions is destroyed by ultraviolet irradiation, the effective wavelengths being below 300 mμ; however, since the source used was a Phillips HP lamp with the glass filtering envelope removed, the effective radiation must have been well to the violet of 230 mμ.

In agreement with this is the observation of Laurent and Wertheim[107] that neutral solutions of various sugars are unaffected by exposure to a source from which the shorter wavelengths were eliminated. Carter[108] has found adenylic acid to be remarkably resistant to radiation from a battery of germicidal lamps. The stability to irradiation of carbohydrate moieties is further testified to by the high degree of reversibility prevailing for the photoproducts of pyrimidine nucleosides and nucleotides (Section V, 4).

In alkaline solution, on the other hand, irradiation of various sugars even at 253.7 mμ, produces decomposition with formation of a variety of products

[103] L. Kwiecinski, J. Meyer, and L. Marchlewski, *Z. physiol. Chem.* **176,** 292 (1928); L. Marchlewski and W. Urbanczyk, *Biochem. Z.* **262,** 248 (1933).
[104] P. Holtz, *Arch. exptl. Pathol. u. Pharmakol.* **182,** 141 (1936).
[105] E. W. Rice, *Science* **115,** 92 (1952).
[106] E. Hvidberg, S. A. Kvorning, A. Schmidt, and J. Schon, *Nature* **181,** 1338 (1958).
[107] T. C. Laurent and E. Wertheim, *Acta Chem. Scand.* **6,** 678 (1952).
[108] C. E. Carter, *J. Am. Chem. Soc.* **72,** 1835 (1950).

exhibiting characteristic absorption in the range 240–290 mμ.[107, 109] However, since analogous changes may be observed in solutions of monosaccharides and polysaccharides under the influence of alkali alone,[110, 111] the enhanced effect resulting from irradiation at this pH is most likely due to excited hydroxyl ions. It is of some interest that analogous products are produced by X-rays[112, 113] and α-irradiation[114] at both neutral and alkaline pH (for detailed discussion and literature on this subject see Phillips *et al.*[115]).

Purine and pyrimidine nucleoside-bound carbohydrates, however, do not exhibit instability in alkali and their resistance to ultraviolet even under these conditions is testified to by the fact that irradiation of a 10^{-4} *M* solution of adenosine in 0.01 *M* NaOH with a resonance lamp, filtered to remove radiation below 240 mμ, at an intensity of 10^{17} quanta/cm.^{2}/min. for 1 hour is without effect on the orcinol reaction for ribose.[116]

One may therefore conclude that, under the pH conditions normally used in photobiological studies and at wavelengths at least above 220 mμ, the carbohydrate components of nucleic acids are not directly affected by radiation; and that, in those instances where destruction of sugar residues does occur this is a consequence of secondary reactions, such as energy transfer, following absorption of light by the aromatic rings.

2. Purine Derivatives

With one exception the photochemistry of purines has been investigated largely from a qualitative viewpoint, thus frequently rendering difficult a comparison of results from different laboratories. The commonly applied criterion has been the loss of characteristic absorption between 220 and 300 mμ; e.g., overnight irradiation with a germicidal lamp of 0.5×10^{-4} *M* solutions of various purines suffices in all cases completely to disrupt the absorption spectrum.[117]

It has been reported by Christensen and Giese[118] that prolonged irradiation of adenine (but not guanine or guanylic acid) leads to an increase in absorption over the range 230–290 mμ. An analogous increase (about 7%)

[109] T. C. Laurent, *J. Am. Chem. Soc.* **78**, 1875 (1956).
[110] F. Petuely and N. Meixner, *Chem. Ber.* **86**, 1255 (1953).
[111] W. G. Berl and C. E. Feazel, *J. Agr. Food Chem.* **2**, 37 (1954).
[112] P. Holtz and J. P. Becker, *Arch. exptl. Pathol. u. Pharmakol.* **182**, 160 (1936).
[113] C. T. Bothner-By and E. A. Balazs, *Radiation Research* **6**, 302 (1957).
[114] M. A. Khenokh, *Doklady Akad. Nauk S.S.S.R.* **104**, 746 (1955).
[115] G. O. Phillips, G. J. Moody, and G. L. Mattock, *J. Chem. Soc.* pp. 3522, 3534 (1958); G. O. Phillips and G. J. Moody, *ibid.* pp. 754, 762 (1960).
[116] D. Shugar and K. L. Wierzchowski, *Biochim. et Biophys. Acta* **23**, 657 (1957).
[117] D. Rapport and A. Canzanelli, *Science* **112**, 469 (1950).
[118] E. Christensen and A. C. Giese, *Arch. Biochem. Biophys.* **51**, 208 (1954).

in absorption at wavelengths above 250 mμ during irradiation of adenine is shown in Table I of a paper by Canzanelli *et al.*,[36] but no significance was apparently attached to this. It is claimed by Garay and Guba[119] that irradiated ATP exhibits an enhanced absorption and that, with time, the spectrum reverts to that of ATP; but no data are presented to substantiate this.

One fact of some importance in relation to the photochemistry of polynucleotides, and upon which all observers are in agreement, is that purine derivatives (particularly of adenine and guanine) are considerably more resistant to irradiation than pyrimidines.[108, 118, 120] Irradiation of a DNA solution to the point where 30% of the pyrimidine bases are destroyed apparently leaves the guanine residues intact while more than 90% of the adenine can be recovered from a hydrolyzate of the photoproduct.[121]

For purposes of comparison with pyrimidine derivatives in studies on the photochemistry of nucleic acids, rough measurements have been made of the quantum yields for adenine and guanine at 253.7 mμ, based on the loss in absorption of the characteristic maxima. The values obtained were 0.6×10^{-4} for adenine and 2×10^{-4} for guanine.[39]

The conclusion of Loofbourow and Stimson,[122] that the lability of purines and pyrimidines to irradiation parallels the number of carbonyl groups in the ring, is perhaps too broad a generalization although it does appear to apply roughly to purines since the stability of these is (in decreasing order) adenine, hypoxanthine, guanine, xanthine, and uric acid. The presence of a carbonyl group in position 2 is probably of greater significance.[36]

The only attempt to examine the kinetics of photodecomposition of purines is that of Kland and Johnson.[123] Irradiation was under nitrogen or oxygen, using a bank of germicidal lamps, and loss of absorption was the criterion for destruction of the purine ring. With the exception of adenine under nitrogen, there is an initial "induction" period; while at high irradiation intensities the rate is proportional to the square of the exposure time, adenine excluded. The kinetic data made possible a division of the five purines examined into two classes: those substituted in position 6, adenine and hypoxanthine, which decompose more slowly under nitrogen; and disubstituted purines (guanine and xanthine) and uric acid, which are less sensitive under oxygen.

The considerably greater sensitivity of adenine (Fig. 9) under aerobic conditions was regarded as supporting evidence for oxidative breakdown at the amino groups

$$RNH_2 \xrightarrow[[O]]{h\nu} [R\cdot NH{:}OH] \xrightarrow[[H_2O]]{h\nu} R\cdot OH$$

[119] K. Garay and F. Guba, *Acta Physiol. Acad. Sci. Hung.* **5**, 393 (1954).
[120] R. L. Sinsheimer and R. Hastings, *Science* **110**, 525 (1949).
[121] M. Errera, *Biochim. et Biophys. Acta* **8**, 30, 115 (1952).
[122] J. R. Loofbourow and M. M. Stimson, *J. Chem. Soc.* p. 844 (1940).
[123] M. J. Kland and L. A. Johnson, *J. Am. Chem. Soc.* **79**, 6187 (1957).

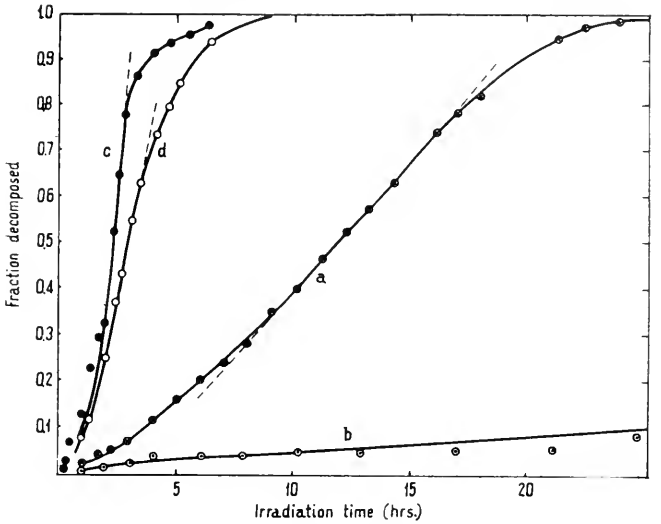


FIG. 9. Relative rates of photodecomposition of $0.8 \times 10^{-4} M$ aqueous, unbuffered solutions of adenine (*a*) under oxygen, (*b*) under nitrogen; and of hypoxanthine (*c*) under oxygen and (*d*) under nitrogen [M. J. Kland and L. A. Johnson, *J. Am. Chem. Soc* **79**, 6187 (1957)].

and hypoxanthine was actually isolated chromatographically from the reaction mixture. On the other hand hypoxanthine was also obtained from adenine irradiated under nitrogen; furthermore the quantities of hypoxanthine actually isolated were extremely small, although this could have been due to the much greater radiation sensitivity of this compound.

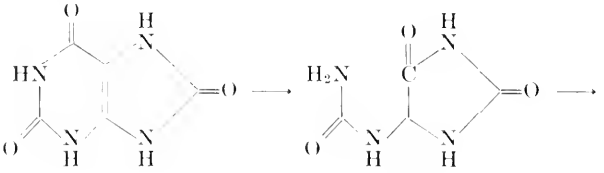
It should also be noted that all the above derivatives were irradiated in unbuffered solutions, the initial pH values of which varied from 5.35 for uric acid to 5.94 for adenine. Since these two compounds possess pK values at 5.4 and 4.15, respectively, a mixture of ionic species with different absorption spectra was present in each case (see Volume I, Chapters 3, 13, 14). Furthermore, the liberation of ammonia during decomposition[36] (see below) undoubtedly led to important pH changes during the course of the reaction.

The decomposition products of irradiated purine derivatives include both urea and ammonia and this question has been extensively investigated for purines and pyrimidines, as well as their nucleosides and nucleotides, by Canzanelli *et al.*[36] who found that liberated urea is derived almost exclusively from the pyrimidine ureide group, and is quantitatively greatest for those compounds with a keto group in position 2, as might be expected. The pyrimidine ureide group is also the source of most of the ammonia.

A significant observation is that the greatest formation of urea is associated with the least yield of ammonia, and conversely, from which it may

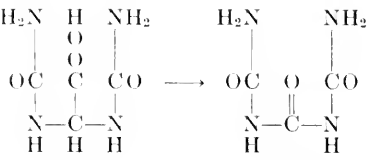
be concluded that the production of each of these involves a different decomposition path. However, since measurements were made only after complete disruption of absorption spectra, no conclusions can be drawn regarding intermediate decomposition products.

Irradiation of uric acid (I) at 253.7 mμ, followed by concentration of the solution, resulted in the isolation in about 10% yield (with respect to the



(I)
Uric acid

(II)
Allantoin



(III)
Allantoic acid

(IV)
Triuret

original substance) of crystalline triuret (IV)[124] and it has been proposed that the reaction may proceed via formation of allantoin (II) and allantoic acid (III), followed by decarboxylation and oxidation.

The isolation of parabanic acid from irradiated uracil[125] (see Section V, 4, *a*) suggests other conceivable pathways for the above reaction. Cyanuric acid was also detected in small amounts in irradiated uric acid.[124]

3. Purine Nucleotides and Nucleotide Coenzymes

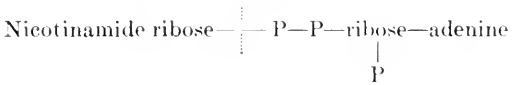
Nucleosides and mononucleotides of adenine and guanine are, like the parent purines, remarkably resistant to irradiation[36, 118] but, under analogous conditions, ATP undergoes considerable degradation to adenine while the photoproducts of diphosphopyridine nucleotide (DPN) include adenine and small amounts of inorganic phosphorus (P), the absence of adenosine indicating that the glycosidic linkage in both adenosine diphosphate (ADP) and ATP is labilized by the pyrophosphate group.[108] The spectral changes resulting from irradiation of DPN are accounted for almost entirely by degradation of the nicotinamide ring.[108, 126]

Irradiation of DPN to the point where its coenzyme activity is completely

[124] J. Fellig, *Science* **119,** 129 (1953).
[125] W. E. Conrad, *Radiation Research* **1,** 523 (1954).
[126] M. W. Seraydarian, A. I. Cohen, and H. Z. Sable, *Am. J. Physiol.* **177,** 150 (1954).

destroyed gives four main degradation products: ADP, adenosine monophosphate (AMP), adenine and nicotinamide, but no adenosine[126, 127] (see above); while about 50% of the total ribose, practically all of it from the nicotinamide moiety, no longer reacts to orcinol.[126]

The behavior of triphosphopyridine nucleotide (TPN) is analogous to that of DPN, with the exception that only about 30% of the ribose (again mainly from the nicotinamide moiety) is destroyed following total loss of coenzyme activity. However, in this case the splitting of the ribose phosphate ester bond of the nicotinamide nucleotide moiety liberates, instead of ADP, a compound similar to the latter but with an additional phosphate



group at position 2′ (or 3′) of adenosine. The identification of this product on the basis of enzymic tests was regarded only as tentative.[128] It is, however, fully in agreement with the structure of TPN proposed by a number of observers since 1950 (see Volume I, Chapter 4) and may, in fact, be regarded as additional supporting evidence for such a structure.

In contrast to the above Shigemoto[129] reports the products of photochemical degradation of DPN at 260 mμ to include nicotinamide-ribose-5′-phosphate, AMP, and adenosine while for ATP the corresponding products are AMP and adenosine. In the absence of the original publication it is not possible to comment on this discrepancy.

In line with what has been pointed out regarding the transparency of carbohydrates at 253.7 mμ (Section V, 1), the destruction of the nicotinamide-bound ribose must be due either to energy transfer or to strain rupture of the ribose ring as a result of interaction between the nicotinamide and adenine rings. That such interaction does indeed exist has been shown by Weber[130] who found that DPNH exhibits a hyperchromicity of about 22% at 260 mμ, in line with an earlier observation of Whitby[131] on flavin adenine nucleotide which was found to exhibit an extinction coefficient 30% less than that of its components. It was also demonstrated by Weber[130] that energy absorbed by the adenine portion of DPNH can appear as fluorescence in the nicotinamide ring with a 30% transfer efficiency.

The resistance of the adenine-bound ribose to destruction is in accord with the fact that considerably higher doses are necessary for destruction of ribose in nucleic acid chains (Section VI, 1).

[127] B. Ekert and R. Monier, *Bull. soc. chim. biol.* **40**, 793 (1958).
[128] M. W. Seraydarian, *Am. J. Physiol.* **181**, 291 (1955).
[129] T. Shigemoto, *Ôsaka Daigaku Igaku Zassi* **10**, 513 (1958); *Chem. Abstr.* **52**, 13832i (1958).
[130] G. Weber, *J. chim. phys.* **55**, 878 (1958).
[131] L. G. Whitby, *Biochem. J.* **54**, 437 (1953).

Seraydarian[132] subsequently showed that complete inactivation of DPN and TPN coenzyme activities could be achieved using only wavelengths to the red of 210 mμ. Actually the influence of wavelength on the photochemical destruction of coenzyme activity had been investigated quantitatively many years earlier by Warburg and Christian,[133] who found what they considered the "apparent" quantum yield to increase with decreasing wavelength so that at 186 mμ it was almost 120 times that at 253.7 mμ. Assuming that ϕ was independent of λ, and that no indirect inactivation resulted from irradiated solvent molecules, they concluded that only a portion of the coenzyme molecule was affected by irradiation; an inference that is surprisingly close to the truth (see above), notwithstanding the objections that could be made to the assumptions on which it is based.

Irradiation of DPNH in the region of its absorption band at 340 mμ is without effect on its coenzyme activity.[134]

4. Pyrimidines and Pyrimidine Nucleosides and Nucleotides

Up to about 5 years ago it was generally accepted that irradiation of pyrimidine derivatives led to irreversible degradation of the aromatic ring; as a result of this, some of the earlier studies are now only of historical interest. The intense flurry of excitement aroused by Kelner's report of photoreactivation in microorganisms in 1949 somehow obscured the discovery in the same year by Sinsheimer and Hastings[120] that the photolysis of uracil, uridine, and cytidylic acid could be reversed in the "dark" by heating or acidification, the criterion for reversal being the reappearance of the original absorption spectra. It was not until Sinsheimer[135] took up this question again five years later that its significance began to be appreciated in connection with the photochemistry of nucleic acids and its bearing on the phenomenon of photoreactivation. Today the first question to resolve in studying the photochemistry of a pyrimidine derivative is whether the reaction leads to the reversible or irreversible formation of a new derivative, or to irreversible degradation.

The first quantitative study of the photolytic degradation of a pyrimidine is that of Uber and Verbrugge[136] who found that the pyrimidine component of thiamine, 2-methyl-5-ethoxymethyl-6-aminopyrimidine, is destroyed by irradiation at 253.7 mμ with a quantum yield of 1.84×10^{-2}, at pH 4.8. Actually the absorption spectra shown by the authors indicate that the compound possesses a pK of about 6.2 so that the amino group was fully protonated. The conclusion that one-third of the amino nitrogen is split off during the reaction to form ammonia is, however, not in accord with

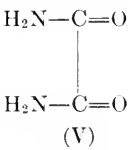
[132] M. W. Seraydarian, *Biochim. et Biophys. Acta* **19,** 168 (1956).
[133] O. Warburg and W. Christian, *Biochem. Z.* **282,** 221 (1935).
[134] D. Shugar, *Experientia* **7,** 1 (1951); *Biochim. et Biophys. Acta* **6,** 548 (1951).
[135] R. L. Sinsheimer, *Radiation Research* **1,** 505 (1954).
[136] F. M. Uber and F. Verbrugge, *J. Biol. Chem.* **134,** 273 (1940).

the reported changes in absorption spectrum; furthermore, ammonia frequently appears as a photolysis product of the pyrimidine ring (see below) as in the case of the purines[36] (Section V, 2).

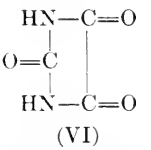
From a comparison of the relative resistance to irradiation of 2-chloro-6-aminopyrimidine, as compared to uracil[137] and barbituric acid,[122] Stimson and Loofbourow[138] concluded that susceptibility to photolysis parallels the number of carbonyl groups in the ring. However, as in the case of purines, it is rather the presence of a carbonyl group in position 2 which is of greater importance.[36] Even this latter generalization is limited in its application if we compare the quantum yield of 1.84×10^{-2} for 2-methyl-5-ethoxymethyl-6-aminopyrimidine, mentioned above, with that for some uracil derivatives (see Table II).

Urea and ammonia are among the products of extensive degradation of pyrimidine derivatives at 253.7 mμ, both originating in the ureido group; e.g., cytosine does not give a greater yield of ammonia than uracil.[36] These results, however, give no information as to the degradation mechanism involved.

Prolonged irradiation of uracil at 253.7 mμ, followed by concentration of the irradiated solution led to the isolation of four unidentifiable products



(V)
Oxamide



(VI)
Parabanic acid

in relatively pure form, as well as crystalline oxamide (V) and parabanic acid (VI).[125] It is clear that neither of these two latter products could result from the rupture of a single bond in the uracil ring, while the existence of four additional products (plus others not isolated) indicates the existence of a number of reactions. We shall return to this question below.

a. Reversible Photolysis of Uracil Analogs

A turning point in our understanding of the photochemistry of pyrimidine nucleotides was the publication of Sinsheimer's[135] investigation on uridylic acid in 1954. Using as source a resonance lamp, from which wavelengths below 210 mμ were filtered out, it was shown that irradiation of uridylic acid results in the disappearance of the characteristic absorption spectrum; at this point removal of the light source followed by acidification of the solution results in the reappearance of the original spectrum with an efficiency of 90–100%. In place of acidification, the same result could be achieved by heating at neutral pH. The photolytic reaction was reported to be first-order with a quantum yield of 0.0216 mole/einstein for both uridine-2′-phosphate and uridine-3′-phosphate; while the reverse reaction was

[137] F. F. Heyroth and J. R. Loofbourow, *J. Am. Chem. Soc.* **53**, 3441 (1931).
[138] M. M. Stimson and J. R. Loofbourow, *J. Am. Chem. Soc.* **63**, 1827 (1941).

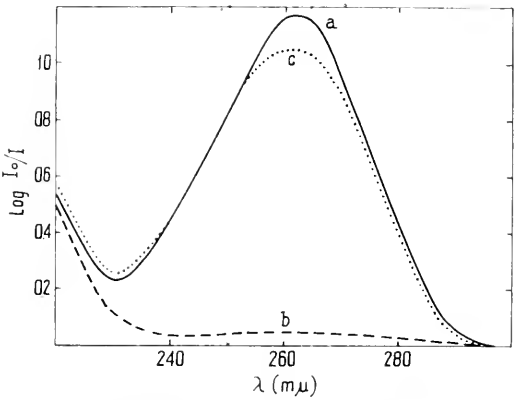


FIG. 10. Reversible photolysis of uridine-3′-phosphate at 253.7 mμ: (*a*) before irradiation, (*b*) after 40 minutes irradiation, (*c*) irradiated solution acidified to pH 0.8 and left 42 hours at room temperature [from R. L. Sinsheimer, *Radiation Research* **1**, 505 (1954)].

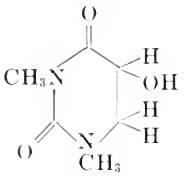
likewise first-order but more rapid for the 3′ isomer. The behavior of uridine was qualitatively similar to that of Up. Additional evidence for reversion of the photoproduct to the original compound is furnished by the observation[139] that irradiated uridine could not satisfy the growth requirements of a pyrimidine-requiring mutant of *Neurospora crassa*, but could after heating at neutral pH, or acidification or slight alkalization at room temperature.

Uracil was found to undergo photodecomposition with a quantum yield of 0.052, with the formation of several products, only 50% of which reverts to uracil on acidification or heating. Rörsch *et al.*[51] have provided additional spectral evidence for the existence of several reactions on irradiation of uracil, of which only one is reversible.

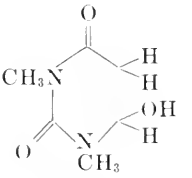
A qualitatively similar behavior is exhibited by 3-methyluracil[140, 141] and 1,3-dimethyluracil.[59, 142-145] Moore and Thomson's[59] choice of dimethyluracil for elucidation of the mechanism of the photochemical reaction was a fortunate one in that its photoproduct exhibits 100% reversibility while its physicochemical properties made it particularly suitable for the isolation of the photoproduct by fractionation and chromatography.

[139] D. Rapport, A. Canzanelli, and R. Sossen, *Federation Proc.* **14**, 386 (1955).
[140] K. Wierzchowski and D. Shugar, *Ann. Congr. Chem. Ges. D.D.R., Leipzig, 1956*, p. 85 (1957).
[141] K. L. Wierzchowski and D. Shugar, *Biochim. et Biophys. Acta* **25**, 355 (1957).
[142] A. M. Moore and C. H. Thomson, *4th Intern. Conf. Radiobiol., Cambridge, 1955* p. 75, (1956).
[143] A. M. Moore and C. H. Thomson, *Can. J. Chem.* **35**, 163 (1957).
[144] S. Y. Wang, M. Apicella, and B. R. Stone, *J. Am. Chem. Soc.* **78**, 4180 (1956).
[145] S. Y. Wang, *J. Am. Chem. Soc.* **80**, 6196 (1958).

Elementary analysis of the photoproduct of 1,3-dimethyluracil, and its molecular weight of 158.3 as against 140 for dimethyluracil, indicated uptake of a water molecule during irradiation; this is further supported by the infrared spectrum of the photoproduct, which exhibits an intense band at 2.98 μ corresponding to —OH frequencies and absent in dimethyluracil. The disappearance of the characteristic absorption maximum, as for irradiated uridine-3′-phosphate (Fig. 10) and dihydrouracil (Fig. 5), plus the absence of the characteristic reaction of unsaturated pyrimidine rings with bromine water, suggested the 4,5 double bond as the point of attachment. It was therefore proposed that the photoproduct is either 5- or 4-hydroxy-1,3-dimethylhydrouracil (VII) or (VIII). Since the properties of the known



(VII)
5-Hydroxy-1,3-dimethylhydrouracil



(VIII)
4-Hydroxy-1,3-dimethylhydrouracil

compound (VII) did not correspond with those of the photoproduct, it was concluded that the latter must be (VIII). By analogy the corresponding reversible photoproduct for uracil should be 4-hydroxyhydrouracil. Both of these deductions were subsequently confirmed by direct chemical syntheses of (VIII) and of 4-hydroxyhydrouracil,[143-146] which exhibited the properties of the photoproducts of dimethyluracil and uracil, respectively, and could be made to revert to the latter by elimination of a water molecule.

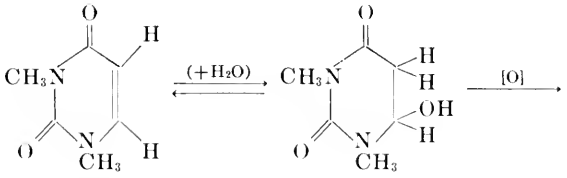
It follows that the photoproducts of uridine and uridylic acid are the corresponding 4-hydroxyhydro derivatives. Analogous reversible photoproducts are formed from isopropylideneuridine and uridine-2′,3′-phosphate[39, 68, 69] as well as uridine diphosphate (UDP), uridine triphosphate (UTP), and UDPG.[101] However, the position of esterification of the carbohydrate moiety markedly affects the stability of the photoproduct, i.e., the rate of reversion to the original compound. The maximum stability of the photoproduct of Up is at pH 5.2[135] or roughly the same as for dimethyluracil.[142] Quantum yields for photoproduct formation, based on the loss of spectral absorption, are shown for a number of derivatives in Table II.

Prolonged irradiation results in gradual destruction of the primary reversible photoproducts, e.g., extensive irradiation of uridine leads to the appearance of uracil as well as other products.[139] A number of colored, unidentified degradation products are formed from extensively irradiated

[146] A. M. Moore, *Can. J. Chem.* **36**, 281 (1958).

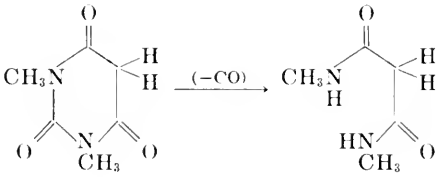
dimethyluracil; one of these is also found following prolonged irradiation of uracil and Up.[142]

Wang has shown[147] that irradiation of the reversible photoproduct of dimethyluracil, 4-hydroxy-1,3-dimethylhydrouracil (VIII), leads to the formation of *N*,*N*′-dimethylmalonamide (XI) via dimethylbarbituric acid (X), so that the reaction sequence starting from dimethyluracil (IX) would be as follows:



(IX) Dimethyluracil

(VIII) 4-Hydroxy-1,3-dimethylhydrouracil

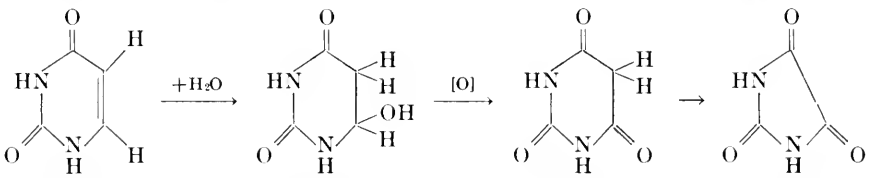


(X) Dimethylbarbituric acid

(XI) *N*,*N*′-Dimethylmalonamide

At starting concentrations of (VIII) higher than 10^{-4} *M*, products other than (XI) were also formed, a not unexpected result (see below).

The demonstration of (X) as the photoproduct of (VIII) enables us to formulate the reaction sequence leading to the formation of parabanic acid (VI) from uracil (XII), mentioned above,[125] and which may be as follows:



(XII) Uracil

(XIII) 4-Hydroxyhydrouracil

(XIV) Barbituric acid

(VI) Parabanic acid

The nature of the final reaction step requires clarification. In addition oxamide (V) is probably formed from (VI) by decarbonylation as in the formation of (XI) from (X) above.

[147] S. Y. Wang, *J. Am. Chem. Soc.* **80**, 6199 (1958).

TABLE II

QUANTUM YIELDS FOR PHOTOLYSIS AT 253.7 mμ OF SOME NUCLEIC ACID CONSTITUENTS AND RELATED COMPOUNDS AT NEUTRAL pH AND ~10^{-4} *M*

Compound	$\phi \times 10^3$	Reversibility	References
Uracil	5.5	Incomplete[i]	*a*, *c*
3-Methyluracil	12.5	High	*c*
1,3-Dimethyluracil	10.4[g]	100%	*b*
Uridine	21.6	90–100%	*a*, *c*
Uridine-2′-phosphate	21.6[h]	90–100%	*a*
Uridine-3′-phosphate	21.6	90–100%	*a*
Uridine-2′,3′-phosphate	21.6	90–100%	*c*
Thymine	0.4	0	*c*
Thymidine	0.65	0	*d*
Thymidylic acid	1.0	0	*d*
Uridine-2′(3′),5′-diphosphate	~20	>90%	*c*
UDP, UTP, UDPG	—	High	*e*
4-Methyluracil	0.8	Very slight[b]	*d*
1,3-Dimethylbarbituric acid	4.6	0[f]	*d*
2-Ethoxyuracil	8.0	0	*d*
2-Thiouracil	1.7	0	*d*

[a] R. L. Sinsheimer *Radiation Research* **1**, 505 (1954).

[b] A. M. Moore and C. H. Thomson, *4th Intern. Conf. Radiobiol., Cambridge, 1955*, p. 75 (1956).

[c] K. L. Wierzchowski and D. Shugar, *Acta Biochim. Polon.* **6**, 313 (1959); *Ann. Congr. Chem. Ges. D.D.R., Leipzig, 1956* p. 85 (1957); *Biochim. et Biophys. Acta* **25**, 355 (1957).

[d] D. Shugar and K. L. Wierzchowski, *Postepy Biochem.* **4**, Suppl., p. 243 (1958).

[e] L. P. Zill, *Federation Proc.* **16**, 276 (1957).

[f] See also S. Y. Wang, *J. Am. Chem. Soc.* **80**, 6199 (1958).

[g] For 10^{-2} *M* concentration; see K. L. Wierzchowski and D. Shugar, *Acta Biochim. Polon.* **6**, 313 (1959), for influence of concentration.

[h] Moore and Thomson[b] report $\phi = 19 \times 10^{-3}$ at 10^{-3} *M* and 37×10^{-3} at 10^{-2} *M* for uridine-2′(3′)-phosphate; see K. L. Wierzchowski and D. Shugar, *Acta Biochim. Polon* **6**, 313 (1959), for more extensive data.

[i] See A. Rörsch, R. Beukers, J. Ijlstra, and W. Berends, *Rec. trav. chim.* **77**, 423 (1958).

b. Reversible Photolysis of Cytosine Analogs

Irradiation at 253.7 mμ of a variety of cytosine derivatives, in particular nucleosides and nucleotides[39, 116, 120, 140-142, 148, 149] as well as some other 6-amino derivatives,[39, 142] also results in the formation of photoproducts which revert to the original products to a greater or lesser degree in the "dark" either by heating or acidification.

For cytosine and 3-methylcytosine in aqueous unbuffered solution, photodecomposition proceeds much more slowly than for uracil but with a similar decrease in absorption; following irradiation, heating restores to a considerable extent the original spectrum[116] (Fig. 11). If, however, irradiation is more prolonged, the degree of reversibility is considerably reduced.[141]

[148] R. L. Sinsheimer, *Radiation Research* **6**, 121 (1957).

[149] D. Shugar and K. L. Wierzchowski, *Intern. Symposium Macromolecules, Prague, 1957*, Paper No. 123; *J. Polymer Sci.* **31**, 269 (1958).

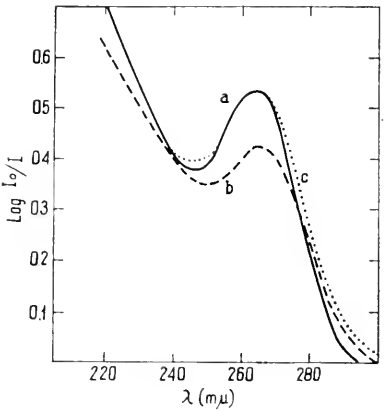


FIG. 11.

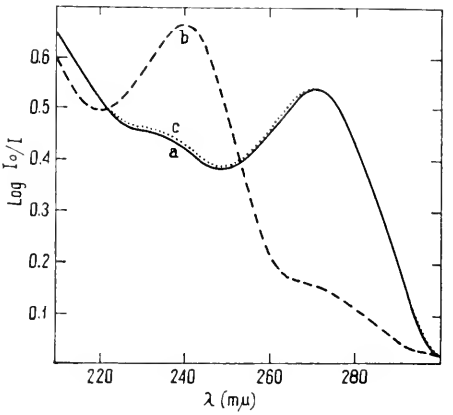


FIG. 12.

FIG. 11. Absorption spectrum of cytosine at neutral pH, unbuffered: (*a*) before irradiation, (*b*) after 85 minutes irradiation, (*c*) irradiated solution heated 10 minutes at 80° [from D. Shugar and K. L. Wierzchowski, *Biochim. et Biophys. Acta* **23**, 657 (1957)].

FIG. 12. Reversible photolysis of cytidine or cytidylic acid at neutral pH: (*a*) before irradiation, (*b*) after irradiation, (*c*) irradiated solution after 16 hours at room temperature or 5 minutes at 80° [from R. L. Sinsheimer, *Radiation Research* **6**, 121 (1957), and K. L. Wierzchowski and D. Shugar, *Biochim. et Biophys. Acta* **25**, 355 (1957)].

In acid medium the effects of irradiation are not reversible, while in alkaline medium a new unknown pyrimidine derivative is formed which is relatively unstable.

For nucleosides (and nucleotides) the disappearance of the maximum at about 270 mμ is accompanied by the simultaneous appearance of a new maximum at about 236 mμ.[116, 140, 141, 148] If irradiation is not unduly prolonged following completion of the reaction, acidification or heating results in 100% reversion to the original compound (Fig. 12).

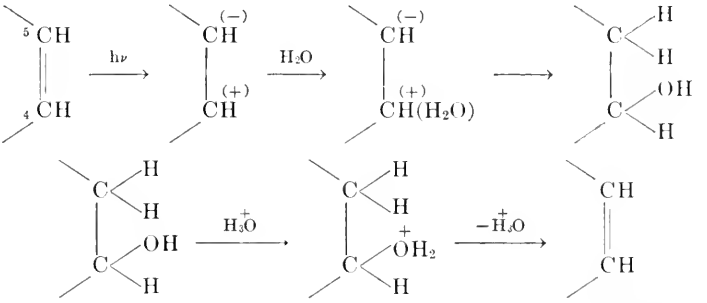
Isolation of the photoproducts of cytosine nucleosides and nucleotides is more difficult than for the corresponding uracil analogs because of the lower stability of the former, some of which revert spontaneously to the parent compound at room temperature.

The reverse reaction is first-order for Cp[148] as well as for all cytidylic acid isomers,[149] and is more rapid than for uridine nucleotides.[39, 116] At neutral pH and 30°C the half-times for the reverse reaction for the 2′ and 3′ isomers of Cp are, respectively, 22 and 96 minutes; at pH 4 these values are decreased to about one-eighth and at pH 13 they are too short to measure.[148]

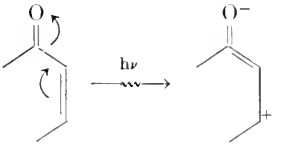
By analogy with the uracil analogs the mechanism may be assumed to involve uptake of a water molecule at the 4,5 double bond. If such were the case the quantum yield for photodecomposition should be lower in

heavy water because of the higher activation energy required, while the reverse reaction should be more rapid since D_2O is less basic than H_2O, as a result of which the photoproduct will more effectively compete for the deuteron in D_2O than for the proton in H_2O; hence the concentration of the conjugate acid, and the rate of the elimination reaction, will be greater.[150]

For 3-methyluracil, cytosine, and cytidine the quantum yields in D_2O were indeed found to be half those in H_2O and the reverse reactions 2–3 times faster in D_2O in support of the above argument[116]; so that the reaction mechanism may be represented as follows:



An essentially similar mechanism has been proposed for the reverse reaction by Moore and Thomson.[143] Wang[145] suggests that the primary process may involve an ionic intermediate



It would undoubtedly be profitable to examine a number of uracil and cytosine derivatives by flash photolysis (Section II, 3). Finally, it should be noted that there is no direct chemical evidence to indicate, as in the case of uracil derivatives, whether the water hydroxyl is taken up at position 4 or 5.

If we compare the absorption spectrum of the photoproduct of cytidine (Fig. 12) with that of dihydrodeoxycytidine (Fig. 6), it will be seen that good correspondence prevails between the two. A similar comparison of the photoproduct of cytosine (Fig. 11) with that of dihydrocytosine (which is similar to that of dihydrodeoxycytidine, Fig. 6) shows that the spectrum of the former cannot be explained on the assumption of simple uptake of a water molecule, and this question remains an open one, which is rendered

[150] K. B. Wiberg, *Chem. Revs.* **55**, 713 (1955).

even more puzzling by the observation that there is no evidence of reversibility following irradiation in *buffered* medium.[39, 141]

The differences in behavior between cytosine and 3-methylcytosine on the one hand, and nucleosides and nucleotides of cytosine on the other are indeed striking. The quantum yields for the latter are approximately one order of magnitude greater at neutral pH, while the degree of reversibility they exhibit varies from 90–100% after complete photodecomposition. For cytosine the rate of the reverse reaction is considerably lower, while the degree of reversibility decreases with increasing formation of the photoproduct.

Since the behavior of 3-methylcytosine is similar to that of cytosine, it follows that the difference in behavior between cytosine glycosides and 3-methylcytosine is not due to substitution on the N_3 ring nitrogen but must result from some interaction between the pyrimidine and carbohydrate rings; and this is clearly placed in evidence by an examination of the rate of the forward reaction for the various cytidylic acid isomers, as well as for the individual isomers in the alkaline pH range (Table III) where dissociation of the carbohydrate hydroxyls is revealed by modifications in spectra.[66] On the basis of these results it has been postulated that the increased facility of reversible photodecomposition of cytosine nucleosides, as compared to 3-methylcytosine, is due to intramolecular hydrogen bonding between the 2-carbonyl of the aromatic ring and one of the sugar hydroxyls[39, 140, 141, 149]; that this is stereochemically possible is shown by the synthesis of *cyclo*-nucleosides.[151, 152] The photochemical data appear to indicate involvement largely of the 5′-hydroxyl, but not necessarily to the exclusion of the others[39, 149]; the 2′-hydroxyl has been suggested as that principally involved, on the basis of spectral evidence.[153]

c. 4- and 5-Substituted Derivatives

There are a number of pyrimidine derivatives which do not form reversible photoproducts,[39, 140-142, 148] such as orotic acid, thymine and its nucleosides and nucleotides, barbituric acid (see above) and methylated barbituric acids, 2-thiouracil, 2,4-diethoxypyrimidine, etc. On the other hand there are compounds such as 2-ethoxyuracil which on irradiation give rise to relatively stable, new pyrimidine derivatives which do not revert to the parent compound; and 2-methoxycytosine, the photoproduct of which exhibits an absorption spectrum incompatible with the existence of a 4,5 saturated bond, but which largely reverts to the original compound on

[151] A. M. Michelson and A. R. Todd, *J. Chem. Soc.* p. 816 (1955).
[152] A. M. Michelson, *Tetrahedron* **2**, 333 (1958).
[153] J. J. Fox, J. F. Codington, N. Yung, L. Kaplan, and J. O. Lampen, *J. Am. Chem. Soc.* **80**, 5155 (1958).

TABLE III

QUANTUM YIELDS (MOLES/EINSTEIN × 10^3) FOR PHOTOLYSIS OF CYTOSINE DERIVATIVES, CONCENTRATION ~10^{-4} *M*, AT 253.7 mμ AND AT VARIOUS pH VALUES[a]

Compound	pH								
	1	2	5.6[b]	7.1	8.9	10.5	11	13	14
Cytosine	—	1.3	1.7[c]	1.3[b, c]	—	—	—	6.3	8.0
1-Methylcytosine	—	1.4	2.1[c]	1.3[b, c]	—	—	—	2.1	—
Cytidine	—	1.6[c]	9.0[c]	10.0[c]	—	8.9[c]	6.8[c]	3.6	2.9
Deoxycytidine	—	1.6[c]	9.5[c]	8.6[c, f]	—	8.3[c]	6.0[c]	0.5	—
Pyranosylcytosines	1.6[c]	—	16.0[c]	13.0[c]	15.0[c]	11.0[c]	7.9[c]	1–2[d]	0.25–2.5[d]
Cytidine-2′-phosphate	—	—	—	14.1[c, e]	—	—	—	—	—
Cytidine-3′-phosphate	—	—	—	9.5[c, e]	—	—	—	—	—
Cytidine-2′(3′)-phosphate	1.1	—	—	12.5[c]	—	9.4[c]	6.3[c]	1.4	—
Cytidine-2′,3′-phosphate	—	—	—	11.5[c]	—	—	—	—	—
Cytidine-5′-phosphate	—	—	—	5.2[c]	—	—	—	—	—
Deoxycytidine-5′-phosphate	—	—	—	3.0[c]	—	—	—	—	—

[a] K. L. Wierzchowski and D. Shugar, *Biochim. et Biophys. Acta* **25**, 355 (1957), and D. Shugar and K. L. Wierzchowski, *Intern. Symposium Macromolecules, Prague, 1957*, Paper No. 123 (1957); *J. Polymer Sci.* **31**, 269 (1958). Values are "effective" quantum yields uncorrected for reverse reaction which is rapid at pH 11–12.

[b] Unbuffered solution; for influence of buffer see *a*, first ref.

[c] Reaction reversible in the dark; for cytosine and 1-methylcytosine only partial reversibility

[d] Quantum yield increases appreciably during course of reaction.

[e] R. L. Sinsheimer [*Radiation Research* **6**, 121 (1957)] reports a quantum yield of 17 × 10^{-3} for cytidine-2′-phosphate and cytidine-3′-phosphate.

[f] A. M. Moore and C. H. Thomson [*Can. J. Chem.* **35**, 163 (1957)] report a quantum yield of 7 × 10^{-3} at a concentration of 10^{-2} *M*.

heating.[39] Work in progress in our laboratory shows that the number of pyrimidine derivatives giving rise to stable photoproducts containing the pyrimidine ring is fairly extensive and should be of interest not only to the physical chemist and biologist, but also to the synthetic organic chemist.

Of special interest are those derivatives substituted in positions 4 or 5 such as thymine, 5-methylcytosine, etc. A number of observers have assumed that such compounds do not give rise to reversible photoproducts although Moore and Thomson[142] reported "slight" reversibility for 4-methyluracil. This question is of paramount importance in relation to the photochemistry of nucleic acids and, particularly, photoreactivation (Section X).

From an investigation of a wide variety of substituted pyrimidines it was concluded that the ability to take up a water molecule reversibly at the 4,5 double bond is dependent on the electron distribution in the pyrimidine ring and is not necessarily attributable to steric hindrance resulting from substitution of one of the positions at 4 or 5.[39] Investigations in progress on this and related problems have now shown that, although no re-

versibility can be observed for 4-methyluracil as reported,[142] a very high degree of reversibility prevails for 1,3,4-trimethyluracil; experiments with 5-hydroxymethyluracil have been negative, but 5-hydroxymethylcytosine gives some indication of formation of reversible photoproducts.

An extremely interesting observation in this connection has been made by Wang,[154] who found that the *final* product of irradiation of 1,3-dimethylthymine is *N*,*N'*-dimethylmethylmalonamide; by analogy with the situation for dimethyluracil (Section V, 4, *a*) the most logical explanation of this is the initial uptake of a water molecule at the 4,5 double bond; the inability to observe this intermediate is due to the greater ease with which it undergoes dehydration as compared to uracil analogs. The possibility is envisaged that in nucleic acid chains the hydroxyhydro intermediate of thymine may be more stable than in the free state. It remains only to add that such a possibility can readily be tested by studying the photochemical behavior of a synthetic polynucleotide containing only thymine, using a procedure similar to that for oligo- and poly-U[69] (Section VII, 1, *b*).

From all that has been said above, it is clear that the behavior of a given derivative is difficult to predict and must be determined experimentally. The significance of the substitution of position 5 in relationship to the photochemistry of the T-even phages will be pointed out in Section X.

d. Kinetics, Concentration, and Solvent Effects

Most of the observations reported in the previous sections were obtained from studies on dilute solutions (about 10^{-4} *M*) transmitting some of the incident energy; under these conditions, the decrease in concentration resulting from irradiation will be exponential, i.e., the reaction will be first-order (assuming that adequate correction is made for any absorption by the photoproduct). If the optical density of the solution is so high that all the incident energy is absorbed for an appreciable part of the reaction, then the reaction will be zero-order. The quantum yield should also be constant for both cases.

Moore and Thomson[142] actually found that ϕ for Up was 0.019 at 10^{-3} *M* and 0.037 at 10^{-2} *M*. A detailed examination of the kinetics of photolysis of a variety of uracil derivatives, conducted in relation to the photochemistry of synthetic oligonucleotides, has now shown that the kinetic behavior of concentrated solutions is not as simple as appears from the behavior of dilute solutions, and that even the rate of the reverse reaction may be markedly affected.[69] For a compound such as 2-methoxycytosine even the nature of the photoproduct is altered in concentrated solution.[39]

Also of considerable interest, mainly to the physical chemist and synthetic organic

[154] S. Y. Wang, *Nature* **184,** B.A. 59 (1959).

chemist, is the behavior of a number of derivatives in nonaqueous solvents, leading to the formation of new, occasionally analogous, photoproducts.[39, 142]

Beukers *et al.*[155] have investigated the reversible photolysis of some uracil and cytosine derivatives in frozen solutions and found marked differences in sensitivity for some compounds as compared to that in aqueous solution. Their conclusion that the behavior in frozen solution more adequately represents that to be expected for the same compounds in nucleotide chains is open to question, although in no way detracting from the interest of their findings which are undoubtedly worth following up.

VI. Photochemistry of Nucleic Acids

1. Physicochemical Studies

Ultraviolet irradiation of DNA is accompanied by a decrease in viscosity,[156] which drops almost to zero[121] before changes in absorption spectrum take place involving a decrease in the height of the principal absorption band at 260 mμ. The decrease in viscosity proceeds with a quantum yield of the order of 10^{-6} while the initial decrease in absorption is due largely to the destruction of pyrimidine rings since irradiation to the point where 30% of the pyrimidines are destroyed leaves the purine rings practically intact.[121] No attempt appears to have been made to determine whether one of the pyrimidine components is more susceptible, although deoxycytidylic acid would be expected to disappear more rapidly than thymidylic acid because of the large difference in quantum yields for photolysis of the free nucleotides (Section V, 4).

Under the influence of high doses of radiation a small percentage of dialyzable products appears,[121] while under more extreme conditions rupture of internucleotide linkages may take place with the appearance of appreciable quantities of inorganic phosphate.[157] No attempts appear to have been made to isolate some of the products of degradation under these conditions.

Analogous high doses of radiation have been used to determine the extent to which DNA is susceptible to degradation under conditions resembling those *in vivo*, by irradiating rat thymocytes and then extracting the DNA.[158] The yield of polymerized DNA from irradiated thymocytes was only one-half that from a nonirradiated control homogenate, additional evidence for fragmentation being based on qualitative determinations of large amounts of thymine in the supernatant fluids from the ultraviolet treated thymocytes during the extraction procedure. It follows from this

[155] R. Beukers, J. Ijlstra, and W. Berends, *Rec. trav. chim.* **77**, 729 (1958).
[156] A. Hollaender, J. P. Greenstein, and W. V. Jenrette, *J. Natl. Cancer Inst.* **2**, 23 (1941).
[157] M. W. Seraydarian, A. Canzanelli, and D. Rapport, *Am. J. Physiol.* **172**, 42 (1953).
[158] S. J. deCourcy, J. O. Ely, and M. H. Ross, *Nature* **172**, 119 (1953).

that DNA is much more sensitive to degradation *in vivo*, a fact which, although perhaps not general, yet derives support from other observations.

Loofbourow *et al.*[159] have claimed, on the basis of optical observations, that ultraviolet-injured yeast cells exhibit an increased production of nucleic acid-like material, and have actually extracted twice as much crude RNA from irradiated yeast cells, as compared to nonirradiated controls. However, the extraction procedure used was not an efficient one, the maximum yield of crude RNA from nonirradiated cells being only 1%; furthermore, the extinction coefficient of the RNA from irradiated yeast was higher than that from nonirradiated, indicating that it was partially degraded (see Section IV, 4) and hence easier to extract.

Ultraviolet treated DNA also exhibits a decreased affinity for methyl green[160] which, while it does not necessarily indicate degradation of the nucleotide chains, does suggest a separation of the twin strands of the two-stranded Watson-Crick structure.[161] A similar loss in staining affinity for methyl green is exhibited by irradiated cell nuclei; rough calculations indicated that this process requires 50 times less energy than for isolated DNA.[162]

This surprising result may be linked to the state of DNA in cellular material. Possibly related to this are the studies of Setlow and Doyle[163] on the irradiation of dry DNA films, as a result of which the DNA forms a gel insoluble in aqueous solution with a quantum yield of about 10^{-2}, or about 4 orders of magnitude greater than that for loss in viscosity. The phenomenon has been examined in greater detail by Kaplan[164] in connection with the effect of humidity on the ultraviolet radiation sensitivity of microorganisms. The rate of gelation of DNA films was found to increase by a factor of 22 as the humidity was decreased from 97.5 to 33%. A quantitatively similar relationship prevailed for the s-mutation and killing rates of *Serratia marcescens* and it was suggested that a common mechanism accounts for all three processes, involving hydrogen-bond rupture in the DNA molecule.

Irradiation of RNA from TMV with fairly high doses results in complete destruction of viscosity and intrinsic viscosity, as well as a loss in ability to stain metachromatically with safranin, which is reasonable evidence for molecular degradation.[98]

The radiation sensitivity of DNA was found to be unaltered at low temperatures while that of proteins is decreased, and it has been sug-

[159] J. R. Loofbourow, *Growth* **12,** Suppl., p. 77 (1948).
[160] S. Devreux, M. Johannson, and M. Errera, *Bull. soc. chim. biol.* **33,** 800 (1951).
[161] H. S. Rosenkranz and A. Bendich, *J. Biophys. Biochem. Cytol.* **4,** 663 (1958).
[162] M. Errera, *Ann. soc. roy. sci. méd. et nat. Bruxelles* **5,** 65 (1952).
[163] R. Setlow and B. Doyle, *Biochim. et Biophys. Acta* **12,** 508 (1953); **15,** 117 (1954).
[164] R. W. Kaplan, *Naturwissenschaften* **42,** 184, 466 (1955).

gested that this may be useful in action spectra studies to distinguish between nucleic acid and protein effects.[165]

Since nucleic acids in living cells exist predominantly in the form of nucleoproteins, it would obviously be desirable to learn something about the effects of irradiation on isolated nucleoproteins. Some early observations[121] on DNA-histone in salt solutions of different concentration suggested little difference in behavior from that of free DNA. An investigation has also been made of the photochemical behavior of nucleoprotein from sea urchin sperm in salt solution, which gradually loses the ability to form long threads on transfer to water.[166] It is nonetheless difficult to draw any definite conclusions from these findings in the absence of some criterion as to the degree of dissociation of the nucleoproteins in the salt solutions in which they were irradiated (Section IV, 5; see also Volume I, Chapter 10).

More definite information on this subject is forthcoming from photochemical studies of TMV and infectious RNA from TMV, from which it appears that the protein portion of a nucleoprotein complex may profoundly affect the behavior of the nucleic acid fraction.

It appears to the writer that, apart from viruses, studies on the behavior of nucleoproteins are likely to be more informative in those instances where the nucleic acid portion exhibits biological activity; the best model for this purpose at the present time being bacterial nucleoproteins containing transforming DNA.

2. Biological Inactivation

Although an understanding of the chemical and physicochemical modifications resulting from irradiation of nucleic acids is of unquestionable value in attempts to interpret photobiological phenomena, it is nonetheless obvious that the usefulness of such information may be considerably enhanced if combined with quantitative data on biological inactivation. The possibility of such studies has, however, hitherto been limited by the fact that the known biological activities of free nucleic acids, susceptible to quantitative study, are relatively few.

a. Infectious RNA from TMV

The inactivation of infectious RNA from TMV is first-order[167-170] to a residual activity of about 1% which is highly resistant to further irradiation.[168] The reaction has been found to proceed with a quantum yield of about 3×10^{-4} assuming a molecular weight of 2.5×10^6. Particularly instructive is the fact that following complete inactivation there is no reduction in viscosity or intrinsic viscosity, but only a less than 1% drop in extinction of the maximum at 260 mμ which is about that to be ex-

[165] R. Setlow, *Advances in Biol. and Med. Phys.* **5**, 37 (1958).
[166] A. C. Giese, *Anat. Record* **99**, 116 (1947).
[167] A. Siegel, S. G. Wildman, and W. Ginoza, *Nature* **178**, 1117 (1956).
[168] A. D. McLaren and W. N. Takahashi, *Radiation Research* **6**, 537 (1957).
[169] A. Siegel and A. Norman, *Virology* **6**, 725 (1958).
[170] F. C. Bawden and A. Kleczkowski, *Nature* **183**, 503 (1959).

pected if it is assumed that only the uridylic and cytidylic acid residues are involved. It is also rather striking that the dose required for total inactivation is only about 1 % of that required to depolymerize the RNA molecule.[168]

Finally, attention should be drawn to the fact that samples of infectious RNA, isolated from two different TMV strains which differed more than five-fold in radiation sensitivity, were equally sensitive to ultraviolet irradiation,[167] a finding which has proved useful in studies on the nature of interaction between the protein and nucleic acid components of viruses and to which we shall return below.

b. Transforming DNA

The inactivation at 253.7 mμ of transforming DNA (TDNA) for capsule *b* formation in *Haemophilus influenzae* has been reported to follow a "multi-

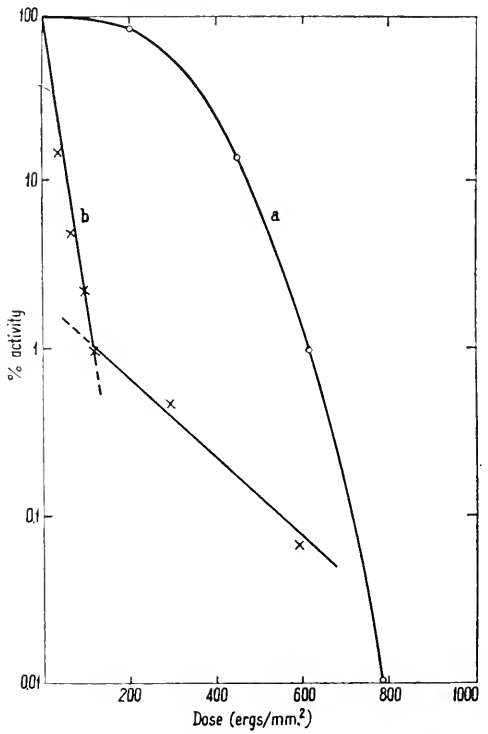


FIG. 13. Course of ultraviolet inactivation of *H. influenzae* transforming DNA (*a*) for type *b* capsule formation [from S. Zamenhof, G. Leidy, E. Hahn, and H. E. Alexander, *J. Bacteriol.* **72,** 1 (1956)] and (*b*) for streptomycin resistance in type *d* [from R. Pakula, E. Hulanicka, and W. Walczak, *Bull. acad. polon. sci., Classe II,* **7,** 217 (1959)].

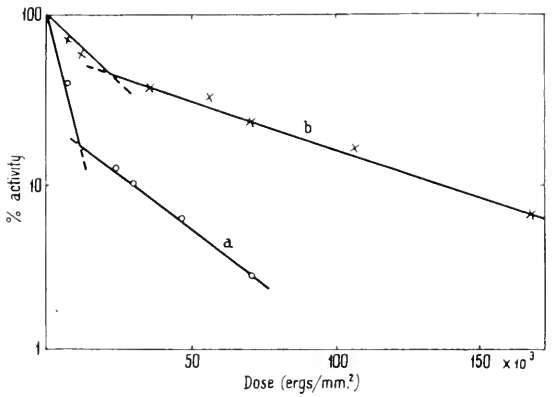


FIG. 14. Ultraviolet inactivation of transforming DNA from hemolytic streptococci group H, strain Challis: (*a*) for intraspecific transformation (of streptomycin resistance) and (*b*) for interspecific transformation, receptor strain *sbe* type I/II [from R. Pakula, E. Hulanicka, and W. Walczak, *Bull. acad. polon. sci., Classe II*, **7**, 217 (1959)].

hit" type of process[171] but this is apparently not a general phenomenon (see Fig. 13). Complex inactivation curves have been reported for two genetic markers of pneumococcal TDNA, but it is claimed that a "one-hit," or first-order, process is involved.[172]

Figure 14 exhibits the course of ultraviolet inactivation of TDNA from hemolytic streptococci group H for homologous and heterologous transformation of streptomycin resistance.[173] The differences in sensitivity are very marked; but were much less so for pneumococcal TDNA, where the heterologous receptor was the *sbe* type I/II strain of hemolytic streptococci group H. [173]

Similar types of inactivation curves exhibiting two exponentials have been reported by others[172, 174] for pneumococcal TDNA; and Beiser and Ellison[174] have actually fractionated pneumococcal TDNA on ECTEOLA columns into components with sensitivities corresponding to the two segments of the inactivation curve for the unfractionated DNA.

It has been suggested that the inactivation rate of TDNA is markedly dependent on the concentration, but since the results are based only on the measurement of residual activities—and some of the solutions were op-

[171] S. Zamenhof, G. Leidy, E. Hahn, and H. E. Alexander, *J. Bacteriol.* **72**, 1 (1956).
[172] L. J. Tolmach and L. S. Lerman, *Radiation Research* **7**, 128 (1957).
[173] R. Pakula, E. Hulanicka, and W. Walczak, *Bull. acad. polon. sci., Classe II* **7**, 217 (1959).
[174] S. M. Beiser and S. A. Ellison, *7th Intern. Congr. Microbiol., Stockholm, 1958* Paper No. 4f (1958).

tically "thick" so that complete absorption of the incident light must have prevailed[171]—this requires re-examination.

Latarjet and Cherrier[175] have emphasized the remarkable resistance of pneumococcal TDNA to irradiation and calculate a quantum yield of about 10^{-5} (molecular weight of DNA not given). Some idea regarding the differences in sensitivity of different TDNA's may be gained by comparing the doses in Fig. 14 with results for *H. influenzae* DNA (Fig. 13) which requires about 500 ergs/mm.^{2} for 99% destruction of activity for capsule transformation and about 100 ergs/mm.^{2} for destruction of activity for streptomycin resistance.

For *H. influenzae* TDNA the dose required to produce a noticeable decrease (3%) in viscosity is 500-fold greater than that required for 90% inactivation.[171] In conjunction with analogous observations on infectious RNA from TMV[168] (Section VI, 2, *a*) it follows that the doses required to provoke important biological effects in nucleic acids are much lower than those resulting in degradation of nucleotide chains and that the biological effects are highly localized in character and most likely involve the pyrimidine nucleotide residues. It is of some interest in this connection that the RNA core is highly resistant to biological inactivation (Section VI, 2, *c*).

Attempts to demonstrate differences in stability of several pneumococcal transforming factors towards ionizing radiations were unsuccessful.[176] Nor is there any difference in sensitivity of various TDNA's if loss of viscosity is used as a criterion of radiation sensitivity.

Different markers of the same transforming DNA, on the other hand, may exhibit different sensitivities to ultraviolet[172, 177] and, of a variety of agents possessing the ability to produce differential inactivation of more than one marker, ultraviolet is by far the most effective.[177] Figure 15 illustrates the differences in stability of different heredity determinants in *H. influenzae* TDNA.

The high radiation resistance of the 1% residual activity in curve 3 of Fig. 15 suggests that some molecules might be more stable than others, possibly owing to the presence of several heredity determinants. This was excluded by preparing new TDNA from receptor cells transformed with the residual activity; the new TDNA exhibited similar radiation sensitivity. It should be recalled that irradiated infectious RNA from TMV also exhibits a residual activity highly resistant to radiation and the same is true for many viruses (Section IX). A similar phenomenon has been observed for pneumococcal TDNA inactivated by X-rays.[178]

[175] R. Latarjet and N. Cherrier, *2nd Intern. Congr. Photobiol., Turin, 1957* p. 131 (1957).

[176] J. Marmur and D. J. Fluke, *Arch. Biochem. Biophys.* **57,** 506 (1955).

[177] S. Zamenhof, G. Leidy, S. Greer, and E. Hahn, *J. Bacteriol.* **74,** 194 (1957).

[178] H. Ephrussi-Taylor and R. Latarjet, *Biochim. et Biophys. Acta* **16,** 183 (1955).

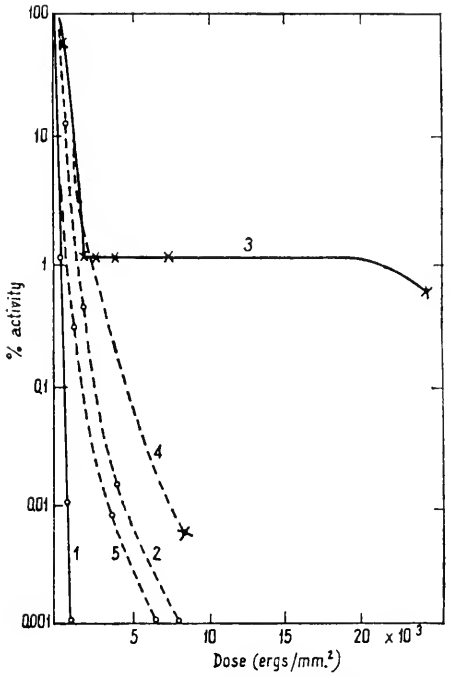


FIG. 15. Ultraviolet inactivation of transforming DNA from *H. influenzae* for: (1) capsule *b* or *c* formation, (2) capsule *d* formation, (3) streptomycin resistance in type *b*, (4) streptomycin resistance in type *d*, (5) streptomycin resistance in type *a* [from S. Zamenhof, G. Leidy, S. Greer, and E. Hahn, *J. Bacteriol.* **74**, 194 (1957)].

It is as yet not feasible to define the reasons for this differential stability of different markers, especially since TDNA preparations are by no means homogeneous. Ravin[179] objects to the use by Zamenhof *et al.*[177] of the phrase "differential stability of heredity determinants" on the grounds that it presupposes the mechanism of the effect, and suggests there may be no difference in stability of various markers but only a variation in the ability of different markers in a given irradiated TDNA to be incorporated into the host genome. It is consequently worth noting that the ability to transform is apparently much more sensitive to radiation than the ability to be taken up irreversibly, from which it has been concluded that ultraviolet inactivated determinants can at least enter the bacterial cell.[172]

c. RNA Core

The ability of the ribonuclease-resistant "core" of RNA (Volume I, Chapter 15) to promote streptolysin S formation in streptococci (Volume

[179] A. W. Ravin, *Ann. Rev. Microbiol.* **12**, 309 (1958).

II, Chapter 28) provides a small oligonucleotide suitable for photochemical studies, although less useful from a general point of view. It has been found that the RNA core is inactivated very slowly, with a quantum yield certainly not greater than 10^{-4} and probably lower than this. Following a 50% loss in activity there is only a 10% decrease in metachromasia against basic dyes, indicating relatively little chain degradation. Since we are dealing here with a polypurine oligonucleotide containing a terminal pyrimidine nucleotide residue which is very susceptible to irradiation, it may be concluded from the above that the presence of this terminal pyrimidine nucleotide is unnecessary for biological activity, a conclusion arrived at independently by direct removal of the terminal pyrimidine nucleotide.[180] The above result confirms biologically the physicochemical evidence for the high radiation resistance of purine nucleotide residues in polynucleotide chains.

VII. Reversibility of Nucleic Acid Photolysis

In view of the fact that the photolysis of uracil and cytosine nucleotides is almost completely reversible in the dark, one is logically led to an examination of the likelihood of such reversal by these constituents when they are incorporated into nucleotide chains. It should be recalled that the quantum yields for these components are from 10–100 times greater than for purine nucleotides, so that one would expect them to be first affected during biological inactivation; and supporting evidence for this is forthcoming from the observation that even extensive degradation of DNA leaves the purine residues almost intact. Moreover, proposed schemes of nucleic acid structure do not involve either positions 4 or 5 of the pyrimidine rings in secondary bonds, so that the double bond involving these linkages is, theoretically, still capable of reversible uptake of a water molecule, albeit to a modified extent as a result of involvement of other positions of the pyrimidine ring in secondary bonds as well as mutual interaction between adjacent rings. We shall now summarize the evidence, based on physicochemical and biological properties of nucleic acids, which demonstrates that at least direct partial reversal of the effects of photolysis may indeed be achieved.

1. Physicochemical Studies

Physicochemical investigations of reversal of photochemical effects in nucleic acids have hitherto been confined to observations of spectral changes. Such data could be objected to on the grounds that hyperchromicity is difficult to account for, and for this reason no experiments have yet been undertaken with DNA where the uncertainty from this source might be considerable.

[180] K. Tanaka, *J. Biochem.* (*Japan*) **45**, 109 (1958).

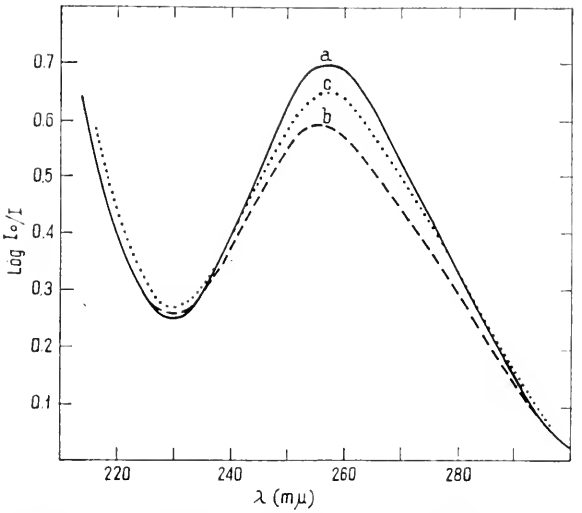


FIG. 16. Irradiation and subsequent partial reversibility of photolysis of RNA at neutral pH: (*a*) prior to irradiation, (*b*) after 65 minutes irradiation, (*c*) irradiated solution after 5 minutes at 92° [from D. Shugar and K. L. Wierzchowski, *Intern. Symposium Macromolecules, Prague, 1957*, Paper No. 123 (1957); *J. Polymer Sci.* **31**, 269 (1958)].

a. Natural Nucleic Acids

Initial experiments,[39, 149] which were confined to RNA, were subsequently extended to apurinic acid (APA) since this provides a chain containing only two components, thymidylic and deoxycytidylic acids, for which only the latter is subject to reversibility. A typical experiment is illustrated in Fig. 16 for RNA, from which it will be observed that somewhat more than 50% of the drop in absorption due to irradiation may subsequently be restored by heating. The results for APA are analogous; but, assuming that the drop in absorption is due mainly to the deoxycytidylic acid component, the quantum yield is found to be about 3 times that to be expected. It will be observed from Fig. 16 that the "regenerated" spectrum exhibits a small permanent increase in absorption at all wavelengths to the violet of 230 mμ; this phenomenon is fairly general and suggests that some permanent damage has resulted which may explain the failure to observe 100% reversibility.

Deaminated RNA and APA, which at first failed to reveal any signs of reversibility, have since been found to exhibit reversal under appropriate conditions.

b. Synthetic Oligo- and Polynucleotides

An extensive series of experiments, along the lines outlined in the preceding section, has now been completed with homopolymers of uridylic

acid, in which hyperchromicity is too small to raise any doubts as to the validity of the findings. Only a brief outline of these results, which substantiate and considerably extend those outlined above, will be given here.[69]

The rate of photolysis of UpUp corresponds to a reaction order of about 2.5 up to the point where about 50% of the aromatic rings have reacted, following which it drops to nearly unity; the initial quantum yield is about twice that prevailing for an equal concentration of Up alone. The extent to which the reaction may be reversed in the dark is, however, dependent on the degree of photolysis. Up to the point where 50% of the uracil rings have reacted, only 50% of the loss in absorption can be restored by heat; further decreases in absorption as a result of irradiation are completely reversible, so that total thermal reactivation may attain over 75%.

It follows from the above that in a dinucleotide, one of the rings will react more readily than in the free state but that if the second ring has not reacted, the chances of reversal for the photolysed ring are considerably reduced. Hence despite the practical absence of hyperchromicity in poly-U (Section IV, 4) it must be concluded that interaction does prevail between the aromatic rings. That this is not due simply to incorporation in the chain, as suggested by Warner,[72] is shown by the fact that the quantum yield for uridine-2′(3′),5′-diphosphate is the same as for other uridylic acid isomers and the degree of thermal reversibility over 90% (Table II).

The behavior of UpU and UpUp! is entirely analogous to that for UpUp, as is also that of the isomer of UpUp[79] in which the internucleotide linkage is 2′,5′.

The behavior of longer chains is similar qualitatively; when we get to highly polymerized poly-U the maximum extent of thermal reactivation following photolysis drops to about 55%, as compared to over 75% for a dinucleotide.

It can therefore be considered as reasonably well established that reversible photodecomposition of free pyrimidine nucleotide residues is duplicated when these are incorporated in polynucleotide chains. It remains to establish what has happened to that portion of the residues which apparently does not undergo reversibility, i.e., whether they have been irreversibly photolysed or whether adjacent unaltered rings inhibit reversal.

The desirability of extending the above results to poly-C as well as copolymers of pyrimidine and purine nucleotides, particularly di- and trinucleotides, is obvious (see Addendum).

2. Biological Studies

It has long been known that ultraviolet inactivated bacteriophage cannot be photoreactivated outside of their host cells, a fact suggestive of the involvement of some cellular factor or mechanism.[181] Attempts have been made to photoreactivate ultraviolet-inactivated transforming DNA with near ultraviolet light, but without success.[171, 177] It would indeed have been surprising if the results had been positive since DNA does not exhibit specific absorption in the near ultraviolet.

No trials as yet appear to have been made to reactivate ultraviolet inactivated transforming DNA by heating at temperatures which do not normally result in heat inactivation, although this would be well worth trying.

[181] J. Jagger, *Bacteriol. Revs.* **22,** 99 (1958).

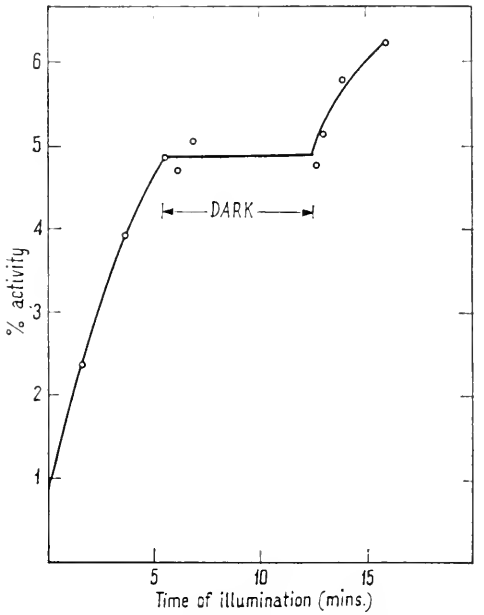


FIG. 17. Increase in activity of *H. influenzae* transforming DNA (previously inactivated by exposure to ultraviolet) in presence of *E. coli* extract under influence of visible light; during period indicated by arrows the source of illumination was removed [from C. S. Rupert, S. H. Goodgal, and R. M. Herriot, *J. Gen. Physiol.* **41**, 451 (1958)].

It has now been demonstrated[182] that ultraviolet inactivated *H. influenzae* transforming DNA may be partially reactivated by visible light in the presence of a cell-free *E. coli* B extract (Fig. 17). The degree of reactivation is proportional to the concentration of the extract, light intensity, and temperature, and varies from 10–50% of the activity of a nonirradiated control. The extract consists of a dialyzable, heat-stable fraction and a nondialyzable thermolabile component.

It appears most likely from the evidence thus far presented that the reaction system involves a light-activated enzymic reaction and it is tempting to speculate about the possibility of enzymic removal of a water molecule from the saturated 4,5 double bond of pyrimidine residues in the ultraviolet inactivated DNA molecule.

It is instructive that the active extract is derived from *E. coli* B, an organism which itself is photoreactivable, whereas an extract from *H. influenzae* (an organism not susceptible of photoreactivation following ultraviolet inactivation) is ineffective in the above *in vitro* system.

An illuminated *E. coli* B extract has been reported capable of reactivating to a

[182] C. S. Rupert, S. H. Goodgal, and R. M. Herriot, *J. Gen. Physiol.* **41**, 451 (1958).

small extent *Diplococcus pneumoniae* transforming DNA (R. Latarjet and N. Rebeyrotte, cited in Jagger[181]). Pakula (personal communication) confirms this and also finds only slight photoreactivation for streptococcal transforming DNA under conditions where *H. influenzae* DNA exhibits photoreactivation; in fact, it was found that the DNA of the coli extracts inhibits competitively the pneumococcal and streptococcal DNA's. A yeast extract, on the other hand, exhibits the same reactivating ability as an *E. coli* extract on *H. influenzae* DNA.[183]

The above system has been applied in an ingenious manner to demonstrate ultraviolet damage, as well as photoreversal of such damage, in DNA which exhibits no biological transforming activity.[183, 184] The technique is based on the fact that photoreversal of irradiated transforming DNA is inhibited in the presence of irradiated, but not nonirradiated, nontransforming DNA. If, however, ultraviolet-inactivated nontransforming DNA is first subjected to photoreactivation, it then no longer inhibits photoreactivation of irradiated transforming DNA.

It is worth noting that the maximum degree of photoreactivation achieved, about 50%, is approximately equal to the maximum degree of thermal reactivation obtained with various nucleotide chains, using spectral changes as the criterion for reversibility.

VIII. Radiation Receptors in Living Organisms

Although we have been dealing in this chapter almost exclusively with the effects of irradiation at one wavelength, 253.7 mμ, the application of different wavelengths to the same system, coupled with a knowledge of the absorption spectra of the various components of the system, has proved an extremely useful tool in gaining additional information about the mechanism of action of radiation and, in particular, regarding the component(s) of the system responsible for the initial absorption process leading to an observed physicochemical or biological effect. The theoretical basis of action spectroscopy has been described by Loofbourow[159] and Blum[185] and the subject has recently been comprehensively reviewed.[165]

The correspondence between action spectrum and absorption spectrum for a given substance is based, among others, on the assumption that quantum yields are independent of wavelength. It is consequently necessary to emphasize that for nucleic acids and their derivatives there is very little information available regarding the dependence of ϕ on wavelength. An action spectrum has been obtained for the polymerization or cross-linking of dry DNA films and it corresponds reasonably well with the

[183] C. S. Rupert, *Federation Proc.* **17**, 301 (1958).

[184] C. S. Rupert and R. M. Herriot, *7th Intern. Congr. Microbiol., Stockholm*, **1958** Paper No. 4s (1958).

[185] H. Blum, *in* "Biophysical Research Methods" (F. M. Uber, ed.), Chapter 13. Interscience, New York, 1950.

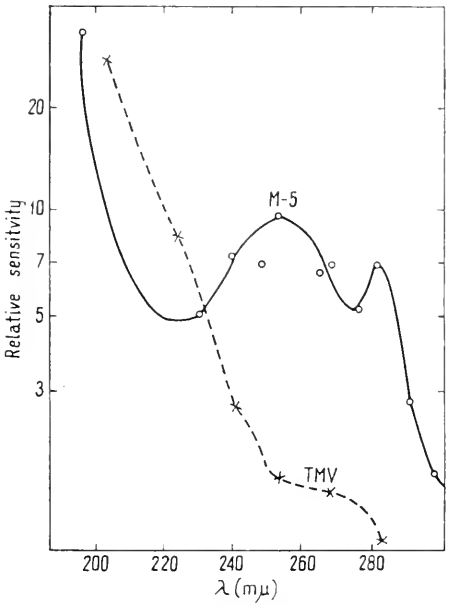


FIG. 18. Action spectrum for *B. megaterium* bacteriophage M-5 [from R. M. Franklin, M. Friedman, and R. B. Setlow, *Arch. Biochem. Biophys.* **44**, 159 (1953)] and of the common strain of TMV [from A. Hollaender and J. W. Oliphant, *J. Bacteriol.* **48**, 447 (1944)].

absorption spectrum;[165] but this is not as satisfactory as observations based on biological activity such as infectivity of RNA from TMV or transforming DNA.

Nevertheless, it must be admitted that the evidence from action spectra is strongly in favor of nucleic acids as the primary receptors of radiation in many biological phenomena. The extent to which proteins are involved cannot, however, be considered as equally well established. This is due largely to the experimental difficulty of obtaining a sufficient number of points on the action spectrum. In at least one instance where this has been achieved,[186] for a *Bacillus megaterium* bacteriophage, the use of additional wavelengths in the neighborhood of 280 mμ resulted in an action spectrum suggestive of the involvement of the protein component (Fig. 18). By contrast, the action spectrum for the common strain of TMV,[187] shown in the same figure, is inexplicable in terms of either the protein or nucleic acid moieties. We shall return to this question below in connection with viruses.

[186] R. M. Franklin, M. Friedman, and R. B. Setlow, *Arch. Biochem. Biophys.* **44**, 159 (1953).

[187] A. Hollaender and J. W. Oliphant, *J. Bacteriol.* **48**, 447 (1944).

An important consideration is the extent to which the energy absorbed is utilized at the absorption site or transferred to some other cell component where its effect is manifested. Action spectra alone are incapable of resolving this problem and recourse must be had to supplementary experimental data.

The subject of transference of absorbed energy in excited biological systems has only recently begun to attract attention. A well-known example of such transfer is the dissociation of CO-myoglobin with equal efficiency by light absorbed either in the protein or heme components.[2, 3] An illustration of energy transfer from a nucleotide coenzyme (DPN) to a protein to which it is bound is provided by triosephosphate dehydrogenase, where light absorbed at the 340 mμ band of enzyme-bound DPNH results in an increase of enzymic activity, presumably by reduction of enzyme —S—S— groups[134]; an analogous phenomenon *in vivo*[188] has been interpreted on the basis of this mechanism[134] and attention has been drawn to its possible significance in photoreactivation.[94, 181]

Due to the complexity of biological systems, synthetic models have been used to obtain quantitative data. Broser and Lautsch[189] coupled poly-DL-(phenylalanine-glutamic acid) with carbonyl-mesohemin-IX and irradiated the complex at a wavelength corresponding to the phenylalanine absorption band. The resulting dissociation of the CO from the hemin group with a quantum yield of unity was regarded as evidence of highly efficient energy transfer along a polypeptide chain due to overlapping amplitude functions of adjacent groups in a helix; but resonance transfer has been suggested as an equally likely interpretation.

An interesting study has been made of electronic energy transfer by proteins and nucleic acids by preparing dye conjugates of each and measuring the efficiency with which light absorbed by protein or nucleic acid excites dye fluorescence.[190] No such energy transfer by either RNA or DNA could be detected and this was ascribed to the assumed nonfluorescent nature of nucleic acid derivatives in solution. The evidence on this latter point is, however, conflicting[39] and requires clarification, particularly in view of its importance in resonance transfer of energy, i.e., the transfer of energy by electrodynamical interaction from an excited oscillator to an oscillator in resonance with it and so close to it that their separation is small by comparison with the wavelength of the vibrating electromagnetic field emitted by the former. An excellent presentation of this subject has been given by Karreman and Steele[191] (cf. Bücher[192]).

From a study of the fluorescence efficiency of TMV and its protein moiety alone,[190] it was concluded that energy transfer from protein to nucleic acid is nonexistent but that up to 25% transfer from nucleic acid to protein may exist. However, such is not necessarily the case at short wavelengths (Section IX). Indirect evidence for low energy transfer efficiency from nucleic acid to protein is the well-known fact that virus infectivity may be destroyed by irradiation without appreciably affecting antigenic properties.

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[188] G. Calcutt, *Nature* **166**, 443 (1950).

[189] W. Broser and W. Lautsch, *Z. Naturforsch.* **11b**, 453 (1956).

[190] V. G. Shore and A. B. Pardee, *Arch. Biochem. Biophys.* **60**, 100 (1956); **62**, 355 (1956).

[191] G. Karreman and R. H. Steele, *Biochim. et Biophys. Acta* **25**, 280 (1957).

[192] T. Bücher, *Advances in Enzymol.* **14**, 1 (1953).

The subject of energy transfer in photobiological and radiobiological systems has been dealt with in several recent publications and symposia.[193]

IX. Viruses

Viruses are undoubtedly the most useful and fascinating systems for photochemical studies, as well as those which have most profited from such investigations since they are, in effect, nucleoproteins exhibiting a variety of biological activities and may be irradiated either *in vitro* or in the intracellular state where they behave as components of more complex integrated biological systems. The limited scope of this chapter permits only a brief discussion of the photochemistry of viruses in relationship to the nucleic acid components. Broader aspects of virus photochemistry have been dealt with by Luria,[194] Pollard,[195] and Kleczkowski.[196]

Ultraviolet inactivation of virus infectivity usually follows a first-order reaction, but numerous exceptions are known;[39, 194] some of them are quite puzzling, e.g., inactivation of T1 phage is first-order in the dry state, but not in solution.[197] For a polio virus the complex inactivation curve has been interpreted as due to the presence of two strains of varying sensitivity.[198] The effect of irradiation conditions on the shape of the survival curves of T1 phage has been extensively investigated by Hill and Rossi[199]; although no definite conclusions could be drawn, the results are nonetheless of considerable value for future investigations. In a number of instances a small percentage of the residual activity has been found remarkably resistant to irradiation[194, 200]; for T1 and T2 phages this has been ascribed[200] to multiplicity reactivation,[194] but it is unlikely that such is the case for infectious RNA from TMV of which about 1% of the residual activity is remarkably radiation resistant. It should also be recalled that transforming DNA exhibits a similar behavior.

Quantum yields have been obtained for only a few viruses and are shown in Table IV.[201-203] In general, these values are based on corrections for scattered light which may be subject to considerable error, e.g., Oster and

[193] (a) "Basic Mechanisms in Radiobiology" (J. L. Magee, M. D. Kamen, and R. L. Platzman, eds.), Natl. Research Council Publ. No. 305, Washington, D. C., 1953; (b) I. A. Vladimirov and S. Konev, *Biofizika* **2**, 3 (1957); (c) Symposium Soc. de chim. phys., in *J. chim. phys.* **55**, (11–12) (1958); (d) Symposium Faraday Soc., Nottingham, 1959 (*Discussions Faraday Soc.* in press).

[194] S. E. Luria, *in* "Radiation Biology" (A. Hollaender, ed.), Vol. II, Chapter 9. McGraw-Hill, New York, 1955.

[195] E. C. Pollard, "The Physics of Viruses." Academic Press, New York, 1953.

[196] A. Kleczkowski, *Advances in Virus Research* **4**, 191 (1957).

[197] D. J. Fluke, *Radiation Research* **4**, 193 (1956).

[198] J. Fogh, *Proc. Soc. Exptl. Biol. Med.* **89**, 464 (1955).

[199] R. F. Hill and H. H. Rossi, *Radiation Research* **1**, 282, 358 (1954).

[200] M. R. Zelle and A. Hollaender, *J. Bacteriol.* **68**, 210 (1954).

[201] F. M. Uber, *Nature* **147**, 148 (1941).

[202] A. Kleczkowski, *Biochem. J.* **56**, 345 (1954).

[203] D. E. Lea, "Actions of Radiations on Living Cells." Cambridge Univ. Press, London and New York, 1955.

TABLE IV

QUANTUM YIELDS FOR VIRUS INACTIVATION

Virus	Irradiation wavelength (mμ)	ϕ	References
TMV strain U1 (common strain)	253.7	2.6×10^{-5}	*a*
	253.7	4.3×10^{-5}	*b*
	253.7	$3\text{–}6 \times 10^{-5}$	*c*
	253.7	7×10^{-5}	*d*
TMV strain U2	253.7	20×10^{-5}	*e*
T1 phage	260	3×10^{-4}	*f*
T1 phage	222–302	6×10^{-4}	*g*
T2 phage	222–302	3×10^{-4}	*g*

[a] F. M. Uber, *Nature* **147**, 148 (1941).

[b] G. Oster and A. D. McLaren, *J. Gen. Physiol.* **33**, 215 (1950).

[c] A. Kleczkowski, *Biochem. J.* **56**, 345 (1954).

[d] D. E. Lea, "Actions of Radiations on Living Cells." Cambridge Univ. Press, London and New York, 1955.

[e] From A. Siegel and A. Norman [*Virology* **6**, 725 (1958)] assuming ϕ for U1 strain is about 4×10^{-5}.

[f] D. J. Fluke, quoted by E. C. Pollard, "The Physics of Viruses." Academic Press, New York, 1953.

[g] M. R. Zelle and A. Hollaender, *J. Bacteriol.* **68**, 210 (1954); values are approximate means of values obtained at various wavelengths in range indicated.

McLaren's[98] value for TMV is based on a 50% scattering correction; this is certainly too large and the quantum yield is therefore too high, perhaps by as much as 30%. The apparent independence of ϕ with wavelength for T1 and T2 phages in Table IV is in serious disagreement with the marked wavelength dependence reported by Fluke[197] for T1 irradiated either in the wet or dry state. Zelle and Hollaender[200] present a valuable discussion of the possible influence of experimental conditions on these discrepancies. Luria's[194] emphasis on the importance of quantum yield measurements for action spectra determinations is no less valid today. But experimental difficulties are still formidable and relative sensitivities at different wavelengths continue to be used.

The action spectra of the common (U1) strain of TMV (Fig. 18) and, to a lesser extent, Rous sarcoma virus, differ appreciably from those for other viruses, and cannot be readily accounted for. Siegel and Norman[169] therefore compared the relative sensitivities of TMV strains U1 and U2 at three different wavelengths with those of the infectious RNA components from these strains, the latter of which exhibit identical sensitivities. U2 is about 5 times as sensitive as U1 at 280 mμ and 253.7 mμ, but both strains exhibit equal sensitivity at 226 mμ; at 254 mμ the RNA components extracted from each virus, following partial inactivation, were found to have been reduced in infectivity to the same extent as the parent virus. It was therefore concluded that at 254 mμ inactivation is due solely to RNA absorption and this is considered to be substantiated by the fact

that U2 and RNA_2 are equally sensitive at 254 mμ.[167] Since the difference in sensitivities between U1 and U2 at 254 mμ had previously been shown to be due to protection of the RNA of the former by its protein component, it was concluded that the equal sensitivities of both strains at 226 mμ may be ascribed to energy absorption by the protein components alone. While issue may be taken with the conclusion that inactivation of virus infectivity at 226 mμ is due *entirely* to protein absorption and that at 254 mμ entirely to nucleic acid, this method of approach is of great value, particularly as infectious nucleic acids from other viruses are becoming available for such studies.

Probably the most striking illustration of the role of nucleic acid-protein interaction in virus activity is the demonstration by Bawden and Kleczkowski[170] that infectious RNA from TMV may be photoreactivated. Previous work by the same authors[204] had shown that a variety of plant viruses exhibited photoreactivation, several TMV strains being the sole exceptions.

It has been variously shown that the sensitivity of a virus bears little, if any, relation to its nucleic acid content and it now is apparent that this is not necessarily due to possible differences in the structure of the nucleic acid components alone, but to the structural integrity of the virus as a whole.

When we attempt to correlate physicochemical changes with loss of infectivity for a given virus as a result of irradiation, we encounter a situation similar to that for transforming DNA and infectious RNA. Completely inactivated TMV exhibits the same X-ray pattern, isoelectric point, optical rotation, molecular weight, and ability to crystallize, although the crystals are slightly opalescent; the appearance of the inactivated virus under the electron microscope is unchanged,[98, 205] and its absorption spectrum is unaffected. It follows that inactivation must result from highly localized injury, a conclusion also derived from studies of "cross-reactivation" in bacteriophage.[206]

The original observation of Stanley[205] that ultraviolet-inactivated TMV retains the serological properties of the original virus first pointed to the now generally recognized feasibility of separating the infective from the antigenic properties of a virus by irradiation. The practical application of irradiation to the production of vaccines has now been investigated for a number of viruses and in at least some instances the products obtained appear to be entirely satisfactory.[207a-d] Ultraviolet irradiation has also been

[204] F. C. Bawden and A. Kleczkowski, *J. Gen. Microbiol.* **8**, 145 (1953); **13**, 370 (1955).
[205] W. M. Stanley, *Science* **83**, 626 (1936).
[206] G. S. Stent and C. R. Fuerst, *Advances in Biol. and Med. Phys.* **7**, in press (1959).
[207] (a) S. O. Levinson, A. Milzer, H. J. Shaughnessy, J. L. Neal, and F. Oppenheimer, *J. Am. Med. Assoc.* **125**, 531 (1944); *J. Immunol.* **50**, 317 (1945); (b)

successfully applied to the freeing of plasma products from the viruses causing hepatitis and jaundice.[207e] The literature on this subject has been briefly reviewed recently.[39]

X. Photoreactivation

The reversal of ultraviolet damage to a biological system by exposure to visible or near ultraviolet radiation has been observed for a wide variety of ultraviolet radiation effects in every type of living organism, with only a few exceptions.[181] These exceptions are of considerable interest from the standpoint of a general theory to account for photoreactivation (PR) and have, for instance, included TMV and many bacteria. The reason for previous failures to photoreactivate TMV has now been shown to be due to the nature of the linkage between the RNA and protein components of the virus, and infectious RNA from TMV is photoreactivable.[170] A similar explanation may apply to some bacteria where the intracellular state of binding of DNA may likewise inhibit PR.

Another interpretation is suggested by the several observations cited above (Section VI) with regard to the greater radiation sensitivity of some DNA'S *in vivo*; and particularly the finding that pneumococcal transforming DNA is much more sensitive to irradiation prior to its extraction from these microorganisms,[174] which could well account for lack of PR in pneumococci. Still another possibility is photosensitized inactivation in the primary irradiation process; as in the case of X-rays, it is unlikely that PR would then be possible, as illustrated by Galston's[208] failure to demonstrate PR for photooxidized T6r bacteriophage.

These examples suffice to underline the importance of investigations on the reasons for the absence of PR in specific cases,[181] not only from the point of view of photochemical mechanisms *in vivo* but also for the information they may provide as to the state of the intracellular components involved.

The earlier review of Dulbecco[209] devoted exclusively to this subject has been brought up to date in an excellent and comprehensive survey by Jagger.[181] Before going on to a discussion of a possible mechanism for PR, it is perhaps worthwhile to summarize a few of its salient features:

K. Habel and B. T. Sockrider, *J. Immunol.* **56**, 273 (1947); (c) L. H. Collier, D. McLean, and H. Vallet, *J. Hyg.* **53**, 513 (1955); (d) H. J. Shaughnessy, A. M. Wolf, M. Janota, J. L. Neal, F. Oppenheimer, A. Milzer, H. Naftulin, and R. A. Morrissey, *Proc. Soc. Exptl. Biol. Med.* **95**, 251 (1957); (e) K. B. McCall, F. H. Gordon, F. C. Bloom, L. A. Hyndman, H. L. Taylor, and H. D. Anderson, *J. Am. Pharm. Assoc. Sci. Ed.* **46**, 295 (1957).

[208] A. W. Galston, *Science* **111**, 619 (1950).

[209] R. Dulbecco, *in* "Radiation Biology," (A. Hollaender, ed.), Vol. II, Chapter 12. McGraw-Hill, New York, 1955.

(*a*) Most photoreactivable phenomena include reproduction, mutation or transformation, which involve the nucleus and therefore DNA-protein; there are also two authentic examples of cytoplasmic PR. In the case of bacteriophage there can be no doubt that PR involves DNA-protein and the evidence appears to indicate that the PR site is DNA. Insofar as plant viruses are concerned, any previous doubts[181] as to their general susceptibility to PR has now been resolved by the demonstration that infectious RNA from TMV is photoreactivable.[170] Coupled with the demonstration of PR in transforming DNA, reasonably good evidence exists that the site of PR in living organisms is DNA and/or RNA.

(*b*) Ultraviolet damage *in vivo* may be restored or repaired by agents other than visible light and, of these, the one that most closely resembles PR is heat, or thermal restoration (TR).

(*c*) PR is not a 100% phenomenon and is usually of the order of 50% or less.

(*d*) The available evidence implies that PR operates via reversal of damage caused by ultraviolet and not through the formation of new metabolic pathways.

Facts (*a*) and (*d*) have stimulated a number of authors to propose general theoretical models to account for PR on the basis of such physical principles as electron capture in crystals (Herschel effect), the formation of metastable states, internal conversion, etc.,[6, 210] all of which are conceivable in rigid macromolecules, such as DNA, where conditions similar to those in the solid state may possibly exist.

The demonstration, on the other hand, that the reversible photolysis of pyrimidine nucleotides is likewise exhibited by these constituents in nucleotide chains, provides an experimental model which is reasonably consistent with all the above facts, if due consideration is given to the possibility that TR and PR merely represent two different sources of energy for the reactivation process. It must be borne in mind that TR has received considerably less attention, experimentally, than PR; it is also less specific.

It would obviously be desirable to learn something about the specificity requirements of the light-activated enzymic mechanism responsible for PR in transforming DNA; and we have made several attempts to observe PR for the reversible photoproducts of dimethyluracil and uridylic acid with illuminated *E. coli* B extracts (under conditions where *H. influenzae* transforming DNA exhibits PR) but with negative results (Janion and Shugar, unpublished). It is, however, quite possible that the specificity requirements of this enzyme include the presence of an internucleotide linkage.

A number of attempts have been made to reverse the effects of ultraviolet radiation on model substances by exposure to light of longer wavelength, which we shall now summarize.

Kita *et al.*[211] report that the decrease in absorption of ATP resulting from exposure

[210] (a) G. Stein, *J. chim. phys.* **51**, 133 (1954); (b) J. Duchesne and J. Garsou, *ibid.* **54**, 789 (1957); (c) J. Duchesne and B. Rosen, *ibid.* **56**, 76 (1959).

[211] H. Kita, H. Maede, B. Hanazaki, F. Shimizu, and H. Fujita, *Bull. Tokyo Med. Dental Univ.* **I**, 37 (1954).

to ultraviolet could be reversed by visible radiation; but this is not substantiated in the text of their article. It would indeed be surprising if there were any effect of visible light on the photoproduct of ATP, since neither one nor the other absorb in the visible region of the spectrum; and this is most likely the reason for the failure of Wells and Johnson[212] to find any such effect for ATP. A similar explanation applies to the unsuccessful efforts of Ekert and Jagger[181] to photoreactivate, with visible light, the photoproducts of dimethyluracil, uridylic acid, and some other analogs of these compounds.

Wells[213] claims to have observed PR for DPN, but the following considerations suggest the necessity for independent verification of this observation. The supposed PR was obtained by illumination with a GE CH-4 spotlamp through a Corning 3060 filter. This filter transmits only 0.5% at 365 mμ and zero below this wavelength; but neither DPN nor its photoproducts absorb at 365 mμ. It is therefore likely that the effect observed resides in the crude tissue extracts used to test for DPN activity. Ekert and Monier[127] attempted to duplicate Wells' experiments, using an established enzymic assay procedure for DPN activity; the results were negative, as would be expected from the fact that the PR wavelengths used were far to the red of the DPN absorption band. For this same reason their proposal that Wells' results may have been true PR involving some enzyme system in the crude extracts, similar to that responsible for PR in transforming DNA, is open to some doubt.

The photoproduct of 2-methoxycytosine, which is largely reversible to the parent compound by TR, exhibits an absorption band at 310 mμ and extending to 330 mμ.[39] Irradiation of this photoproduct with wavelengths just above 300 mμ gave no reversal; nor has PR been found for several photoproducts, including irradiated RNA, exposed to visible light in the presence of photosensitizing dyes (Wierzchowski and Shugar, unpublished).

It is clear that some of the attempts to demonstrate PR for model substances have ignored the first law of photochemistry, and future efforts should take this into account. It would obviously be desirable to attempt TR trials with infectious RNA from TMV and with transforming DNA. No efforts appear to have been made to obtain TR in viruses and bacteriophage, but such experiments are undoubtedly worth trying, due consideration being given to possible denaturation at the temperatures employed.

Attention should be drawn to at least one potential argument against the proposal that PR involves reversible photolysis of pyrimidine nucleotides. The T-even phages exhibit PR but their pyrimidine components include only thymine and 5-hydroxymethylcytosine.[214] It was pointed out in Section V, 4, *c*, that not all 5-substituted pyrimidines exhibit reversibility; but that thymine incorporated in a nucleotide chain conceivably may,[154] while some evidence exists that 5-hydroxymethylcytosine does exhibit reversibility. The question of thymine is common to all nucleic acids but that of 5-hydroxymethylcytosine is unique for the T-even phages in that they contain 5-hydroxymethylcytidylic acid as well as glucosylated

[212] P. H. Wells and H. Johnson, *Anat. Record* **117**, 644 (1953).
[213] P. H. Wells, *Science* **124**, 31 (1956).
[214] G. R. Wyatt and S. S. Cohen, *Nature* **170**, 1072 (1952).

5-hydroxymethylcytidylic acid.[215] If these derivatives fail to exhibit reversible photolysis, serious doubts exist as to the participation of pyrimidine derivatives in PR. Sinsheimer[215b] mentions that 5-hydroxymethylcytidylic acid and glucosylated 5-hydroxymethylcytidylic acid differ notably in sensitivity and nature of response to ultraviolet, but gives no details. The significance of a marked difference in behavior of these two substances may, however, be appreciated when it is realized that it may provide an important clue to the site of action of radiation in these viruses, as well as to the mechanism involved.

If, now, PR is due to reversible photolysis of pyrimidine rings, the modifications in biological properties of an irradiated single-stranded nucleic acid chain would result from (*a*) a "change" in base sequences, and (*b*) changes in secondary structure due to modifications in intrachain hydrogen bonds and to weakening of the interaction between adjacent rings, at those sites where a pyrimidine ring has been transformed to the corresponding 4-hydroxy-5-hydro derivative. For a double-stranded chain we should also expect some modification in the number of hydrogen bonds between base pairs involving those pyrimidine rings which have reacted photochemically. It is assumed that internucleotide bonds are unaffected, an assumption which is reasonable if the radiation doses have not exceeded those which are biologically effective.

It is consequently of interest that 90% ultraviolet inactivation of transforming DNA leads to a detectable drop in denaturation temperature or of the transition profile (i.e. of the mean temperature for the thermally induced transition of the DNA chain from a helix to a random coil).[216] The fact that the decrease is small (1.5°C.) indeed suggests that only a few hydrogen bonds were either broken or weakened by the irradiation, as would be expected, so that reformation of such bonds may be readily visualized at those points where the original pyrimidine ring is subsequently restored by PR or TR. It might be argued that a few hydrogen bonds could be broken by the absorbed energy without photolysis of pyrimidine rings, but in such an event one might expect spontaneous reformation of these bonds.

Extension of the above observation of Marmur and Doty[216] to transforming DNA containing several markers with different radiation sensitivities (Section VI, 2, *b*) might provide valuable information regarding the relationship between biological activity and some types of base-pair sequences.

[215] (a) E. Volkin, *J. Am. Chem. Soc.* **76,** 5892 (1954); (b) R. L. Sinsheimer, *Science* **120,** 551 (1954); (c) S. S. Cohen, *in* "The Chemical Basis of Heredity" (W. D. McElroy and D. Glass, eds.). Johns Hopkins, Baltimore, 1957; (d) M. R. Loeb and S. S. Cohen, *J. Biol. Chem.* **234,** 364 (1959).

[216] J. Marmur and P. Doty, *Nature* **183,** 1427 (1959).

XI. Addendum

One example has been found of thermal reactivation of a bacteriophage. Partial reactivation of phage T1, adsorbed on *E. coli*, was obtained by warming at 45°C.[217] Independent confirmation of this observation, as well as its extension to other phages, particularly the T-even phages (see next paragraph), would be extremely useful.

An extensive investigation of the photochemistry of a variety of substitututed pyrimidines included some preliminary observations on the photochemical behaviour of 5-substituted cytosines and their nucleosides and nucleotides, in relation to photoreactivation of the T-even phages[218] (see Section X). Although no definite evidence for reversibility was obtained, these derivatives proved to be relatively radiation resistant; in addition the primary photoproducts are relatively stable and contain the intact pyrimidine ring, indicating that irreversible damage has not necessarily occurred.

Beukers *et al.*[219, 220] report some remarkable observations on the behavior of orotic acid in the presence of various gases and of thymine in the frozen state (cf. Section V, 4, *c*). Orotic acid, e.g., if irradiated in nitrogen, exhibits partial reversibility on subsequent irradiation in oxygen. The decrease in absorption of an irradiated frozen solution of thymine may be almost completely restored by thawing under continued irradiation; the resulting product differs chromatographically from thymine, but the fact remains that the pyrimidine ring has been regenerated. An adequate explanation for these findings requires additional data, but they emphasize once more the importance (Section II,3) of investigating the excited states of pyrimidines by flash photolysis.

The foregoing authors[221] have also shown that irradiation of APA leads to destruction of thymine residues at a rate considerably greater than that to be expected from the behavior of free thymidylic acid. A similar finding has been obtained[222] by irradiation of an oligonucleotide consisting mainly (90%) of thymine isolated from thymus DNA[223] but efforts to demonstrate reversibility (see Section V,4,*c*) were unsuccessful. The observation of Griffin *et al.*[224] that polyribothymidylic acid exhibits up to 33% hyperchromicity may be related to this increased sensitivity of thymine, when incorporated in a nucleotide chain, if it is recalled (Section IV,4) that poly-

[217] C. Z. Bresch, *Z. Naturforsch.* **5b**, 420 (1950).
[218] K. L. Wierzchowski and D. Shugar, *Acta Biochim. Polon.* **7**, 63 (1960).
[219] R. Beukers, J. Ijlstra, and W. Berends, *Rec. trav. chim.* **78**, 879 (1959).
[220] R. Beukers, J. Ijlstra, and W. Berends, *Rec. trav. chim.* **78**, 883 (1959).
[221] R. Beukers, J. Ijlstra, and W. Berends, *Rec. trav. chim.* **78**, 247 (1959).
[222] K. L. Wierzchowski and D. Shugar, *Acta Biochim. Polon.* **7**, No. 2-3, in press (1960).
[223] A. Adamiec and D. Shugar, *Naturwissenschaften* **46**, 356 (1959).
[224] B. E. Griffin, A. R. Todd, and A. Rich, *Proc. Natl. Acad. Sci. U. S.* **44**, 1123 (1958).

ribouridylic acid (poly-U) exhibits only 5–6% hyperchromicity. The synthetic small linear and cyclic thymine oligonucleotides of Tener *et al.*[225] would be ideal compounds for clarification of this problem. Aside from the photochemical aspects, the source of the above difference in hyperchromicity merits investigation from the point of view of nucleotide chain structure.

The enormous increase in sensitivity (on a survival basis) of *E. coli* cells containing 5-bromouracil, in place of thymine, in their DNA suggested that such DNA is itself less stable to irradiation.[226] An interesting sequel to this observation is the finding that the sensitivity of phage ϕX174, which contains a single-stranded DNA molecule, is increased when it incorporates 5-bromouracil in place of thymine, while that of phage T2 is unaffected. Increased radiation sensitivity following bromouracil incorporation is suggested as a feasible test for the single-stranded character of DNA in multiplying cells, and has been utilized to follow the fate of DNA during its transfer from parental to progeny phage.[227]

It would obviously be of interest to examine the action spectrum for brominated ϕX174. A preliminary investigation has shown that the action spectrum for ordinary ϕX174 resembles the spectrum of cytosine plus thymine, and not that of ordinary DNA.[228] In view of this result, the action spectrum of TMV (Fig. 18) merits re-examination.

Procedures have now been worked out for studying the photochemistry of model heterologous oligonucleotides which exhibit high hypochromicity that must be corrected for in quantitative work[222] (see Section VII,1,*b*). The extensive data of Michelson[79, 229] on hyperchromicity in model oligonucleotides is very useful in such investigations. The results substantially clarify the role of base sequences in reversible photolysis in nucleotide chains, suggest a different interpretation of the kinetics of photolysis of UpUp,[69] and make available a new tool for investigations of hypochromicity and structure in oligonucleotides. A related study[230] on absorption spectra and structure of dihydropyrimidines (Section IV,3) and hydrogenated uracil-containing oligonucleotides provided useful supplementary data in the foregoing investigations.

The relatively stable hydroperoxides formed by the X-irradiation of pyrimidines (but not purines) and nucleic acids[231, 232] and which, in the

[225] G. M. Tener, H. G. Khorana, R. Markham, and E. H. Pol, *J. Am. Chem. Soc.* **80,** 6223 (1958).
[226] S. Zamenhof, *Ann. N. Y. Acad. Sci.* **81,** 784 (1959).
[227] A. W. Kozinski and W. Szybalski, *Virology* **9,** 260 (1959).
[228] R. Setlow, *Biochim. et Biophys. Acta* **39,** 180 (1960).
[229] A. M. Michelson, *Acta Biochim. Polon.* **6,** 335 (1959); *J. Chem. Soc.*, p. 3655 (1959).
[230] C. Janion and D. Shugar, *Acta Biochim. Polon.*, **7,** No. 2-3, in press (1960).
[231] G. Scholes, J. Weiss, and C. M. Wheeler, *Nature* **178,** 157 (1956); **185,** 305 (1960).
[232] B. Ekert and R. Monier, *Ann. inst. Past.* **92,** 556 (1957).

case of thymine, were postulated as being the *cis* and *trans* isomers of 4-hydroxy-5-hydroperoxythymine[233] (and therefore involving saturation of the 4,5 double bond as for photoproducts of many pyrimidines) have now been shown to be so by direct chemical synthesis.[234] These peroxides may be of some significance in the photochemistry of nucleic acids because of the observation[231] that they are also formed by ultraviolet irradiation of nucleic acids and their derivatives in an oxygen atmosphere. In view of the frequent references in this text to the effects of unfiltered radiation, it becomes desirable to delineate the upper wavelength limit for such peroxide formation which probably is below 210 mμ.

The value of simultaneous investigations with ultraviolet and ionizing radiations (Section I) is reflected in the increasing number of publications devoted to such studies; particular interest attaches to those of Elkind and Sutton[235] wherein evidence is presented for the existence, in dividing yeast cells, of an overlap in sites sensitive to X-rays, ultraviolet, PR and, most important of all, ultraviolet reactivation of, or protection against, X-ray lethality. It is difficult to avoid the temptation to explain the latter effect by the formation, by ultraviolet light, of 4-hydroxy-5-hydropyrimidine derivatives which would subsequently not form hydroperoxides upon X-irradiation (see above).

Increasing attention is being devoted to the origin of the complex, or multicomponent, nature of the inactivation curves for transforming DNA (Section VI,2,*b*) by both ultraviolet and ionizing radiations and special attention is drawn to the comprehensive papers on this subject by Latarjet *et al.*[236] and Lerman and Tolmach.[237]

Irradiation of air-dried DNA films (see Section VI,1) at 253.7 mμ has been found to result in formation of a photoproduct which forms fibers insoluble in water or salt solution and resistant to the action of deoxyribonuclease.[238] The irradiated films exhibit a considerable increase in specificity for methyl green. Staining techniques with labeled dyes[239] were therefore used for following the effects of irradiation on films of a variety of natural and synthetic polynucleotide chains, the results indicating that the resultant cross-linking is rather an ordered, than a haphazard, process involving the pyrimidine rings.[238] No similar effect could be observed with γ-radiation although deuteron irradiation had previously been shown to induce slight

[233] G. Scholes and J. Weiss, *Radiation Research*, Suppl. 1, 177 (1959).
[234] B. Ekert and R. Monier, *Nature* **184**, B.A. 58 (1959).
[235] M. M. Elkind and H. Sutton, *Radiation Research* **10**, 283, 296 (1959).
[236] R. Latarjet, H. Ephrussi-Taylor, and N. Rebeyrotte, *Radiation Research*, Suppl. 1, 417 (1959).
[237] L. S. Lerman and L. J. Tolmach, *Biochim. et Biophys. Acta* **33**, 371 (1959).
[238] D. Shugar and J. Baranowska, *Nature*, **135**, 33 (1960); J. Baranowska and D. Shugar, *Acta Biochim. Polon.*, in press (1960).

cross-linking in DNA films,[163] while extensive cross-linking of DNA in sperm heads has been produced by electron irradiation.[240] Since a DNA film more nearly resembles nuclear DNA than does a solution, cross-linking by ultraviolet and ionizing radiations may be involved in some cellular effects. It would be of interest to examine the photochemistry of DNA films in the vacuum ultraviolet, as has been done for proteins,[241] in order to obtain a better comparison of the effects of ultraviolet and ionizing radiations.

The apparent temperature independence of quantum yield for cross-linking of dry DNA was proposed[165] as a tool for distinguishing between protein and nucleic acid effects in action spectra (Sections VI,1 and VIII). This suggestion may require revision in view of the finding of Lerman and Tolmach[237] that the quantum yield for inactivation of transforming DNA at 77°K. is much lower than at 275°K. The latter authors calculate a chain length of 1.6×10^3 nucleotide residues for the critical segment of transforming DNA (cf. Guild and DeFilippes[242]) and find a quantum yield at 253.7 mμ for this segment of about 3×10^{-3}, or more than an order of magnitude greater than that for infectious RNA from TMV (SectionVI,2,*a*).

This chapter would hardly be complete without at least some reference to the growing interest in the role of radiations in the origin of life on the earth.[243] In a recent review of this subject Miller and Urey[244] point out that the greatest source of energy for amino acid synthesis on the primitive earth was ultraviolet. It would be of interest to examine the possibilities of purine and pyrimidine synthesis in such a system, perhaps via dihydro, hydroperoxy, or hydrated pyrimidines, all of which are more radiation resistant than the pyrimidines themselves and, once formed, could under more favorable conditions be transformed to nucleic acid building blocks.

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[241] R. Setlow, *Radiation Research*, in press (1960).

[242] W. R. Guild and F. M. DeFilippes, *Biochim. et Biophys. Acta* **26**, 241 (1957).

[243] A. I. Oparin, "The Origin of Life," 2nd ed. Dover Publications, New York, 1953.

[244] S. L. Miller and H. C. Urey, *Science* **130**, 245 (1959).

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Chapter 31

Chemical and Enzymic Synthesis of Polynucleotides

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I. Abbreviated Formulations and Nomenclature of Polynucleotides[1]

1. Abbreviated Formulations

Shorthand formulations for ribo-oligonucleotides were first introduced by Markham and co-workers.[2, 3] These are convenient and useful and have been largely accepted. The letters A, U, G and C represent the nucleosides of, respectively, adenine, uracil, guanine, and cytosine. The letter "p" to the left of the nucleoside initial indicates a 5′-phosphomonoester group, while the same letter to the right, a 3′-phosphate. The 2′-deoxyribo-oligonucleotides may be distinguished from their ribo-counterparts by prefixing

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[1] Polynucleotides derived from the ribose and deoxyribose series have often been named polyribonucleotides and polydeoxyribonucleotides. It seems more appropriate to use the alternative names ribopolynucleotides and deoxyribopolynucleotides. These names are adopted in this article.

[2] R. Markham and J. D. Smith, *Biochem. J.* **52**, 558 (1952).

[3] L. A. Heppel, P. R. Whitfeld, and R. Markham, *Biochem. J.* **60**, 8 (1955).

them with the letter "d." It may be noted that according to this method the polynucleotide chain is specified in the direction $C_{3'}$–$C_{5'}$. The reverse procedure, in which the letter "p" at the right terminus indicates a 5′-phosphate group, has also been used.[4] The former method has recently been adopted by the Editors of *The Journal of Biological Chemistry*[5] and is used here as well. Such abbreviated names for three oligonucleotides are shown along with the full names below.

2. Diagrammatic Representations

The above procedure for the naming of the polynucleotide chain in the direction $C_{3'}$–$C_{5'}$ can be incorporated in diagrammatic representations of polynucleotide chains. These are again very useful in illustrating many chemical and enzymic reactions.[6, 7] In these diagrams, a nucleoside is depicted by a vertical line (the sugar residue) capped with the initial letter of its purine or pyrimidine base. A diagonal line broken by the letter "p," joining the midpoint (C-3′ position) of one vertical line with the bottom (C-5′ position) of the next vertical line to the right, represents the phosphodiester linkage. A letter "p" at either end of the chain indicates a terminal phosphomonoester group. Furthermore, the 2′-hydroxyl groups in ribopolynucleotides as well as any terminal hydroxyl groups in all polynucleotides may be designated by horizontal lines attached to the vertical lines. This designation is consistent with the diagrammatic formulations often used for sugars.

3. Nomenclature

A system of nomenclature which is finding wide acceptance[5] has been proposed.[8] In this system, following after the generally accepted polypeptide nomenclature, a polynucleotide is considered as a chain in which each nucleotide esterifies the hydroxyl function of the succeeding one rather than as a number of nucleosides joined together by phosphodiester bonds. Further elaboration is, however, necessary, because although the naturally occurring polynucleotides probably all have the $C_{3'}$–$C_{5'}$ internucleotide linkage, synthetic compounds can be made which contain a variety of types of linkage. For the mononucleotides the trivial names, e.g., thymidylic-(5′) acid and adenylic-(3′) acid are used; the number in parentheses refers to the carbon atom to which the phosphoryl group is attached. The internu-

[4] H. S. Shapiro and E. Chargaff, *Biochim. et Biophys. Acta* **26,** 596 (1957).

[5] See "Use of Abbreviations," *J. Biol. Chem.* **233** (1958).

[6] L. A. Heppel and J. C. Rabinowitz, *Ann. Rev. Biochem.* **27,** 613 (1958).

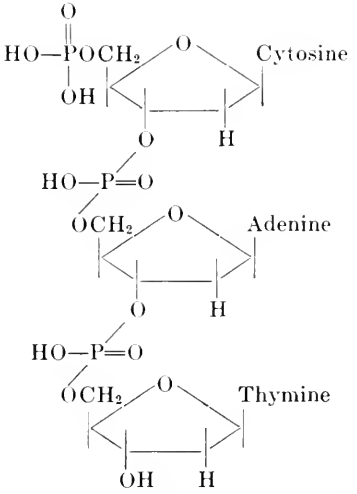
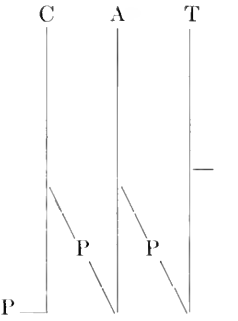
[7] L. Shuster, H. G. Khorana, and L. A. Heppel, *Biochim. et Biophys. Acta* **33,** 452 (1959).

[8] P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.* **80,** 6212 (1958).

cleotide linkage is designated by a method analogous to that already in use for the specification of oligosaccharides. The name of each nucleotide residue is followed by two numbers, separated by an arrow, in parentheses inserted into the name by hyphens. The first number refers to the carbon atom by which the phosphodiester is joined to the preceding nucleoside. The second number denotes the point of linkage to the succeeding nucleoside. The use of the system is illustrated in the examples given below. Obviously, the system permits equally well the naming of a polynucleotide chain in one or the other direction and for maximum simplicity the choice of naming may be influenced by the nature of the groups present at one or the other terminus of the chain.

A further modification may be introduced in that the terminal nucleotide, bearing the phosphomonoester group or a 2′,3′-cyclic phosphate group in the ribopolynucleotide series may be designated, e.g., as nucleoside-5′-phosphate or nucleoside-2′,3′-cyclic phosphate (see Examples 1, 2, and 3).

EXAMPLE 1

Structure	Diagrammatic representation	Abbreviation
		d-pCpApT

Full names: 5′-*O*-Phosphoryl-deoxycytidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-thymidine

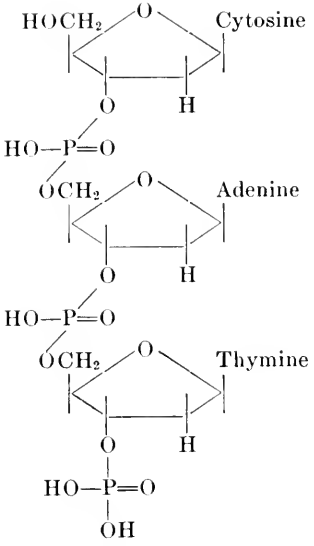
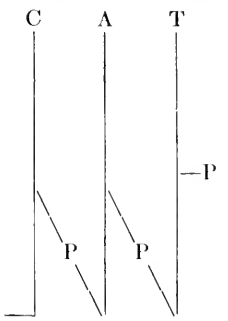
or

Thymidylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-deoxycytidylic-(5′) acid

or

Thymidylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-deoxycytidine-5′ phosphate

EXAMPLE 2

Structure	Diagrammatic representation	Abbreviation
		d-CpApTp

Full names: Deoxycytidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-thymidylic-(3′) acid

or

Deoxycytidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-thymidine-3′ phosphate

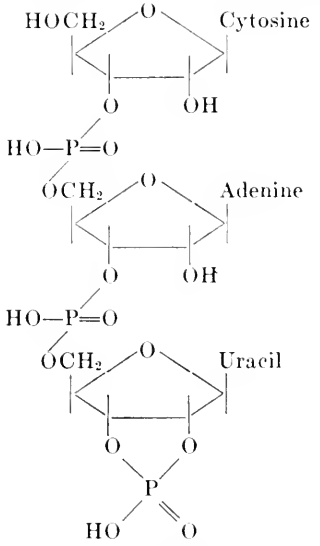
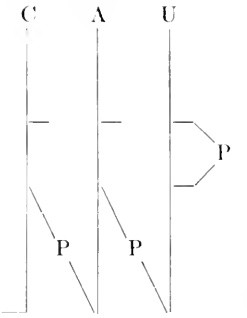
or

3′-*O*-Phosphoryl-thymidylyl-(5′ → 3′)-deoxyadenylyl-(5′ → 3′)-deoxycytidine

II. Chemical Synthesis of Polynucleotides

It is now known with certainty that both the ribo- and deoxyribonucleic acids consist of polynucleotide chains in which the individual nucleosides are joined together by $C_{3'}$–$C_{5'}$ phosphodiester bonds (Volume I, Chapter 12). As a logical development in the chemistry of these macromolecular substances, the chemical synthesis of polynucleotides has been undertaken during the last few years. There the major problems are (1) the development of phosphorylation methods for the formation of phosphodiester bonds and (2) the preparation of suitably protected nucleosides or nucleotides so as to leave only the desired (3′- or 5′-hydroxyl) function free for a phosphorylation reaction. Because of the complexity of these classes of compounds it is inevitable that much effort will be expended on the problems of the protecting groups.

EXAMPLE 3

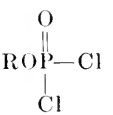
Structure	Diagrammatic representation	Abbreviation
		CpApU-Cyclic p

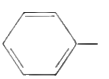
Full name: Cytidylyl-(3′ → 5′)-adenylyl-(3′ → 5′)-uridine-2′,3′ cyclic phosphate

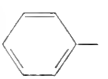
1. METHODS FOR THE SYNTHESIS OF INTERNUCLEOTIDE (PHOSPHODIESTER) BONDS

a. The Use of Bifunctional Phosphorylating Agents

The reagent monophenylphosphorodichloridate (I) has been used by



(I), R = 

(II), R = O_2N 

Baer[9] and his colleagues in their extensive synthetic work in the phospholipid field and involves the successive phosphorylations of two different hydroxylic compounds as shown below.

[9] E. Baer, *Can. J. Biochem. Physiol.* **34**, 288 (1955).

$$R'OH + (I) + \text{limited base} \rightarrow R'O-\underset{\underset{Cl}{|}}{\overset{\overset{O}{\|}}{P}}-OR$$

(III)

$$(III) + R''OH + \text{base} \rightarrow R'O-\underset{\underset{OR}{|}}{\overset{\overset{O}{\|}}{P}}-OR'' \rightarrow R'O-\underset{\underset{OH}{|}}{\overset{\overset{O}{\|}}{P}}-OR''$$

In the nucleotide field the reagent was used in the synthesis of what is now known to be uridylyl-(5′ → 5′)-uridine by Gulland and Smith[10] as early as 1947. Two molar equivalents of 2′,3′-*O*-benzylidene uridine (then regarded as 3′,5′-*O*-benzylidene uridine) were made to react with one equivalent of the reagent and the phenyl group was removed from the presumed initially formed neutral ester by treatment with alkali. More recently, by using the crystalline and highly reactive, mono-*p*-nitrophenylphosphorodichloridate[11] (II), thymidylyl-(5′ → 5′)-thymidine was prepared from 3′-*O*-acetyl thymidine.[12] The approach has, however, proved unsatisfactory so far in the successive phosphorylations of two different nucleoside derivatives to form $C_{3'}$–$C_{5'}$ internucleotide bonds.[8] In any case, in the synthesis of the unsymmetrical phosphodiester bonds, the formation of some of the symmetrical products would appear to be unavoidable.

b. The Use of Halogeno and Other Activated Nucleosides

This approach utilizes the principle that has been used in the synthesis of sugar-1-phosphates, namely the reaction of an alkyl halide with the salt of a phosphate ester. It was used in the synthesis of adenylyl-(5′ → 5′)-uridine[13] by treating a suitably protected derivative of the silver salt of adenosine-5′-phosphate with 2′,3′-*O*-isopropylidene-5′-deoxy-5′-iodouridine and the subsequent removal of the protective groups. The approach is severely limited by the inaccessibility of the corresponding halogenated derivatives of other nucleosides. An alternative method for the activation of hydroxylic

$$ROH + Ar-N=C=N-Ar \rightarrow Ar-N=\underset{\underset{O-R}{|}}{C}-NH-Ar$$

(IV)

$$(IV) + (R'O)_2-\overset{\overset{O}{\|}}{P}-OH \rightarrow (R'O)_2\overset{\overset{O}{\|}}{P}-OR + Ar-NH\overset{\overset{O}{\|}}{C}NH-Ar$$

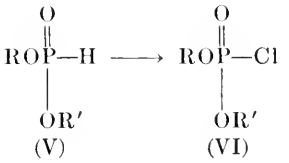
Ar = Aromatic

[10] J. M. Gulland and H. Smith, *J. Chem. Soc.* p. 1532 (1948).
[11] A. F. Turner and H. G. Khorana, *J. Am. Chem. Soc.* **81**, 4651 (1959).
[12] W. E. Razzell and H. G. Khorana, *J. Biol. Chem.* **234**, 2105 (1959).
[13] D. T. Elmore and A. R. Todd, *J. Chem. Soc.* p. 3681 (1952).

compounds has been proposed as shown below and the reaction of these derivatives with phosphoric acid esters leads to the formation of tertiary phosphate esters.[14] The approach remains to be investigated in the nucleotide field but it would appear to be much less attractive than the methods described below.

c. The Use of Phosphorochloridates and Other Mixed Anhydrides

In analogy with the method of phosphorylation of hydroxyl groups using dialkylphosphorochloridates, the aim of this approach has been to (1) convert a suitably protected nucleoside to the corresponding nucleoside phosphorochloridate (VI) via the nucleoside phosphite (V) and (2) subject it to reaction with a second suitably protected nucleoside. The approach was used in the first synthesis of a dinucleoside phosphate [thymidylyl-(3′ → 5′)-thymidine, (IX)][15] containing the naturally occurring internucleotide



(V), (VI), R = nucleoside, R′ = benzyl

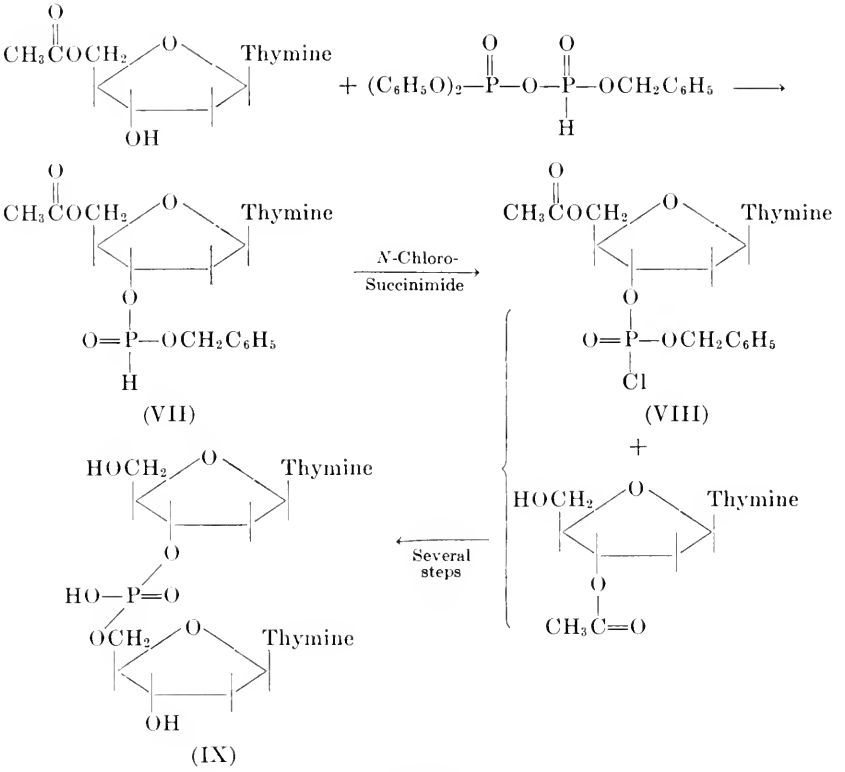
linkage. The steps are shown in Scheme 1. Worthy of note is the preparation of nucleoside-3′-phosphite (VII) and its subsequent conversion to the phosphorochloridate (VIII).

Although the approach has successfully been used in two further syntheses (see below), the yields were uniformly low. The difficulties encountered in the preparation and purification of the neutral intermediates in this approach make it unlikely that the method could be extended to the synthesis of higher oligonucleotides.

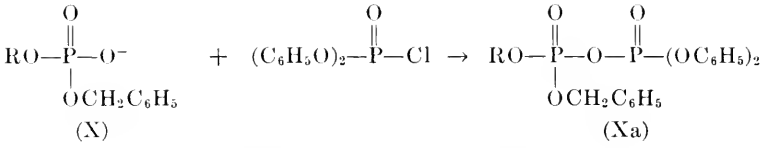
Another approach consists in the preparation of an anhydride (Xa) of a mononucleotide ester (X) and a stronger acid such as diphenyl hydrogen phosphate, as shown, and its use in the phosphorylation of a second nucleoside.[16]

This procedure was very satisfactory in the synthesis of the C_5'–C_5' linked substance adenylyl-(5′ → 5′)-uridine but was ineffective in the synthesis of C_3'–C_5' internucleotide bonds.[15] The same approach has, however, been used for the activation of ribonucleoside-2′,3′-cyclic phosphates

[14] H. G. Khorana, *Can. J. Chem.* **32**, 227 (1954).
[15] A. M. Michelson and A. R. Todd, *J. Chem. Soc.* p. 2632 (1955).
[16] R. H. Hall, A. R. Todd, and R. F. Webb, *J. Chem. Soc.* p. 3291 (1957).



SCHEME 1



(X) (Xa)

(X), (Xa), R = nucleoside radical

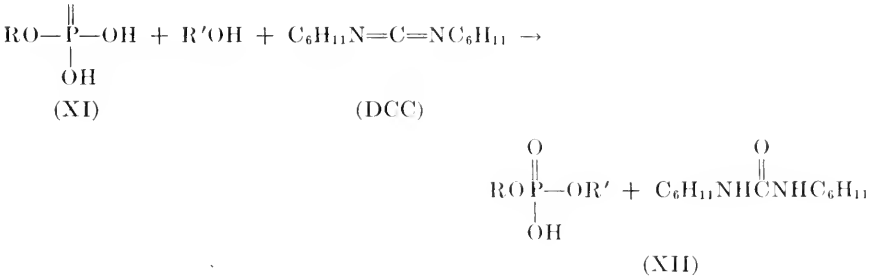
to form random mixtures of C_2'–C_5' and C_3'–C_5' linked ribo-oligonucleotides (see below).

d. Direct Activation of Monoesters of Phosphoric Acid (Mononucleotides)

The approach which has so far proved the most satisfactory and has been used in most of the work described below, consists of the reaction of a monoalkyl phosphate (XI) and an alcohol under anhydrous conditions with dicyclohexylcarbodiimide[8, 17] (DCC) or reactive anhydrides such as *p*-tolu-

[17] H. G. Khorana, W. E. Razzell, P. T. Gilham, G. M. Tener, and E. H. Pol, *J. Am. Chem. Soc.* **79**, 1002 (1957).

enesulfonyl chloride.[18] The diester of phosphoric acid (XII) is thus obtained directly and the yields are consistently high. The mechanism of the



(XI) (DCC) (XII)

reaction appears to be complex,[8, 19] but presumably involves the activation of the monoester to poly- or metaphosphates. In the nucleotide field, the reaction was first applied to the synthesis of thymidylyl-(3′ → 5′)-thymidine, (XVIa) by the reaction of 3′-*O*-acetylthymidine-5′-phosphate (XIVa) and 5′-*O*-tritylthymidine (XIII) (Scheme 2) and the desired product was obtained in about 65% yield when equimolar amounts of the two components were used. When a 100% excess of one component over the second is used, the yield of the product is quantitative with respect to the latter. The same method has been applied to syntheses of two more dinucleoside phosphates (XVIb and XVIc) by reacting 5′-*O*-tritylthymidine (XIII) with (XIVb) and (XIVc), respectively. Both compounds are obtained in satisfactory yields.[8] The method is, thus, readily and generally applicable in the deoxyribonucleotide field.

2. Stepwise Synthesis of Deoxyribo-oligonucleotides

The work in this field is still in its infancy and only limited progress has been reported. The requirements in the stepwise synthesis of oligonucleotides, higher than dinucleotides and containing different mononucleotide units, are, first, selectively to expose an hydroxyl function at one end of the fully protected dinucleoside phosphate (XV) and, then, to treat it with another suitably protected nucleotide.

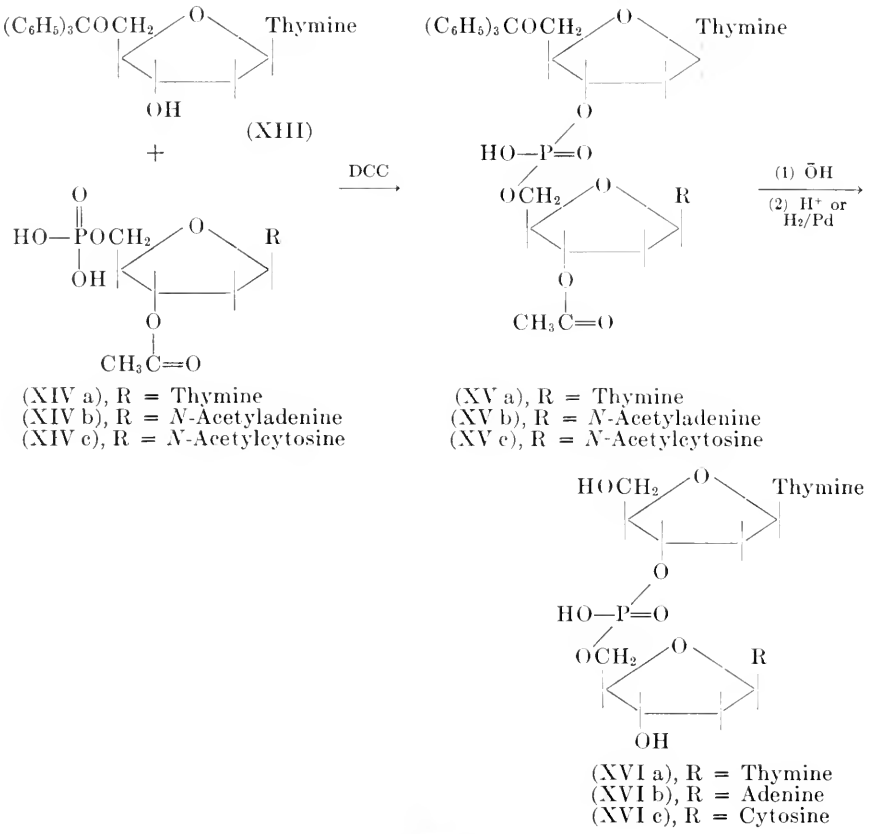
a. Thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine (XVIII)[20]

Mild alkaline treatment of (XVa) gave (XVIIa), which was permitted to react with two molar equivalents of 3′-*O*-acetylthymidine-5′-phosphate

[18] H. G. Khorana, G. M. Tener, J. G. Moffatt, and E. H. Pol, *Chem. & Ind.* p. 1523 (1956).

[19] M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.* **80**, 6204 (1958).

[20] P. T. Gilham and H. G. Khorana, *J. Am. Chem. Soc.* **81**, 4647 (1959).



(XIV a), R = Thymine
(XIV b), R = *N*-Acetyladenine
(XIV c), R = *N*-Acetylcytosine

(XV a), R = Thymine
(XV b), R = *N*-Acetyladenine
(XV c), R = *N*-Acetylcytosine

(XVI a), R = Thymine
(XVI b), R = Adenine
(XVI c), R = Cytosine

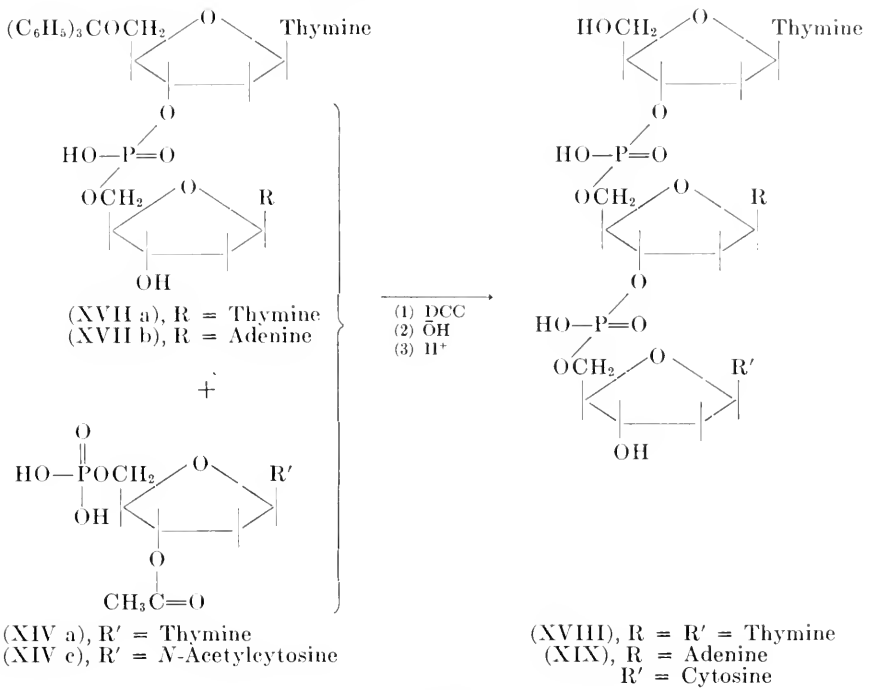
SCHEME 2

(XIVa) (Scheme 3). After removal of the protecting groups by successive alkaline and acidic treatments, the desired product (XVIII) was obtained in 68% yield.

b. Thymidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-deoxycytidine (XIX)[20]

Analogously to the above synthesis, *N*,3′-*O*-diacetyldeoxyadenosine-5′-phosphate was condensed with 5′-*O*-tritylthymidine and the product obtained after alkaline treatment and purification (XVIIb) was made to react with *N*-3′-*O*-diacetyldeoxycytidine-5′-phosphate (XIVc). The mixed trinucleoside diphosphate (XIX) was obtained after removal of the protecting groups.

The synthesis of mixed and higher oligonucleotides is certainly feasible and the next few years should see progress in this field. The major problems to be overcome are (1) insolubility in anhydrous organic solvents (2) separa-



SCHEME 3

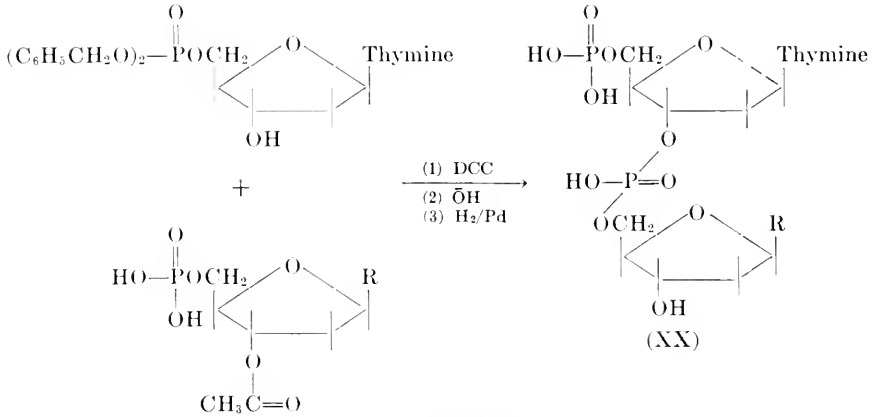
tion of products (3) extreme acid lability of glycosyl bonds in the purine deoxyribonucleosides and (4) the interference from the amino groups on the adenine, guanine, and cytosine ring.

c. Introduction of Phosphomonoester Groups at Ends of Oligonucleotide Chains

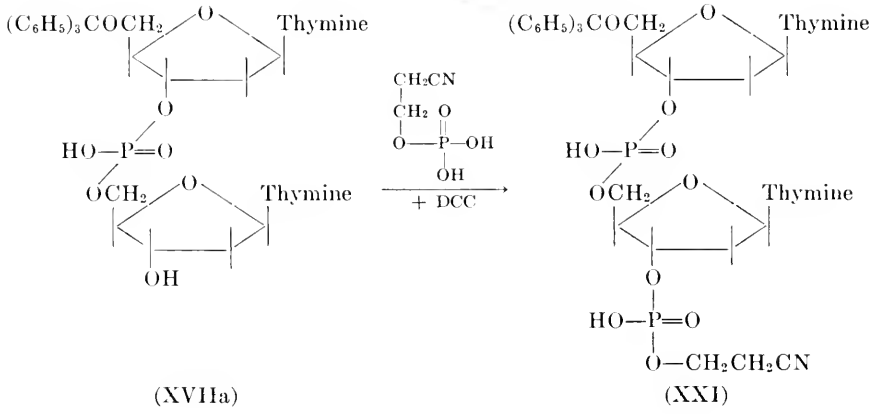
Linear polynucleotides usually carry a phosphomonoester group at one or the other end of the chain and the above methods can be adapted to yield such true oligonucleotides. Two approaches are available. The first one is that in which a protected phosphoryl group is used as a blocking group at the outset of the synthesis. The synthesis of two dinucleotides (XX, R = thymine or adenine) by this method has been recorded[8] (Scheme 4). The synthesis of 5′-*O*-phosphoryl-thymidylyl-(3′ → 5′)-thymidine (XX, R = thymine) by Michelson and Todd[15] by the route illustrated in Scheme 1 also used the same principle.

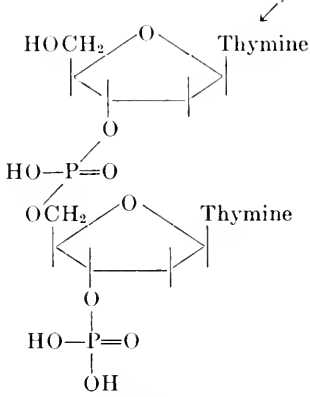
In the second approach the phosphomonoester end group is introduced after the formation of the internucleotide bond(s) as shown below for the synthesis of thymidylyl-(3′ → 5′)-thymidylic-(3′) acid (XXII)[21] (Scheme

[21] P. T. Gilham and G. M. Tener, *Chem. & Ind.* p. 542 (1959).



SCHEME 4





SCHEME 5

5). The method of phosphorylation using β-cyanoethyl phosphate and dicyclohexylcarbodiimide gives the β-cyanoethyl phosphate ester (XXI), from which the cyanoethyl group is removed by mild alkaline treatment.

3. The Specific Synthesis of Interribonucleotide Bonds

The problem of the specific synthesis of the $C_{3'}$–$C_{5'}$ or $C_{2'}$–$C_{5'}$ interribonucleotide linkages is complicated by the presence of the vicinal *cis*-hydroxyl group in ribonucleotides. It is the same factor which caused so much difficulty in the elucidation of the nature of the internucleotide linkage in the ribonucleic acids (Volume I, Chapter 12). The requirement is to prepare appropriately blocked ribonucleoside derivatives, the protecting groups used being such that they do not migrate to the adjacent hydroxyl group during synthesis and can be removed at the end without damage to the phosphodiester bonds synthesized.

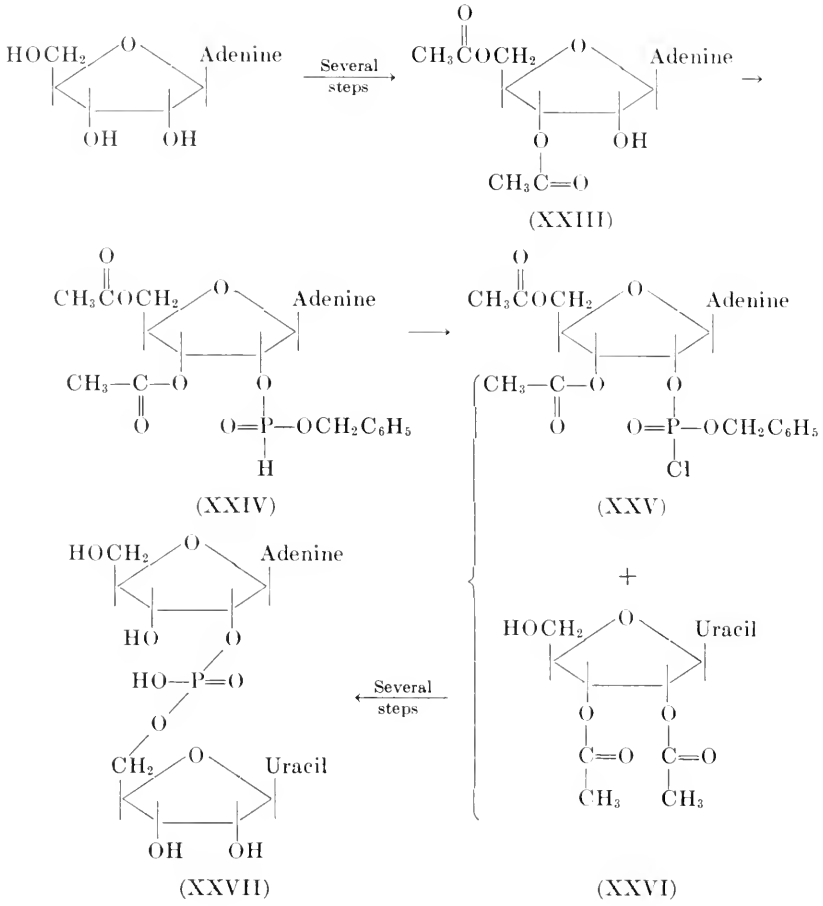
a. Adenylyl-(2′ → 5′)-uridine (XXVII)[22]

The synthesis of this substance in low (8%) yield was accomplished as illustrated below (Scheme 6). 3′,5′-Di-*O*-acetyladenosine (XXIII) was prepared from adenosine and converted to the phosphite (XXIV). Chlorination gave the corresponding phosphorochloridate (XXV) which served as the phosphorylating agent for 2′,3′-di-*O*-acetyluridine (XXVI).

b. Uridylyl-(3′ → 5′)-uridine (XXXIII)

Much greater interest clearly resides in the development of approaches to the synthesis of ribo-oligonucleotides containing specifically the naturally occurring $C_{3'}$–$C_{5'}$ interribonucleotide linkages. A promising approach is outlined in Scheme 7 and has been used in the synthesis of uridylyl-(3′ → 5′)-uridine (XXXIII).[23] Uridine-5′-phosphate is converted in high yield to uridine-3′,5′-cyclic phosphate (XXVIII) in extremely dilute solution by reaction with dicyclohexylcarbodiimide. Reaction of the cyclic phosphate, as the free acid, with dihydropyran in dioxane gives the 2′-*O*-tetrahydropyranyl ether (XXIX) quantitatively, which hydrolyses readily on being heated in barium hydroxide, to a mixture of the 3′- and 5′-phosphates (XXX and XXXI) respectively, the former predominating. On treatment of the mixture with triphenylmethyl chloride in pyridine, only the 3′-phosphate derivative (XXX) reacts and the resulting (XXXII) can be readily separated, by partition chromatography, from (XXXI), which itself is a useful synthetic intermediate. The condensation of (XXXII) with 2′,3′-di-*O*-acetyluridine (XXVI) followed by mild alkaline and acidic treatments gives uridylyl-(3′ → 5′)-uridine (XXXIII) in high yield.

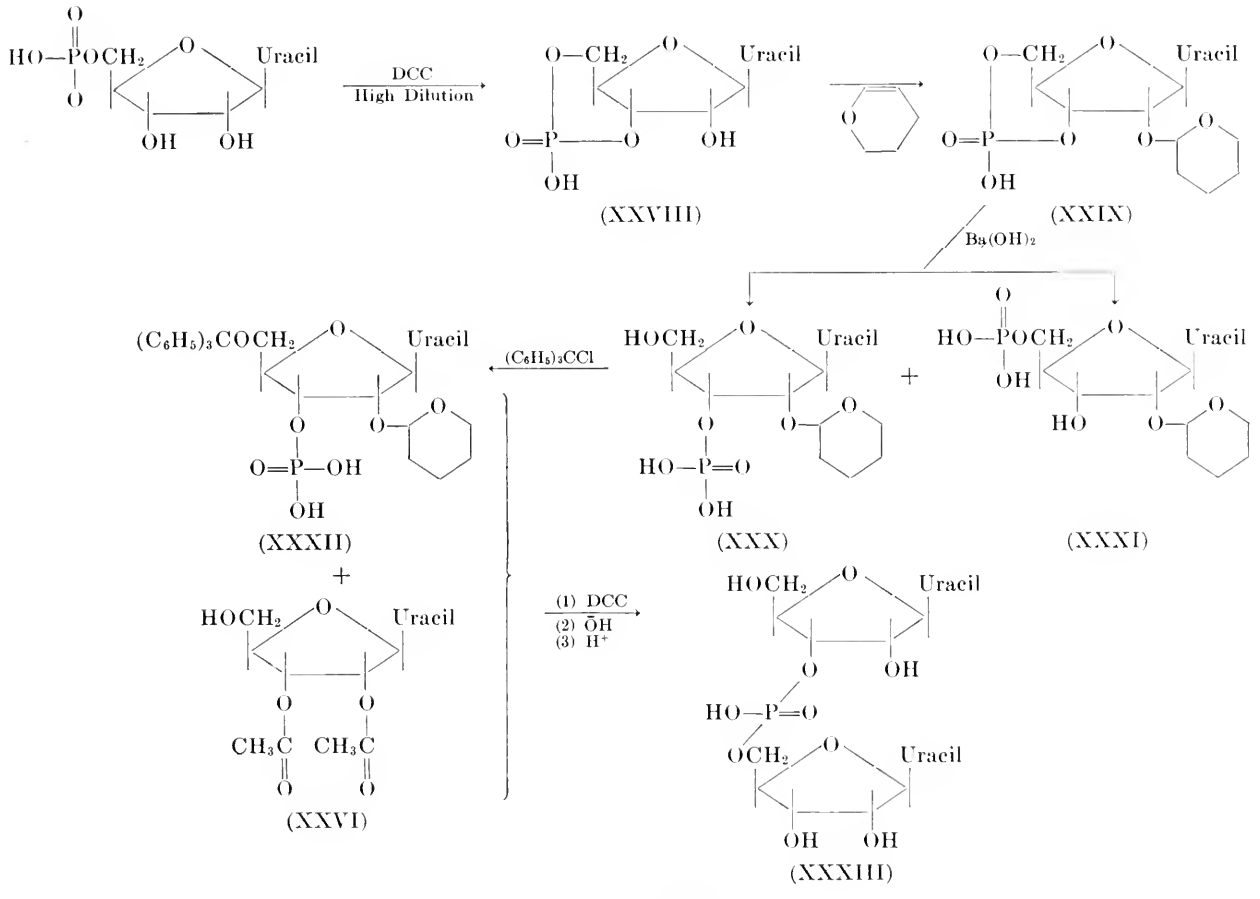
[22] A. M. Michelson, L. Szabo and A. R. Todd, *J. Chem. Soc.* p. 1546 (1956).
[23] M. Smith and H. G. Khorana, *J. Am. Chem. Soc.* **81**, 2911 (1959).



Scheme 6

4. Polymerization of Mononucleotides

Stepwise synthesis of mixed oligonucleotides will undoubtedly be required ultimately but it is beset with great difficulties. Polymerization of mononucleotides is an alternative more expedient means of obtaining a range of simple polynucleotides consisting of only one type of mononucleotide. Such polymers serve admirably for a host of chemical, physicochemical, and enzymic studies. An excellent analogy is provided by the great use that has been made of synthetic polypeptides in recent years for studies in the protein field. Similarly, ribopolynucleotides which have been prepared enzymically (following section) are proving useful in current studies of the nucleic acids.

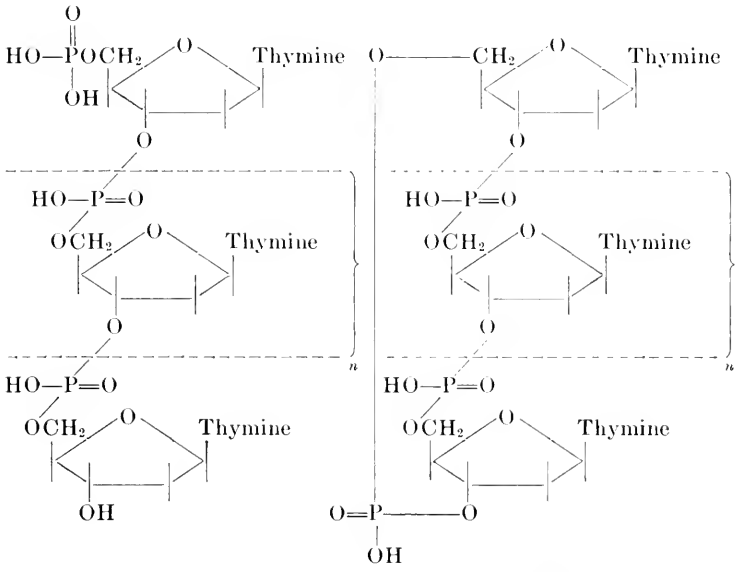

DCC
High Dilution
(XXVIII)
(XXIX)
Ba(OH)2
(C6H5)3CCl
(XXXII)
(XXX)
(XXXI)
(1) DCC
(2) OH
(3) H+
(XXVI)
(XXXIII)

SCHEME 7

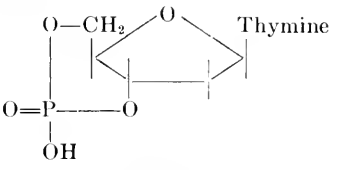
Chemical methods for the polymerization of mononucleotides would be of similar value especially since they could add to the range of the polymers available. Furthermore, in the deoxyribonucleotide field, polymers consisting of one type of nucleotide have not been prepared enzymically as yet.

a. Thymidine Oligonucleotides

The reaction of thymidine-5′-phosphate with dicyclohexylcarbodiimide or *p*-toluenesulfonyl chloride in anhydrous pyridine gives a mixture of polymeric products.[18] The separation and isolation of pure components from the mixture has been achieved by chromatography on cellulose anion exchangers often followed by preparative paper chromatography.[24] In the main, two series of homologous oligonucleotides were obtained.[24] The first are the linear oligonucleotides, the general structures of which are represented by (XXXIV). Thus, they contain the repeating naturally occurring



(XXXIV) (XXXV)

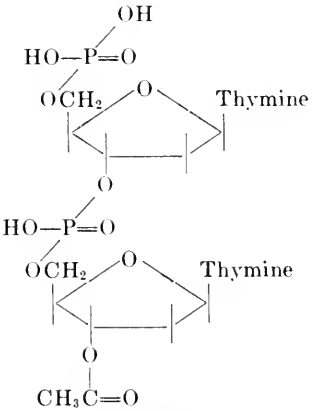


(XXXVI)

[24] G. M. Tener, H. G. Khorana, R. Markham, and E. H. Pol, *J. Am. Chem. Soc.* **80**, 6223 (1958).

3′ → 5′ phosphodiester linkage, a 5′-phosphomonoester group at one end, and a 3′-hydroxyl group at the other end. The second series of compounds are the cyclic oligonucleotides (XXXV) which arise by end to end intramolecular cyclization. The extent of the cyclization process decreases with increase in chain length. In addition to these major series of compounds, minor byproducts are also produced and there is invariably present, in small amount, the monomeric thymidine-3′,5′-cyclic phosphate (XXXVI).

Chemical polymerization of thymidine-5′-phosphate has so far furnished only small sized polymers; the longest polymer contains about twelve units in a chain and the amounts fall off after tri- and tetranucleotides. It is clear that in order to investigate formation of higher linear polymers at least two problems must be solved. These are (1) the inhibition of the intramolecular cyclization process which competes with linear polymerization and (2) the elimination of side product formation so as to induce the polymerization to go further and to facilitate the isolation of pure linear polymers. With respect to the first problem, the concentration used in the reaction will greatly influence the proportion of the linear and cyclic products. Furthermore, a procedure which does, in fact, greatly reduce the cyclization reaction has been devised. This consists of adding some (25–50%) 3′-*O*-acetylthymidine-5′-phosphate to thymidine-5′-phosphate before polymerization.[25, 26] The protected nucleotide forms the terminating unit of the greater portion of polymeric mixture and the compound (XXXVII) and higher homologs cannot undergo intramolecular cyclization.



(XXXVII)

The minor side products in the polymerization reactions have been incompletely investigated[11, 26] as yet but they appear to arise from the direct

[25] H. G. Khorana, *J. Cellular Comp. Physiol.* **54**, Suppl. 1, 5 (1959).
[26] H. G. Khorana, and J. P. Vizsolyi, *J. Am. Chem. Soc.* in press.

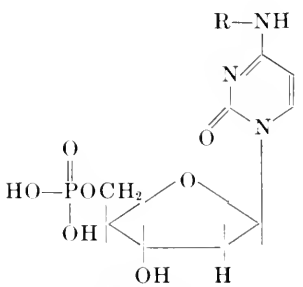
attack of the polymerization reagent (dicyclohexylcarbodiimide or *p*-toluenesulfonyl chloride) on the 3′-hydroxyl function of the nucleotide. Comparative studies of rates of polymerization using different activating agents should throw light on the mechanism of polymerization itself and on the avoidance of side products.

In extension of the above work with thymidine-5′-phosphate, the polymerization of thymidine-3′-phosphate has also been carried out and has furnished moderate amounts of the expected new series of linear thymidine oligonucleotides which bear 3′-phosphomonoester groups at one end and 5′-hydroxyl groups at the other.

b. Other Deoxyribo-oligonucleotides

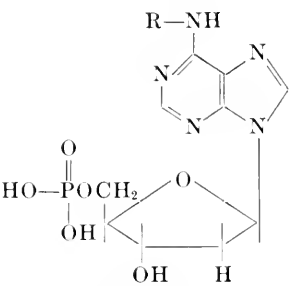
The principle of adding a protected nucleotide, which can serve only as an activated phosphate donor, to a different free nucleotide can be utilized to obtain useful series of compounds, namely, homopolymers terminated in a different nucleotide at one of the ends. Thus copolymerization of *N*,*O*-diacetyldeoxycytidine-5′-phosphate and thymidine-5′-phosphate, followed by alkaline treatment, yielded linear thymidine oligonucleotides, each bearing a terminal deoxycytidylic acid residue.[25, 26]

In investigation of the polymerization of other deoxyribonucleoside-5′-phosphates, serious interference was encountered from the amino groups on the heterocyclic rings of these compounds. Methods have recently been devised for the preparation in virtually quantitative yields of the protected deoxyribonucleotides, N^6-benzoyl and N^6-anisyldeoxycytidine-5′-phosphates (XXXVIII)[26a] and N^6-benzoyldeoxyadenosine-5′-phosphate (XXXIX).[26b] Their polymerization by the methods discussed above, followed by removal of the *N*-acyl groups by treatment with ammonia gave, respectively, deoxycytidine and deoxyadenosine oligonucleotides. The homologous linear oligonucleotides of both series were shown to contain the typical C_3'–C_5' internucleotide bonds.



(XXXVIII)

R = Benzoyl or anisyl



(XXXIX)

R = Benzoyl

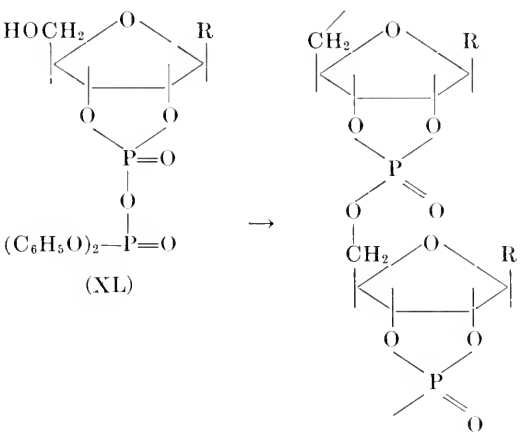
[26a] H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *J. Am. Chem. Soc.* in press.

[26b] R. K. Ralph and H. G. Khorana, *J. Am. Chem. Soc.* in press.

c. Polymerization of Ribonucleotides

The specific synthesis of ($C_{3'}$–$C_{5'}$)-linked ribo-oligonucleotides requires ribonucleotide intermediates in which the 2′-hydroxyl group is protected, e.g., 2′-*O*-tetrahydropyranyluridine-3′-phosphate (XXX). So far, however, only polymerizations to form random mixtures of $C_{2'}$–$C_{5'}$ and $C_{3'}$-$C_{5'}$ internucleotide linkages have been reported. Thus, the direct application of the procedure used for polymerizing thymidine-5′-phosphate to uridine-5′-phosphate gave a mixture of products whose elution pattern from cellulose anion exchangers indicated that they consisted of homologous oligonucleotides.[19]

An alternative method for the formation of random mixtures of ($C_{2'}$–$C_{5'}$)- and ($C_{3'}$–$C_{5'}$)-linked ribo-oligonucleotides has been described.[27] This involves the further activation of ribonucleoside-2′,3′-cyclic phosphates by reaction with diphenylphosphorochloridate or tetraphenyl pyrophosphate.



(XL) (XLI)

The reactive species thus is the mixed anhydride of the type (XL), and the tertiary esters as illustrated in (XLI) must be the initial products. These would then rapidly hydrolyze[28] during work-up in any one of the three possible ways to give (1) a C_2'–C_5' linkage or (2) a C_3'–C_5' linkage or (3) a 2′,3′-cyclic phosphate group which would form the terminus of the oligonucleotide chain. Although there is little doubt that oligonucleotides were formed in this procedure, the isolation of pure components from the mixtures and their adequate characterization has not been reported as yet.

In conclusion, it must be emphasized that studies on chemical polymerization have only begun and progress should be expected in several directions.

[27] A. M. Michelson, *J. Chem. Soc.* p. 1371 (1959).
[28] D. M. Brown, D. I. Magrath, and A. R. Todd, *J. Chem. Soc.* p. 4396 (1955).

III. Enzymic Synthesis of Ribopolynucleotides[29]

1. Introduction

Mechanisms for the enzymic synthesis of mononucleotides are discussed in Chapters 35 and 36. Progress in the understanding of the enzymic pathways for the synthesis of polynucleotides from mononucleotide precursors has been striking during the last five or six years and there has been a marked changed in the views on the subject since a related review (Volume II, Chapter 25) was written. *In vitro* studies on the incorporation of nucleotide precursors into polynucleotides and the discoveries of the nucleoside-5′-mono-, di-, and triphosphates corresponding to all of the naturally occurring purines and pyrimidines in the acid-soluble nucleotide pools of different tissues[30] heightened the supposition that nucleoside-5′-di- or triphosphates might be the biological precursors of polynucleotides. Discrete enzymic reactions utilizing nucleoside-5′-polyphosphates as substrates have indeed been discovered in the synthesis of both series, the ribo- and deoxyribopolynucleotides. It should be emphasized that in the following, the review of this progress is limited to the *in vitro* enzymic studies of the formation of polynucleotides and, in particular, to those reactions where the nature of the starting materials and products is known with reasonable certainty.

2. Ribonucleoside-5′-Diphosphates as Precursors of Ribopolynucleotides

The discovery of an enzyme which catalyzes the synthesis of high molecular weight ribopolynucleotides from ribonucleoside-5′-diphosphates with the release of inorganic phosphate was made by Grunberg-Manago and Ochoa[31] in 1955. The reaction in so far as it may be simply represented by Eq. (1), is reminiscent of the reaction of polysaccharide phosphorylase and the enzyme was therefore called polynucleotide phosphorylase. The enzyme was first isolated from the microorganism *Azotobacter vinelandii*,[31, 32] but soon similar enzymes were described by Littauer and Kornberg[33] in *Escherichia coli* and by Beers[34, 35] in *Micrococcus lysodeikticus*.

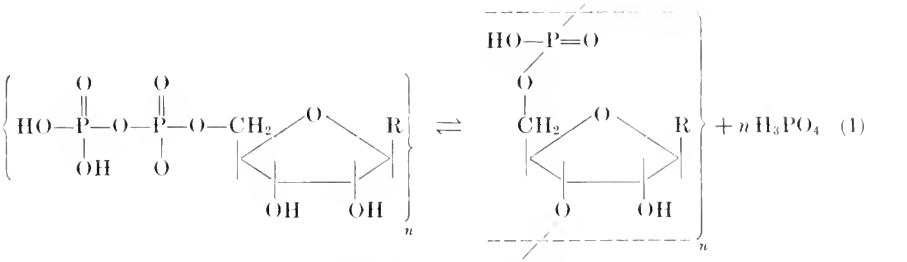
[29] In the following sections some additional abbreviations, which are commonly accepted, are used. These are: ribonucleic acid, RNA; deoxyribonucleic acid, DNA; adenosine-5′ diphosphate, ADP; guanosine-5′ diphosphate, GDP; cytidine-5′ diphosphate, CDP; uridine-5′ diphosphate, UDP; uridine-5′ triphosphate, UTP; guanosine-5′ triphosphate, GTP; the triphosphates of the corresponding deoxyribonucleosides are prefixed by the letter 'd'; inorganic pyrophosphate, PP.

[30] H. Schmitz, R. B. Hurlbert, and V. R. Potter, *J. Biol. Chem.* **209,** 41 (1954).

[31] M. Grunberg-Manago and S. Ochoa, *J. Am. Chem. Soc.* **77,** 3165 (1955).

[32] M. Grunberg-Manago, P. J. Ortiz, and S. Ochoa, *Biochim. et Biophys. Acta* **20,** 269 (1956).

[33] U. Z. Littauer and A. Kornberg, *J. Biol. Chem.* **226,** 1077 (1957).



a. Some Properties of Polynucleotide Phosphorylase

Good balance between the disappearance of nucleoside-5′-diphosphates, with concomitant formation of polynucleotides, and the release of inorganic phosphate can be demonstrated[31, 33] as would be expected for Eq. (1). The reaction requires magnesium and the rate is sensitive to the ratio of its concentration to that of nucleoside diphosphate. (Optimal rates of reaction with the *E. coli* enzyme[33] as well as with a highly purified *Azotobacter* enzyme[36] were obtained when the ratio of the nucleoside diphosphate to magnesium ions was 1:1.5-2.) As regards the pH optimum, different results were obtained with the *Azotobacter* enzyme using two different assays.[32] It is possible that the different ratio of magnesium ion concentration to ADP concentration used in the two assays was a major cause of the variation in the result.

Beers[35, 37] has also observed rather complex interrelationships between the relative concentrations of ADP and magnesium chloride and the pH optimum of the reaction for the *M. lysodeikticus* enzyme. He[35, 37] has also noted the requirement for high salt concentration for this enzyme. No such requirement was found (in fact, some inhibition by the high concentration of salt was observed) by Singer *et al.*[36] for the purified *Azotobacter* enzyme.

The affinity of the enzyme for nucleoside-5′-diphosphates is very low[32, 33] (*Km*'s of the order of 10^{-2} *M*) and the enzyme is apparently devoid of specificity towards the purine or pyrimidine base in the ribonucleoside-5′-diphosphates. A notable exception is that of guanosine-5′-diphosphate[32, 33] which by itself does not polymerize (see below). At various stages of purification (up to 300-fold) of the *Azotobacter* enzyme, the specific activity with respect to different nucleoside diphosphates increased roughly to the same extent.[38] These results as well as the formation of copolymeric chains of

[34] R. F. Beers, Jr., *Nature* **177**, 790 (1956).
[35] R. F. Beers, Jr., *Biochem. J.* **66**, 686 (1957).
[36] M. F. Singer, L. A. Heppel, and R. J. Hilmoe, *J. Biol. Chem.* **235**, 738 (1960).
[37] R. F. Beers, Jr., *Arch. Biochem. Biophys.* **75**, 497 (1958).
[38] S. Ochoa, S. Mii, and M. C. Schneider, *Proc. Intern. Symposium on Enzyme Chem.* p. 44 (1957).

adenylic and uridylic acids and also of adenylic, guanylic, uridylic, and cytidylic acids point to there being a single enzyme. In contrast, Olmsted[39,39a] working with *M. lysodeikticus* preparations has presented some evidence in favor of the view that different enzymes may be involved in handling different nucleoside diphosphates.

As shown in Eq. (1) the reaction is reversible, although the point of equilibrium may vary somewhat with different nucleoside diphosphates. Under favorable conditions, the forward reaction (Eq. 1) may proceed so far as to give 50–80% of the acid-labile phosphate as orthophosphate. The reaction thus favors polynucleotide synthesis.[32, 33]

With the partially purified enzymes[32, 33, 35] linear rates of polynucleotide synthesis were reported without the addition of any polynucleotide primers. Further purification of the *Azotobacter* enzyme led to the recognition of a lag in the reaction[40] and to the finding that a variety of polynucleotides[36, 40, 41] abolished this lag (Section *c*, below).

b. The Structure and Size of Ribopolynucleotides Synthesized by Polynucleotide Phosphorylase

All the enzymically synthesized polymers contain the repeating $C_{3'}$–$C_{5'}$ interribonucleotide linkage which has previously been established to occur in the ribonucleic acids (Volume I, Chapter 12). Thus, the products formed upon hydrolysis of the synthetic products with alkali and with different phosphodiesterases (venom and spleen phosphodiesterases and pancreatic ribonuclease) are as expected from the established mode of action of these agents.[42, 43]

The alkaline hydrolysis of some polymers gives in addition to a mixture of nucleoside-2′ and 3′-phosphates, small amounts of free nucleosides and nucleoside-2′(3′),5′-diphosphate.[42, 44] The result indicates a polynucleotide structure containing a 5′-phosphomonoester group at one end of the chain and free 2′- and 3′-hydroxyl groups at the other end.[45] The origin of the 5′-phosphomonoester group at the terminus cannot be uniquely explained because of uncertainties regarding the mode of action of polynucleotide phosphorylase. Firstly, if the chain formation begins at the 3′-hydroxyl end

[39] P. S. Olmsted, *Biochim. et Biophys. Acta* **27**, 222 (1958).
[39a] P. S. Olmsted and G. L. Lowe, *J. Biol. Chem.* **234**, 2971 (1959).
[40] S. Mii and S. Ochoa, *Biochim. et Biophys. Acta* **26**, 445 (1957).
[41] M. F. Singer, L. A. Heppel, and R. J. Hilmoe, *Biochim. et Biophys. Acta* **26**, 447 (1957).
[41a] M. F. Singer, R. J. Hilmoe, and L. A. Heppel, *J. Biol. Chem.* **235**, 751 (1960).
[42] L. A. Heppel, P. J. Ortiz, and S. Ochoa, *J. Biol. Chem.* **229**, 679 (1957).
[43] L. A. Heppel, P. J. Ortiz, and S. Ochoa, *J. Biol. Chem.* **229**, 695 (1957).
[44] S. Ochoa, *Federation Proc.* **15**, 832 (1956).
[45] R. Markham, R. E. F. Matthews, and J. D. Smith, *Nature* **173**, 537 (1954).

of a primer, then it is possible that the 5′-phosphomonoester group was either present in the primer itself or it arises by the action of a nuclease present in the enzyme preparation used. (The nuclease would be of the type that is present in guinea pig liver nuclei.[42, 46]) Alternatively, if a chain is synthesized *de novo* then the pyrophosphate group of the terminal nucleoside-5′-diphosphate must be hydrolyzed by some enzymic means.

So far the preparation of five different types of polymers containing only one kind of nucleoside residue (homopolymers) has been reported.[32, 33, 35, 47] These are polyadenylic acid, polyuridylic acid, polycytidylic acid, polyinosinic acid, and poly-(ribofuranosylthymine-phosphate).[47] The notable omission is that of polyguanylic acid. Neither the *Azotobacter* enzyme[32] nor the *E. coli* enzyme[33] is able to polymerize guanosine-5′-diphosphate, although guanosine nucleotide is readily incorporated into a polymer[32, 44] when the diphosphate is present among other nucleoside diphosphates. Of the polymers containing more than one kind of nucleotide residue (heteropolymers), adenylic-uridylic acid polymers as well as polymers containing all the four common ribonucleotides[32, 44] have been prepared. Detailed degradative evidence has been presented[42, 43, 48, 48a] to show that the products obtained by using mixtures of nucleoside-5′-diphosphates contain chains in which the different nucleotides are interlinked with each other.

Polymers synthesized by polynucleotide phosphorylase are large, even though molecular weight determinations by different methods have given varying results. Thus, while the physical methods indicate molecular weights of 30,000 to over a million,[34, 44, 48] determinations of average chain length by end group assays gave lower values (an average chain length of 30 for mixed polymers of adenylic, guanylic, uridylic, and cytidylic acids, 120 for an adenylic-uridylic acid polymer, and 230 for polyadenylic acid).[44, 48] Certainly, there is great latitude in the size as well as the type of polymers that can be prepared enzymically and the scope has been enlarged greatly by the studies of Heppel and co-workers as described below.

c. *Primers for Polynucleotide Phosphorylase*

(*1*) *High Molecular Weight Polynucleotides as Primers.* As mentioned above, using a highly purified *Azotobacter* enzyme preparation, Mii and Ochoa[40] demonstrated a lag phase in the reaction. The lag phase could be overcome by certain high molecular weight polynucleotides. However, a number of interesting but intricate relationships were noted between the

[46] L. A. Heppel, P. J. Ortiz, and S. Ochoa, *Science* **123,** 415 (1956).

[47] B. E. Griffin, A. R. Todd, and A. Rich, *Proc. Natl. Acad. Sci. U. S.* **44,** 1123 (1958).

[48] S. Ochoa and L. A. Heppel, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 615. Johns Hopkins Press, Baltimore, 1957.

[48a] P. J. Ortiz and S. Ochoa, *J. Biol. Chem.* **234,** 1208 (1959).

priming capacity of different polynucleotides and the nucleoside diphosphates being polymerized.[38, 40] The synthesis of homopolymers could be primed by the addition of the corresponding homopolymers; thus, for example, the polymerization of ADP was primed by polyadenylic acid. The synthesis of polyuridylic acid as well as the formation of the mixed polymers of adenylic, guanylic, cytidylic, and uridylic acids was primed somewhat by yeast RNA, and more effectively by preformed mixed polymer or *Azotobacter* RNA. However, the synthesis of polyuridylic acid was inhibited by the addition of polyadenylic acid and, correspondingly, the synthesis of polyadenylic acid was inhibited by polyuridylic acid. Again, the synthesis of polycytidylic acid was primed only by polycytidylic acid but was strongly inhibited by polyadenylic acid, polyuridylic acid, RNA, or the mixed synthetic polymer of the four nucleotides. On the other hand, polycytidylic acid primed not only its own synthesis but also that of all other polynucleotides, including polyadenylic acid, polyuridylic acid, and the mixed polymer of all the four nucleotides.

(*2*) *Oligonucleotides as Primers.* The extensive and definitive studies of Heppel and co-workers[36, 41, 41a] on the utilization of oligonucleotides as primers have not only thrown light on the mode of action of the enzyme but have also made available several new types of polynucleotides in which the end groups and environment near one terminus are known with certainty. The main results may be briefly summarized.

Homologous series of oligonucleotides (pApA, pApApA, pApApApA) as well as those bearing no 5′-phosphomonoester end groups (ApA, ApU, ApApA) serve as primers and are effectively incorporated into the polymers synthesized. All evidence points to the initiation of the synthesis of polynucleotide chains onto the ends of these primers bearing the 3′-hydroxyl group.

The minimum sized primer is a dinucleoside phosphate containing a $C_{5'}$–$C_{3'}$ diester bond. Thus ApA and ApU serve as primers, although not as effectively as the higher homologs. The concentrations required for maximal stimulation of the rate of synthesis decreases with increase in chain length of the primer. Approximate concentrations for such rates are 2.4×10^{-3} *M*, 4.2×10^{-4} *M*, and 1.7×10^{-4} *M*, respectively, for pApA, pApApA, and pApApApA. Similar results have been obtained with the corresponding compounds lacking 5′-phosphomonoester groups. The rates using optimal concentrations of the homologous series of oligonucleotides were the same in the synthesis of polyadenylic acid as well as polyuridylic acid, although the optimal rates using the two nucleoside diphosphates differed.

Unlike the results described above for priming by high molecular weight polynucleotides, no evidence of specificity in the priming by oligonucleotides was obtained. Thus pApApA and related compounds served as primers and

were effectively incorporated into chains of polyadenylic and polyuridylic acids and poly-(ribofuranosylthymine-phosphate).

The oligonucleotides initiated polynucleotide chain formation by utilization of their 3′-hydroxyl groups even with enzyme preparations which did not show primer requirement. In experiments with preparations of highly purified *Azotobacter* enzyme, the oligonucleotides were effectively incorporated even when their concentration far exceeded that required for maximal rate of synthesis.

Using the oligonucleotides as primers, and especially by adjusting the primer to nucleoside diphosphate ratio, the stepwise addition of nucleoside-5′-phosphoryl units to the 3′-hydroxyl ends of preformed oligonucleotide chains could be convincingly demonstrated and the products obtained were, in general, of much lower chain length than those obtained in the absence of primers.

By a mechanism as yet obscure, small oligonucleotides bearing 3′-phosphomonoester end groups and thus containing no free 3′-hydroxyl groups, also served to abolish the lag period in the polymer synthesis, although they were not incorporated into the polymers formed. [It may be noted that working with the enzyme from *M. lysodeikticus*, Hendley and Beers[49] have recorded the inhibition of polynucleotide synthesis by certain mixtures of larger oligonucleotides bearing 3′-phosphomonoester end groups (cf. Beers[50])].

d. Phosphorolysis of Polynucleotides Catalyzed by Polynucleotide Phosphorylase

(*1*) *Phosphorolysis of High Molecular Weight Polymers and Ribonucleic Acids.* Studies of the phosphorolysis of enzymically synthesized polynucleotides and a variety of ribonucleic acids have been reported.[32, 33, 51, 52, 53] Phosphorolysis of enzymically synthesized polynucleotides containing one kind of nucleotide is facile.[32, 33, 51] In contrast, mixing of polyadenylic acid and polyuridylic acid confers great resistance to phosphorolysis.[51] This is obviously due to the interaction to form multistranded chains.[54-56, 56a] The mixed synthetic polymer of the four nucleotides as well as highly polymerized yeast, leaf, and rat liver ribonucleic acids are also phosphorolyzed

[49] D. D. Hendley and R. F. Beers, Jr., *Federation Proc.* **18**, 245 (1959).
[50] R. F. Beers, Jr., *Nature* **183**, 1335 (1959).
[51] S. Ochoa, *Arch. Biochem. Biophys.* **69**, 119 (1957).
[52] D. D. Hendley and R. F. Beers, Jr., *Federation Proc.* **17**, 240 (1958).
[53] P. Lengyel and S. Ochoa, *Biochim. et Biophys. Acta* **28**, 200 (1958).
[54] R. C. Warner, *J. Biol. Chem.* **229**, 711 (1957).
[55] G. Felsenfeld and A. Rich, *Biochim. et Biophys. Acta* **26**, 457 (1957).
[56] L. A. Heppel, M. F. Singer, and R. J. Hilmoe, *Ann. N. Y. Acad. Sci.* **81**, 635 (1959).
[56a] M. Grunberg-Manago, *J. Mol. Biol.*, **1**, 240 (1960).

very slowly.[33, 51, 53] Tobacco mosaic virus ribonucleic acid, in contrast, was found to be phosphorolyzed fairly readily.[51]

(*2*) *Phosphorolysis of Ribo-oligonucleotides.* While the macromolecular structure has an important influence on the phosphorolysis reaction, as has further been shown by Grunberg-Manago,[56, 56a] the studies using well-defined substrates by Singer[57] have shown the requirements of the end group and size for phosphorolysis to occur. Thus, oligonucleotides bearing 3′-phosphomonoester or 2′,3′-cyclic phosphate end groups are resistant, while oligonucleotides bearing 3′-hydroxyl groups are readily phosphorolyzed. The presence of a 5′-phosphomonoester group at the other terminus of the chain is not essential, both series with or without such groups being phosphorolyzed at comparable rates. With equivalent concentrations, a tetranucleotide is more rapidly phosphorolyzed than the corresponding trinucleotide. The smallest oligonucleotide susceptible to phosphorolysis is a trinucleotide or a trinucleoside diphosphate, the dinucleotides and dinucleoside monophosphates being completely resistant. These results are complementary to the findings in the primer work discussed above. The phosphorolysis reaction has further been shown to be stepwise from the end bearing the 3′-hydroxyl group by a kinetic study of homologous compounds of the type ApApU and ApApApU. In this respect, then, the mode of action is completely similar[58] to that of the venom diesterase.[59]

The maximal rate of phosphorolysis occurs at 10^{-2} M concentration of inorganic phosphate and requires magnesium ions.

c. Remarks on the Mechanism of Action of Polynucleotide Phosphorylase

The studies of Heppel and co-workers, as described above, have established the stepwise synthesis as well as the phosphorolysis of phosphodiester bonds from the 3′-hydroxyl end of the oligonucleotide chains. The reaction,[60] which is depicted below, is formally analogous to the other two enzymic reactions described in later sections, namely, the utilization of a ribonucleoside-5′-triphosphate to attach a nucleoside-5′-phosphoryl group to the 3′-hydroxyl end of a preexisting ribopolynucleotide chain, and likewise, the addition of deoxyribonucleotide units to deoxyribopolynucleotide chains.

Despite the above clarification much remains unknown about the reactions catalyzed by polynucleotide phosphorylase (cf., Heppel *et al.*[56]). Since none of the enzyme preparations hitherto available have shown an

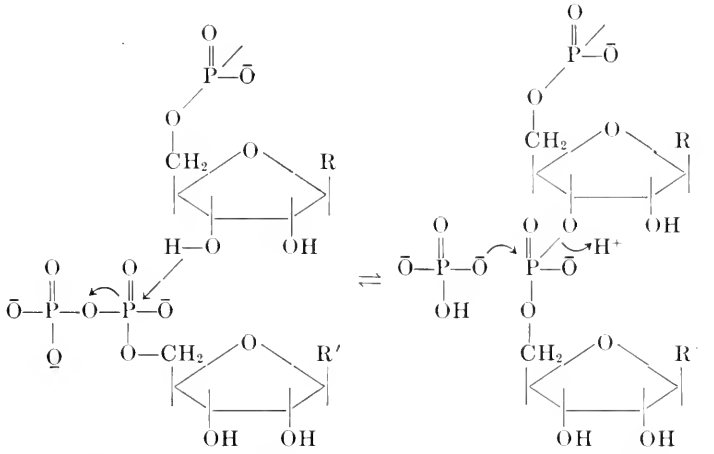
[57] M. F. Singer, *J. Biol. Chem.* **232,** 211 (1958).

[58] R. J. Hilmoe. *Ann. N. Y. Acad. Sci.* **81,** 660 (1959).

[59] W. E. Razzell and H. G. Khorana, *J. Biol. Chem.* **234,** 2114 (1959).

[60] Its reversibility indicates that the free energy of hydrolysis of the phosphodiester bond cannot be very different from that of the pyrophosphate bond.

absolute requirement for a primer, what is the meaning of the lag period? One interpretation might be that the small amount of polynucleotide material which is still present in the enzyme preparations[38] has to be transformed to a suitable primer, perhaps by the action of a contaminating nuclease. Further, what are the mechanisms of the synthetic reactions when polynucleotides and oligonucleotides bearing 3′-phosphomonoester end groups serve as primers? Can a polynucleotide chain be synthesized *de novo* through the agency of this enzyme and can the primers which may not actually be incorporated into the synthetic polymers influence the composition of the latter?



Stepwise Reaction Catalyzed by Polynucleotide Phosphorylase

The behavior of GDP in the enzyme system is unique. No polymerization occurs when present alone even with the crude preparation which readily polymerizes other nucleoside diphosphates. However, incorporation in a polymer chain readily occurs when other nucleoside diphosphates are also present. Interesting are the findings of Heppel and co-workers[41a, 56] on the polymerization of GDP using oligonucleotides such as pApApA as primer. The reaction although slower than that with other nucleoside diphosphates does lead to the stepwise addition of guanosine-5′-phosphoryl residues to the expected end of the primer to give pApApApG and higher polymers.

Another poorly understood reaction catalyzed by polynucleotide phosphorylase is the exchange of the terminal phosphate in nucleoside-5′-diphosphates with inorganic phosphate (P^{32}-labeled) of the medium.[32, 33, 35] Does this reaction proceed via the true reversal of polynucleotide synthesis? The specific case of GDP is again worth noting. With the *Azotobacter* enzyme, relatively crude[32] as well as highly purified,[56] exchange takes place under appropriate conditions just as with other nucleoside diphosphates.

(Under suitable conditions, good rates of exchange of the terminal phosphate group of GDP with the phosphate in the medium have also been ob-obtained with the purified *E. coli* enzyme.[60a])

There are other factors which make the enzymic reaction much more complex than it might appear at first sight. These factors stem from the metal requirement of the reaction. Of the three reaction components involved, a primer, nucleoside diphosphate, and an enzyme, one or more may be required as the metal complex.

It is clear that a prerequisite for an attack on some of these questions is the availability of a completely pure enzyme.

f. Distribution of Polynucleotide Phosphorylase

In addition to the sources mentioned above, the occurrence of the enzyme has been established in extracts of a number of bacterial species.[61] A small activity was found in spinach leaf extracts.[61] Net phosphorolysis of enzymically synthesized polyadenylic acid by a fraction from guinea pig liver nuclei has been reported but as yet net synthesis of polynucleotide has not been demonstrated.[62]

An enzyme has been purified from yeast, which, like the polynucleotide phosphorylase, catalyzes a rapid exchange between labeled orthophosphate and the terminal phosphate groups of the ribonucleoside-5′-diphosphates, but does not phosphorolyze any polynucleotides prepared by the use of the bacterial enzymes, nor does it catalyze polymer formation.[63]

g. Summary of the Types of Oligo- and Polynucleotides Made Available by Polynucleotide Phosphorylase

The discovery of polynucleotide phosphorylase has made available a variety of polynucleotides which in turn have made possible many physicochemical and enzymic studies of polynucleotides. Further, a variety of oligonucleotides has become available by the combined use of polynucleotide phosphorylase, and other enzymes. While the total range and number of polymers available will no doubt be enlarged, the main types that have already been prepared are listed in Table I.

3. Ribonucleoside-5′-Triphosphates as Precursors of Ribopolynucleotides in Mammalian Systems

a. Incorporation of Ribonucleoside-5′-Phosphates into Ribonucleic Acids

The studies on the acid-soluble nucleotide pools of a variety of tissues by Potter and co-workers[30] and on the incorporation of labeled nucleotides

[60a] M. Grunberg-Manago, R. J. Hilmoe, M. F. Singer, and L. A. Heppel, in press.
[61] D. O. Brummond, M. Staehelin, and S. Ochoa, *J. Biol. Chem.* **225,** 835 (1957).
[62] R. J. Hilmoe and L. A. Heppel, *J. Am. Chem. Soc.* **79,** 4810 (1957).
[63] M. Grunberg-Manago and A. del Campillo-Campbell, unpublished results (1959).

TABLE I

OLIGO- AND POLYRIBONUCLEOTIDES PREPARED BY POLYNUCLEOTIDE PHOSPHORYLASE ALONE AND IN COMBINATION WITH OTHER ENZYMES

Polymers	Remarks and References
A. Homopolymers	
(a) Polynucleotides	
(1) Polyadenylic acid, polyuridylic acid, polycytidylic acid, polyinosinic acid, poly-(ribofuranosylthymine-5′-phosphate)	By polymerization of single nucleoside-5′-diphosphates[a, b, c, d]
(b) Oligonucleotides	
(2) pApApA, pApApApA, and homologs	By the action of liver nuclei enzyme on polyadenylic acid [e, f]
(3) ApApA, ApApApA, and homologs	By the action of phosphomonoesterase on type (2)
(4) UpU-Cyclic P, UpUpU-cyclic P, and homologs	By graded action of pancreatic ribonuclease on polyuridylic acid[e]
(5) UpUpU, UpUpUpU, etc.	By ring opening of the terminal cyclic phosphates in (4) followed by phosphomonoesterase[e]
B. Heteropolymers	
(a) Polynucleotides	
(6) Mixed polymers of adenylic, guanylic, uridylic, and cytidylic acids	By copolymerizing the four nucleoside diphosphates[a]
(7) Mixed polymer of adenylic and uridylic acid	By copolymerizing adenosine and uridine-5′-diphosphates[a]
(8) pApApApUpUpU . . .	By using pApApA as primer for polymerizing UDP[g, h]
(9) ApApApUpUpU . . .	By using ApApA as primer for polymerizing UDP[g, h]
(10) pApApGpG . . .	By using pApApA as primer for polymerization of GDP[i]
(b) Oligonucleotides	
(11) ApApUp, ApApApUp, and homologs	By degradation of adenylic-uridylic acid copolymer with pancreatic ribonuclease[j]
(12) ApApU, ApApApU, and homologs	By the action of phosphomonoesterase on type (11)

[a] M. Grunberg-Manago, P. J. Ortiz, and S. Ochoa, *Biochim. et Biophys. Acta* **20**, 269 (1956).
[b] U. Z. Littauer and A. Kornberg, *J. Biol. Chem.* **226**, 1077 (1957).
[c] R. F. Beers, Jr., *Biochem. J.* **66**, 686 (1957).
[d] B. E. Griffin, A. R. Todd, and A. Rich, *Proc. Natl. Acad. Sci. U.S.* **44**, 1123 (1958).
[e] L. A. Heppel, P. J. Ortiz, and S. Ochoa, *J. Biol. Chem.* **229**, 679 (1957).
[f] L. A. Heppel, P. J. Ortiz, and S. Ochoa, *Science* **123**, 415 (1956).
[g] M. F. Singer, L. A. Heppel, and R. J. Hilmoe, *J. Biol. Chem.* **235**, 738 (1960).
[h] M. F. Singer, L. A. Heppel, and R. J. Hilmoe, *Biochim. et Biophys. Acta* **26**, 447 (1957).
[i] L. A. Heppel, M. F. Singer, and R. J. Hilmoe, *Ann. N. Y. Acad. Sci.* **81**, 635 (1959).
[j] L. A. Heppel, P. J. Ortiz, and S. Ochoa, *J. Biol. Chem.* **229**, 695 (1957).

and nucleotide precursors into ribonucleic acids in cell-free systems reported from a number of laboratories[64-69] have led to the now widely accepted conclusion that ribonucleoside-5′-phosphates are intermediates in the biosynthesis of ribonucleic acids.

b. Ribonucleoside-5′-Triphosphates in Additions to Ends of Existing Ribopolynucleotide Chains

A reaction which is now relatively well understood as a result of the work in a number of laboratories is the addition of nucleoside-5′-phosphoryl units derived from the corresponding triphosphates to the 3′-hydroxyl ends of preexisting rather low molecular weight ribonucleic acids. The original observation was recorded by Heidelberger *et al.*[68] that when adenosine-5′-phosphate labeled with P^{32} was incubated with the cytoplasmic fraction of rat liver homogenates, under suitable conditions the nucleotide unit was incorporated *intact* at the terminus of a ribonucleic acid chain. By alkaline degradation it could be further demonstrated that the addition occurred to the 2′- or 3′-hydroxyl group of a terminal cytidine residue. Subsequent work[70-75] has confirmed this observation and furthermore has shown that ATP is the actual donor of the adenosine-5′-phosphoryl residue for this terminal addition. Further understanding of the nature and significance of this reaction has come from the work of Zamecnik and co-workers.[71, 74, 76, 77] The acceptor of the terminal additions was found to be a rather low molecular weight RNA present in the high speed supernatant (soluble) fractions of the rat and Ehrlich ascites carcinoma cell homogenates, while the mi-

[64] E. Goldwasser, *J. Am. Chem. Soc.* **77,** 6083 (1955).
[65] V. R. Potter, L. I. Hecht, and E. Herbert, *Biochim. et Biophys. Acta.* **20,** 439 (1956).
[66] E. Herbert, V. R. Potter, and L. I. Hecht, *J. Biol. Chem.* **225,** 659 (1957).
[67] E. S. Canellakis, *Biochim. et Biophys. Acta* **23,** 217 (1957).
[68] C. Heidelberger, E. Harbers, K. C. Liebman, Y. Takagi, and V. R. Potter, *Biochim. et Biophys. Acta* **20,** 445 (1956).
[69] A. R. P. Paterson and G. A. LePage, *Cancer Research* **17,** 409 (1957).
[70] E. S. Canellakis, *Biochim. et Biophys. Acta* **25,** 217 (1957).
[71] P. C. Zamecnik, M. L. Stephenson, J. F. Scott, and M. B. Hoagland, *Federation Proc.* **16,** 275 (1957).
[72] M. Edmonds and R. Abrams, *Boichim. et Biophys. Acta* **26,** 227 (1957).
[73] E. Herbert, *J. Biol. Chem.* **231,** 975 (1958).
[74] L. I. Hecht, P. C. Zamecnik, M. L. Stephenson, and J. F. Scott, *J. Biol. Chem.* **233,** 954 (1958).
[75] E. Herbert, *Federation Proc.* **17,** 241 (1958).
[76] M. B. Hoagland, M. L. Stephenson, J. F. Scott, L. I. Hecht, and P. C. Zamecnik, *J. Biol. Chem.* **231,** 241 (1958).
[77] L. I. Hecht, M. L. Stephenson, and P. C. Zamecnik, *Biochim. et Biophys. Acta* **29,** 460 (1958).

crosomal and nuclear RNA are inert. The enzymes responsible for the terminal additions are also present in these soluble fractions (designated pH 5 fraction by these authors).[74, 78] By incubations with labeled CTP and ATP, the additions have been shown to occur in a definite sequence. With CTP alone two cytidine-5′-phosphoryl residues may be added to the 3′-hydroxyl end of the chains and the final addition is that of a single adenosine-5′-phosphoryl residue. The last addition can only occur when the cytidylyl residues are present. This is in agreement with the earlier studies described above. Labeled UTP when incubated in the absence of other nucleoside triphosphates is also capable of adding a uridine-5′-phosphoryl residue to the ends of polynucleotide chains.

The terminal additions are magnesium-dependent and are inhibited by inorganic pyrophosphate. In fact, the above additions have been shown to be reversed by inorganic pyrophosphate.

It seems probable that the RNA-dependent incorporation and the exchange reactions of ribonucleoside-5′-triphosphates in extracts of embryonic chicken livers[79] are another example of the pyrophosphorolysis of the end groups of soluble RNA (see, however, ref. 79[a]).

Hurwitz[80] *et al.* have reported on the purification of an enzyme from calf thymus nuclei which is specific for transferring a cytidine-5′-phosphoryl group from CTP to the ends of ribonucleic acid chains. The requirement for RNA is highly specific in that only that from thymus gland serves as the acceptor and additions can occur to chains bearing all of the four different ribonucleosides at the ends. Since the enzyme is also highly specific for CTP it would appear that different enzymes are required for additions of different nucleotides to the ends.

Hecht *et al.*[77] and a number of other workers[81, 82] have shown that the end unit containing adenosine nucleotide provides a functional grouping in the soluble RNA[76] which is required for its action as a carrier of activated amino acids.

[78] E. B. Keller and P. C. Zamecnik, *J. Biol. Chem.* **221**, 45 (1956).

[79] C. W. Chung and H. R. Mahler, *J. Am. Chem. Soc.* **80**, 3165 (1958).

[79a] *Added in proof:* Chung and Mahler [*Biochem. Biophys. Research Communs.* **I**, 232 (1959)] have presented further evidence showing that soluble enzyme preparations from cytoplasmic fractions of chick embryo hearts or livers are capable of incorporating adenosine-5′-phosphoryl portion of ATP-C^{14} into RNA in nonterminal positions.

[80] J. Hurwitz, A. Bresler, and A. Kaye, *Biochem. Biophys. Research Communs.* **1**, 3 (1959).

[81] H. G. Zachau, G. Acs, and F. Lipmann, *Proc. Natl. Acad. Sci. U. S.* **44**, 885 (1958).

[82] J. Preiss, P. Berg, E. J. Ofengand, F. H. Bergmann, and M. Dreckmann, *Proc. Natl. Acad. Sci. U. S.* **45**, 319 (1959).

c. Incorporation of Ribonucleoside-5′-Triphosphates into the Interior of Ribopolynucleotide Chains

Goldwasser[64] presented evidence that in the pigeon liver homogenates, labeled adenosine-5′-phosphate was incorporated intact into the interior of RNA chains. The incorporation of labeled pyrimidine nucleotide precursors into the nuclear fractions of rat liver homogenates was demonstrated by Herbert *et al.*[66] Edmunds and Abrams[72] working with soluble fractions of Ehrlich ascites tumor cells presented evidence for the utilization of C^{14}-labeled ATP for the inner labeling of RNA and indicated that different enzyme systems are involved in the terminal and inner incorporation of nucleotides. Herbert[73, 75] has demonstrated that the enzymes responsible for the appearance of adenine nucleotides into the interior of RNA are present in the particulate fractions of tissue homogenates.

Using a nuclear fraction of rat liver homogenates, Weiss and Gladstone[83] have recently described the incorporation of labeled CTP into the interior of RNA. The incorporation is dependent on the presence of the other three ribonucleoside triphosphates and magnesium. The triphosphates are more than twice as effective as the corresponding diphosphates. In these properties, the system resembles the polymerases for deoxyribonucleotides described in the following section.

It should be pointed out that in the above studies the evidence for incorporation of ribonucleotides into the interior of ribopolynucleotide chains has been derived only from the alkaline hydrolysis which results in the transfer of the P^{32}-label in the nucleoside-5′-phosphoryl group incorporated to the adjacent nucleotide. It is highly desirable to obtain additional evidence for "extensive" internal incorporation of ribopolynucleotide precursors. A promising tool in this field is provided by the venom phosphodiesterase.

Finally, while the evidence that is accumulating points to ribonucleoside-5′-triphosphates as being the precursors of polynucleotides it is clear that the realization of well-defined enzymic systems from mammalian sources for extensive ribopolynucleotide synthesis has yet to come.

IV. Enzymic Synthesis of Deoxyribopolynucleotides

Deoxyribonucleoside-5′-triphosphates have so far been found to be unique precursors for the enzymic synthesis of deoxyribopolynucleotides. This is in contrast with the synthesis of ribopolynucleotides, which utilizes both the 5′-di- and triphosphates of ribonucleosides as the monomeric units.

[83] S. B. Weiss and L. Gladstone, *J. Am. Chem. Soc.* **81**, 4118 (1959).

1. Microorganisms

The studies of Kornberg and co-workers with a system prepared from *Escherichia coli* (Strain B or ML 30) have provided the lead and all the definitive information that is available on the bacterial systems. The system has already made it possible to test many of the tenets of the well-known Watson-Crick[84] structure for the deoxyribonucleic acids (DNA) and it appears that in the near future much more insight into the chemistry of the biosynthesis of DNA and its replication will be gained from these studies.

a. Basic Requirements of the Enzymic System[85, 86]

The enzyme which has been called polymerase, has been purified 2000–4000-fold.[85] It is inactivated upon heating at neutral pH's and is inhibited by high concentrations of salt. Inhibition of DNA synthesis by sodium ions has also been reported by Walwick and Main.[87] The requirements for maximal activity are the presence of the 5′-triphosphates of all the four deoxyribonucleosides (thymidine, deoxycytidine, deoxyadenosine, and deoxyguanosine), magnesium ions, and a primer. DNA from a variety of sources can serve as the primer. Under these conditions, the reaction may be assayed by conversion of the acid-soluble mononucleotides to an acid-insoluble polymer and extensive net synthesis (2–20 times the amount of the primer) can be realized.[86] Omission of any one of the triphosphates, or of either of the other components, or digestion of primer DNA with crystalline pancreatic deoxyribonuclease (however, see below, Sections *g* and *i*) reduces the reaction to below 1 %.[86] The triphosphates cannot be replaced by the corresponding diphosphates. The over-all reaction may therefore be represented as follows:

$$\begin{matrix} n\ \text{dATP} \\ + \\ n\ \text{dGTP} \\ + \\ n\ \text{dCTP} \\ + \\ n\ \text{TTP} \end{matrix} + \text{DNA} \rightleftharpoons \text{DNA}\text{—}\left[\begin{matrix} \text{dAP} \\ \\ \text{dGP} \\ \\ \text{dCP} \\ \\ \text{TP} \end{matrix}\right]_n + 4n\ \text{PP}$$

[84] J. D. Watson and F. H. C. Crick, *Nature* **171**, 737, 964 (1953).

[85] I. R. Lehman, M. J. Bessman, E. S. Simms, and A. Kornberg, *J. Biol. Chem.* **233**, 163 (1958).

[86] M. J. Bessman, I. R. Lehman, E. S. Simms, and A. Kornberg, *J. Biol. Chem.* **233**, 171 (1958).

[87] E. R. Walwick and R. K. Main, U.S. Navy Radiological Defense Laboratory, Tech. Rept. No. 319 (1959).

By using thymidine-5′-triphosphate labeled in the ring (C^{14}) and in the pyrophosphate (P^{32}), good stoichiometry was demonstrated between the nucleotide incorporated and inorganic pyrophosphate released.[86]

b. The Chemical and Physical Properties of the Synthetic Products

Net synthesis of polymer has provided material for physicochemical studies and comparison with the DNA used as primer. The enzymically synthesized product has essentially the same physical properties as DNA prepared from calf thymus.[88] Thus, the sedimentation and viscosimetric behavior are quite similar. The observed greater polydispersity of the enzymic product was ascribed to the action of contaminating nucleases in the polymerase preparation. From the data, several synthetic products were concluded to have average molecular weights in the neighborhood of 5 to 6 million and to have highly ordered rigid structures with effective volumes greater than would be expected from single polynucleotide chains. The behavior on heating at 100°, namely the collapse of macromolecular structure, was again similar to that of calf thymus DNA. Furthermore, degradation with pancreatic deoxyribonuclease gave the typical hyperchromic effect shown by DNA[89] (Volume I, Chapter 14).

The evidence for the nature of internucleotide linkages in the enzymically synthesized products comes from the degradation with pancreatic deoxyribonuclease.[86] The extent and pattern of degradation is very similar to that previously established for DNA. Furthermore, when one of the four nucleotide substrates, namely thymidine-5′-triphosphate, labeled with C^{14} was used, degradation by pancreatic deoxyribonuclease gave in the appropriate region of the ion exchange elution diagrams labeled dinucleotides which were identified as deoxycytidylyl-(5′ → 3′)-thymidylic-(5′) acid and thymidylyl-(5′ → 3′)-thymidylic-(5′) acid. The labeled starting material was thus incorporated in typical $C_{3'}$–$C_{5'}$ internucleotide linkages in polynucleotide chains. From this and further experiments, as well as the evidence from the limited synthetic reaction discussed below, it may be concluded that the synthetic reaction results in the formation of the internucleotide linkages known to exist in DNA.

c. Reversal of the Synthetic Reaction[86]

Inorganic pyrophosphate in the reaction mixture when present in the same concentration as the deoxyribonucleoside triphosphates (1.6×10^{-5} *M*) has no effect on the rate of the synthetic reaction. However, at approximately one hundred times the above concentration the synthetic rate is

[88] H. K. Schachman, I. R. Lehman, M. J. Bessman, J. Adler, E. S. Simms, and A. Kornberg, *Federation Proc.* **17,** 304 (1958).

[89] M. Kunitz, *J. Gen. Physiol.* **33,** 349 (1950).

inhibited by 50% by the pyrophosphate. Under these conditions when labeled pyrophosphate is used, its incorporation into the terminal pyrophosphate groups of the four nucleoside-5′-triphosphates is found. The rate of this exchange reaction is comparable to the synthetic rate in the absence of the added pyrophosphate. Like the synthetic reaction, the exchange reaction is dependent upon the presence of DNA. DNA alone in the presence of an excess of inorganic pyrophosphate and the polymerase undergoes only a small amount of pyrophosphorolytic cleavage.

There is a striking difference between the synthetic and pyrophosphorolytic reaction in respect of the effect of omitting one or more of the deoxyribonucleoside-5′-triphosphates from the reaction mixture. Whereas synthesis is reduced to a fraction of a per cent (see below), the reversal is affected relatively little. In other words, appreciable exchange of the pyrophosphate grouping occurs when only one of the nucleoside-5′-triphosphates is present.

The above findings have been interpreted to mean[56] that whereas pyrophosphorolysis of a long tightly coiled hydrogen-bonded molecule is slow and difficult, single or a few nucleotide units freshly added to the preexisting chain are readily pyrophosphorolyzed. The stabilization effect must be a cumulative one since it would appear that the selection of the incoming nucleotide is governed by prior hydrogen bonding (see below).

d. The Base Composition of the Product as a Function of the DNA Used as Primer[90]

The extended studies of Chargaff and co-workers (Volume I, Chapter 10) on the analytical composition of samples of DNA from a great variety of sources showed the remarkable generalization that purine content always equals the pyrimidine content. Although among the purines, the adenine content may differ greatly from that of guanine and similarly, among the pyrimidines, thymine from cytosine, the total equivalence (adenine + guanine = cytosine + thymine) is maintained by the fact that adenine content equals that of thymine and, again, guanine corresponds in amount to cytosine. The base equivalence provided originally the strongest chemical support for the Watson-Crick[84] postulate for DNA structure.

Using DNA of different origins (bacterial, thymus, T2 phage) as primers in their system, Kornberg and co-workers have found that the base composition of the enzymically synthesized DNA always resembles that of the primer used. Thus, in the synthetic products, adenine and thymine were present in approximately equal amounts and similarly the guanine content

[90] I. R. Lehman, S. B. Zimmerman, J. Adler, M. J. Bessman, E. S. Simms, and A. Kornberg, *Proc. Natl. Acad. Sci. U. S.* **44**, 1191 (1958).

was the same as that of cytosine. Hence, there was the characteristic equivalence between the purine and pyrimidine content. Furthermore, the ratio of adenine + thymine/guanine + cytosine was always close to that found for the primer used.

The same results were obtained when the net synthesis had proceeded to furnish largely new product as well as at very early stages of the synthetic process. Furthermore, the characteristics of the base composition were maintained even when in the reaction mixtures the relative concentrations of the individual triphosphates were varied.[90]

The above results and the findings reported below on the incorporation of analogs in the enzymically synthesized products[91] lead to the conclusion that the selection of precursors according to their hydrogen bond forming characteristics, is an important feature of the reaction catalyzed by the polymerizing enzyme. Furthermore, the influence of the primer DNA on the composition of the synthetic products provides support for the biologically attractive idea that the primer DNA serves as a template for the synthesis of new DNA.

e. The Incorporation of Purine and Pyrimidine Analogs[91]

Extensive studies *in vivo* have previously shown that certain structural analogs of the naturally found purines and pyrimidines may be incorporated into DNA. Studies with the bacterial polymerase using the triphosphates of several of the familiar analogs have shown again that the enzymic reaction is governed by the specific base-pairing rule in the Watson-Crick model. In each case, the analog substitutes specifically for the base it closely resembles with respect to its hydrogen-bonding properties. Thus, in the complete reaction mixture, 5-bromodeoxyuridine-5′- as well as deoxyuridine-5′-triphosphates can replace thymidine-5′-triphosphate but not any one of the other three essential precursors (deoxyadenosine, deoxyguanosine, and deoxycytidine triphosphates). Similarly 5-bromo- and 5-methyldeoxycytidine-5′-triphosphates replaced only deoxycytidine triphosphate in the full complement of the triphosphates. A purine analog, deoxyinosine triphosphate could replace deoxyguanosine triphosphate but not any one of the other triphosphates. It may be noted that the synthetic rates in the presence of the various analogs varied considerably.

In the case of the synthetic product obtained when labeled deoxyuridine-5′-triphosphate was used in place of the corresponding thymidine compound, it was demonstrated that deoxyuridine was bound in a 3′–5′ phosphodiester linkage with each of the deoxyribonucleosides of adenine, guanine, cytosine, and uracil.

[91] M. J. Bessman, I. R. Lehman, J. Adler, S. B. Zimmerman, E. S. Simms, and A. Kornberg, *Proc. Natl. Acad. Sci. U. S.* **44**, 633 (1958).

f. The Limited Synthetic Reaction[92]

Although maximal synthesis by the polymerase requires the complement of four deoxyribonucleoside-5′-triphosphates, a very limited reaction has been noted when only one of the triphosphates is present. The extent of this reaction is only about one-thousandth of that of the complete reaction and has been shown to result in the addition of a single or sometimes runs of a few nucleotide units to those ends of the primer DNA chains which bear 3′-hydroxyl groups. The conclusion that the incorporated deoxyribonucleotides were indeed in 5′–3′ phosphodiester linkage with primer DNA was established by carrying out the limited reaction in the presence of a single deoxyribonucleoside-5′-triphosphate isotopically labeled in the innermost (stable) phosphate group. Degradation of the product, by the micrococcal deoxyribonuclease[93] followed by spleen phosphodiesterase,[94] to deoxyribonucleoside-3′-phosphates released the labeled phosphate group in each of the nucleoside-3′-phosphates. The location of the incorporated nucleotides at or near the ends of the DNA chains bearing 3′-hydroxyl groups was further demonstrated by graded action of venom phosphodiesterase on the products. In keeping with the mode of action of the latter enzyme as recently elucidated,[59] the radioactivity residing in the added nucleotide(s) was released in the time during which less than 3% of the total polymer had been degraded.

g. The Formation of Deoxyadenylic-Thymidylic Polymer

A brief report has been made[90] of the highly interesting observation that a copolymer of deoxyadenylic acid and thymidylic acid is formed by the polymerase in the absence of any primer after a lag period of 3–6 hours. This synthetic material can, in turn, serve as primer and thus abolish the time lag in the synthesis of more of the same type of material. Even though all the four deoxynucleoside-5′-triphosphates may be present, polymerization occurs so as to form only the deoxyadenylic-thymidylic polymer.

h. DNA Synthesis in T2 Phage-Infected Cells

It is known that a phage-infected *E. coli* cell ceases to produce its own DNA but makes instead the DNA characteristic of the infecting phage.[95]

[92] J. Adler, I. R. Lehman, M. J. Bessman, E. S. Simms, and A. Kornberg, *Proc. Natl. Acad. Sci. U. S.* **44**, 641 (1958).

[93] L. Cunningham, B. W. Catlin, and M. P. deGarilhe, *J. Am. Chem. Soc.* **78**, 4642 (1956).

[94] L. A. Heppel and R. J. Hilmoe, *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. II, p. 547.

[95] A. D. Hershey, J. Dixon, and M. Chase, *J. Gen. Physiol.* **36**, 777 (1952–1953).

Recent studies appearing from different laboratories[96-101] have thrown light on the possible enzymic events occurring in the cell upon phage infection that ensure the synthesis of phage DNA. The results can only be briefly summarized. (1) The first report came from Flaks and Cohen[100-101] who showed that within several minutes after infection by T2, T4, or T6 phages a new enzyme appears which hydroxymethylates deoxycytidine-5′-phosphate to 5-hydroxymethyldeoxycytidine-5′-phosphate. A mechanism is thus present for the formation of 5-hydroxymethylcytosine, a characteristic component of DNA of T-even phages. (2) Since the polymerase requires all the precursors at the triphosphate level of phosphorylation, the second discovery vital to the synthesis of T2 phage DNA was that some minutes after phage infection, an enzyme which phosphorylates 5-hydroxymethyl cytosine-deoxyribonucleotide to the corresponding triphosphate is induced.[96, 98, 98a] In addition, the levels of kinases for several of the other deoxyribonucleoside-5′-phosphates were also increased except for the corresponding enzyme for deoxycytidine-5′-phosphate.[96] (3) A further event occurring upon infection is the induction of an enzyme which destroys deoxycytidine-5′-triphosphate[96, 99] (product of deoxycytidine-5′-phosphate kinase which still can be demonstrated in the infected cell).[96] The products of the action of the new deoxycytidine-triphosphatase are inorganic pyrophosphate and deoxycytidine-5′-phosphate, the latter being returned, as it were, for the hydroxymethylating enzyme mentioned above under (1). It thus appears that the selection of the base hydroxymethylcytosine for T2 phage DNA synthesis is ensured by effective removal of the essential precursor for the incorporation of the normal base, cytosine. (4) The DNA's of T-even phages contain glucose linked to the hydroxymethyl groups of 5-hydroxymethylcytosine.[102-104] An enzyme which transfers glucosyl moieties to such groups, presumably at specific sites on the synthesized DNA has been demonstrated in T2 infected cells.[96] The glucosyl donor is the important uridine diphosphate glucose and the transferase is clearly another enzyme

[96] A. Kornberg, S. B. Zimmerman, S. R. Kornberg, and J. Josse, *Proc. Natl. Acad. Sci. U. S.* **45**, 772 (1959).

[97] J. F. Koerner and M. S. Smith, *Federation Proc.* **18**, 264 (1959).

[98] R. Somerville and G. R. Greenberg, *Federation Proc.* **18**, 327 (1959).

[98a] R. Somerville, K. Ebisuzaki, and G. R. Greenberg, *Proc. Natl. Acad. Sci. U.S.* **45**, 1240 (1959).

[99] J. F. Koerner, M. S. Smith, and J. M. Buchanan, *J. Am. Chem. Soc.* **81**, 2594 (1959).

[100] J. G. Flaks and S. S. Cohen, *Biochim. et Biophys. Acta* **25**, 667 (1957).

[101] J. G. Flaks and S. S. Cohen, *Federation Proc.* **17**, 220 (1958).

[102] M. A. Jesaitis and W. F. Goebel, Cold Spring Harbor *Symposia Quant. Biol.* **18**, 205 (1953).

[103] R. L. Sinsheimer, *Science* **120**, 551 (1954).

[104] E. Volkin, *J. Am. Chem. Soc.* **76**, 5892 (1954).

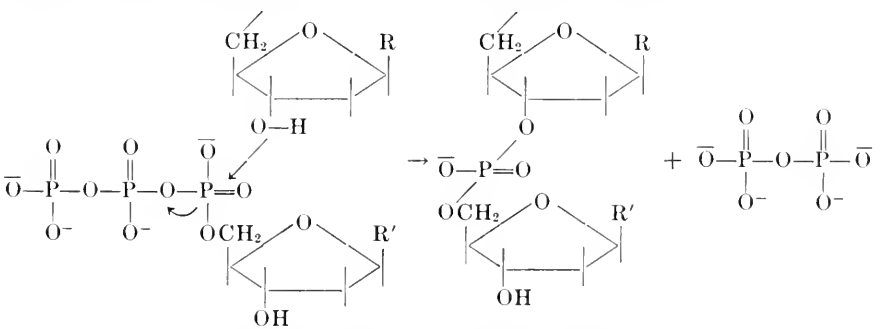
which is induced as a consequence of T-even phage infection. It should be noted that this substitution or alteration of a pyrimidine moiety at polynucleotide level is the first report of its kind. All other interconversions and transformations of the polynucleotide constituents have hitherto been found to occur at the monomeric level.

Intriguing are the observations[96] made on polymerase assays in extracts of *E. coli* after T2 phage infection. When assayed with "native" calf thymus or T2 phage DNA as the primer, the amounts of the polymerase found in normal and infected cells were found to be similar. However, a twelvefold increase of the enzyme in the infected cells was found when DNA previously heated at pH 9 was used as the primer. Thus, it is not clear whether infection results in the formation of a new type of polymerase or in an increase of the normal polymerase with new factors governing its action. As mentioned above, the polymerase from the infected cells can utilize the normal cell DNA as the primer. However, since the DNA synthesized after infection has the base composition of T2 phage DNA, it must be assumed that the injected phage DNA somehow becomes the chosen primer for synthesis after infection. Seen in a different way, the phenomenon may be explained by assuming that the nuclease which has been found to increase[104a] on phage infection destroys selectively in some manner the intrabacterial DNA. (The steady decomposition of bacterial DNA on phage infection had been reported upon previously by Hershey and co-workers.[95])

The work described above may be regarded as being at an initial phase but clearly opens up vistas of investigations, at the enzymic level, of complex phenomena. Much more will no doubt be learned in this field in the near future.

i. The Mechanism of the Synthetic Reaction

In bond-breaking and bond-forming respects the reaction which is illustrated below is similar to those discussed in earlier sections.



[104a] A. B. Pardee and I. Williams, *Ann. inst. Pasteur*, **84**, No. 1, 147 (1953).

Enzymically and biologically the most important question resides in the function of the primer DNA. The information gained in the limited synthetic reaction shows that reaction occurs as above at the existing 3′-hydroxyl end group of the primer chain, similar to the priming of polynucleotide phosphorylase action by ribo-oligonucleotides. Although the relationship between the limited and the extensive synthetic reactions is not clear, it appears likely[92] that the same enzyme is involved in the two reactions. Some support in favor of the idea that 3′-hydroxyl ends are required for the polymerase action may be derived from the observations that already exist. Brief treatment of the primer DNA with pancreatic deoxyribonuclease stimulates[86] about threefold the rate of the synthetic reaction. Also highly pertinent are the observations of Bollum[105] with the calf thymus polymerase (see below) that DNA preparations of rather low viscosity from salmon sperm were effective as primers whereas "native" DNA from calf thymus was not.

The influence of primer DNA on the composition of the product and the incorporation of purine and pyrimidine analogs point to the idea of primer DNA serving as a template for the newly synthesized DNA. As with the polynucleotide phosphorylase, the urgent question that needs to be answered is whether new chains are synthesized by the polymerase systems. The finding of Bollum[105] (see also Lehman[106]) that heating converted non-priming DNA's to effective primers could be interpreted to mean that a single-stranded DNA is in fact required as a primer and that the polymerase catalyzes the synthesis of the complementary strand. The single-stranded DNA of ϕX174 phage[107] serves as primer and has been reported not to require heat activation.[106] The function of unwinding double-stranded DNA to a single-stranded DNA could then also be ascribed to pancreatic deoxyribonuclease in the *E. coli* system.

In the events that the primer is a single-stranded DNA and that the action of the polymerase consists of the synthesis of the complementary chain, then the synthetic reaction should cease when the amount of DNA has been doubled.[105] DNA increases of more than twofold would be possible only if the polymerase systems contained enzyme(s) capable of converting two-stranded non-primer DNA to single-stranded primer DNA.[105] An alternative explanation for extensive synthesis would be that the synthetic reaction involves, as mentioned above, additions to preexisting chains and that the elongated chains are continually broken down by nucleases (phosphodiesterases) with the liberation of new 3′-hydroxyl end groups.

Bollum's recent experiments indicate that synthetic thymidine oligo-

[105] F. J. Bollum, *J. Biol. Chem.* **234**, 2733 (1959).
[106] I. R. Lehman, *Ann. N. Y. Acad. Sci.* **81**, 745 (1959).
[107] R. L. Sinsheimer, *J. Mol. Biol.* **1**, 43 (1959).

nucleotides[24, 26] can serve as primers for the calf thymus polymerase.[107a] These initial observations offer the promise that further insight into the mode of action of the polymerases will be forthcoming in the near future from studies using different types of synthetic oligonucleotides of known structures.

2. Mammalian Systems

a. Rat Liver

Bollum and Potter[108] demonstrated the incorporation of tritiated thymidine into DNA in homogenates from regenerating rat liver. The multienzyme system responsible for the incorporation was found to be present in the soluble high-speed supernatant fraction. Some further properties of the system were reported by these authors as well as by Mantsavinos and Canellakis.[109] In regenerating rat liver the appearance of the enzyme system (after about 18 hours regeneration period) was correlated with the appearance of DNA synthesis *in vivo*.[110] The requirements of the system were established to be[108, 109] the presence of DNA, an energy source, and the incorporation was stimulated by the complementary deoxyribonucleoside-5′-phosphates and inhibited by pyrophosphate. By partial purification of the polymerizing enzyme in the supernatant fraction, Bollum[111] has demonstrated more clearly the requirements for Mg^{++}, DNA, and the presence of all four deoxyribonucleoside-5′-triphosphates, for maximal incorporation of tritium labeled thymidine-5′-triphosphate. In all these properties, therefore, the rat liver enzyme is similar to the bacterial system described above.

b. Thymus

The incorporation of C^{14}-thymidine into DNA by rabbit thymus nuclei was reported by Friedkin and Wood.[112] The presence of the polymerase type of activity has been shown in thymus by Harford and Kornberg[113] and by Bollum.[114] The requirements of this polymerase[115] are, again, the four deoxyribonucleoside triphosphates, DNA and Mg^{++} and Bollum[116] has

[107a] F. J. Bollum, *Federation Proc.* **19,** 305 (1960).
[108] F. J. Bollum and V. R. Potter, *J. Am. Chem. Soc.* **79,** 3603 (1957); *J. Biol. Chem.* **233,** 478 (1958).
[109] R. Mantsavinos and E. S. Canellakis, *J. Biol. Chem.* **234,** 628 (1959).
[110] F. J. Bollum and V. R. Potter, *Cancer Research* **19,** 561 (1959).
[111] F. J. Bollum, *J. Am. Chem. Soc.* **80,** 1766 (1958).
[112] M. Friedkin and H. Wood, *J. Biol. Chem.* **220,** 639 (1956).
[113] C. G. Harford and A. Kornberg, *Federation Proc.* **17,** 515 (1958).
[114] F. J. Bollum, *Federation Proc.* **17,** 193 (1958).
[115] F. J. Bollum, *Ann. N. Y. Acad. Sci.* **81,** 792 (1959).

purified the system to the point that net synthesis of DNA has been achieved.

c. Other Tissues

Bollum and Potter[108] have found significant amounts of the polymerase enzyme in a variety of rat tissues. The enzyme was also found in relatively large amounts in extracts of Flexner-Jobling carcinoma and Walker 256 carcinoma. Harford and Kornberg[113] have shown the presence of polymerase in extracts of HeLa cells, lymph glands, and leukemic blood cells. Mantsavinos and Canellakis[117] have also reported on the preparation and properties of the soluble enzyme from mouse leukemic cells. Net synthesis of DNA in soluble extracts of Ehrlich ascites cells has been demonstrated by Davidson and co-workers.[118, 119]

[116] F. J. Bollum, *Federation Proc.* **18,** 194 (1959).

[117] R. Mantsavinos and E. S. Canellakis, *Cancer Research* in press.

[118] J. N. Davidson, R. M. S. Smellie, H. M. Keir and A. H. McArdle, *Nature*, **182,** 589 (1958).

[119] R. M. Smellie, H. M. Keir, E. D. Gray, D. Bell, J. Richards, and J. N. Davidson, *Biochem. J.* **72,** 17P (1959); *Biochim. et Biophys. Acta* **37,** 243 (1960).

CHAPTER 32

Chemistry of the Nucleic Acids of Microorganisms*

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I. Introduction

As was known long ago,[1-6] bacteria and other microorganisms are characterized by a high nucleic acid content in comparison with that of the cells and tissues of higher organisms. The study of the dynamics of the nucleic acid content in microorganisms showed that the nucleic acid content undergoes considerable variations and is closely connected with the physiological state of the culture, i.e., with its age, activity, and growth.[3, 7-12]

* Translated by Mrs. N. Roomyantseva.

[1] A. N. Belozersky, *Mikrobiologia* **9,** 107 (1940).

[2] R. Vendrely, "Um symposium sur les protéines," p. 165. Masson et Cie, Paris, 1946.

[3] A. N. Belozersky, *Cold Spring Harbor Symposia Quant. Biol.* **12,** 1 (1947).

[4] A. Boivin, R. Vendrely, and R. Tulasne, *Bull. acad. natl. méd.* **131,** 39 (1947).

[5] G. Leonardi, *Compt. rend. acad. sci.* **229,** 393 (1949).

[6] I. Leslie, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. 2, p. 41. Academic Press, New York, 1955.

[7] A. N. Belozersky, *Mikrobiologia* **10,** 185 (1941).

[8] B. Malmgren and C.-G. Hedén, *Acta Pathol. Microbiol. Scand.* **24,** 417, 437, 448, 472 (1947).

[9] B. Malmgren and C.-G. Hedén, *Nature* **159,** 577 (1947).

[10] T. Caspersson, *Symposia Soc. Exptl. Biol.* **1,** 127 (1947).

[11] A. Boivin, *Compt. rend. soc. biol.* **142,** 1258 (1948).

[12] P. C. Caldwell, E. L. Mackor, and C. Hinshelwood, *J. Chem. Soc.* p. 3151 (1950).

In recent years, however, along with a purely quantitative approach, the study of the nucleic acid quality, i.e. of their specificity, acquired an ever greater importance. It was, in fact, in the bacteria that there was revealed the possibility of the existence of qualitative differences between the nucleic acids of different origin, i.e., the possibility of their biological specificity.[13, 14]

Today, therefore, microorganisms attract ever increasing attention as the organisms in which the role of nucleic acid activity is most clearly revealed. Vast experimental material has been obtained with microorganisms, which elucidates the role of nucleic acids in the processes of protein synthesis, hereditary phenomena, etc. That is why this group of organisms seems to be particularly attractive for the study of the general problems of the chemistry and the biochemistry of the nucleic acids.

The present review is restricted, mainly, to the chemical aspects of the problem. The following will be considered: (*a*) various aspects of variations of the nucleic acid content in microorganisms; (*b*) constituents of microbial nucleic acids; and (*c*) composition and specificity of microbial nucleic acids.

II. Nucleic Acid Content and Dynamics

It is well known that the nucleic acid content of microbial cells undergoes great variations depending on the growth phase, the conditions of cultivation, and the physiological and functional state of the cells, as well as on the strain itself or on certain physiological or morphological changes (e.g., acquisition of resistance to an antibiotic or transition into the L-form).

In view of this fact, earlier data on the nucleic acid content in different species of microorganisms[1, 2, 4, 5] are of somewhat limited value since they do not take into consideration these circumstances which are often of decisive importance.

Variations of nucleic acid content with the age of the culture in bacteria were studied for the first time by Belozersky[7] on cultures of *Spirillum*. Later, similar chemical studies were carried out on a number of other strains.[3, 11] It was noted that young cultures were always characterized by a higher nucleic acid content that the old ones. In the process of aging, there always occurred a regular decrease of the nucleic acid content.

A detailed study of the dynamics of the nucleic acid content during growth was, for the first time, carried out by means of spectrography in the ultraviolet range in the investigations of Malmgren and Hedén.[8, 9] These studies demonstrated that an accumulation of nucleic acids takes place in the bacterial cell at the lag-phase preparatory to division. After the nucleic acid content has attained a certain level, the division of cells, i.e. the multiplication of bacteria, becomes possible. From this moment, the amount

[13] O. T. Avery, C. M. MacLeod, and M. McCarty, *J. Exptl. Med.* **79**, 137 (1944).

[14] E. Chargaff, *2nd Intern. Congr. Biochem., Paris* "Symp. sur le métabolisme microbien," p. 41 (1952).

of nucleic acids in each bacterial cell increases proportionally to the rate of increase of growth and cell division, soon attaining its maximum. The amount of nucleic acids in the cells decreases with the slowing down of cell division, and at the end of the growth cycle the nucleic acid content returns to the original level, accompanied by the arrest of the division of the cells.

Thus, all the data obtained suggested that a correlation does exist between the age, the biological activity, and the rate of growth of the cell on the one side, and its nucleic acid content on the other.

However, these data concerned, mainly, the dynamics of the over-all nucleic acid content in microbial cells. Further investigations were aimed at the elucidation of the behavior of each nucleic acid, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), separately.

It should be noted that a review concerning various quantitative and qualitative aspects of microbial DNA was recently published by Vendrely.[15]

1. Deoxyribonucleic Acid

The investigations of Caldwell and Hinshelwood[16] carried out on *Aerobacter aerogenes* showed that the DNA content per cell remains constant when growing the culture under the most varied conditions, in spite of the variation of the size of the cells, the RNA content, and the rate of growth. Webb[17] has also shown that the growth inhibition of *Clostridium welchii* by means of magnesium, which brings about a strong modification of the morphology of the cells, does not alter the constant DNA content in the cell. The constancy of the DNA content in *Hemophilus pertussis*, despite changes in the nutritional medium, was shown by Rumanian authors.[18] Further, Ogur *et al.*[19] have shown on yeasts the constancy of DNA content and the exact correspondence of this content with the ploidy of yeast cells.

Similarly, in the analysis of the dynamics of the DNA content in the cell during growth, as a rule great constancy of the DNA content is observed in the cells in the logarithmic and stationary phases of growth.[17, 20-24]

However, when the lag-phase, i.e. the phase preparatory to cellular divi-

[15] R. Vendrely, *Ann. inst. Pasteur* **94**, 142 (1958).
[16] P. C. Caldwell and C. Hinshelwood, *J. Chem. Soc.* p. 1415 (1950).
[17] M. Webb, *Science* **118**, 607 (1953).
[18] C. Barber and I. Soare, *Studii şi cercetări inframicrobiol., microbiol. şi parazitol.* **8**, 617 (1957).
[19] M. Ogur, S. Minckler, G. Lindegren, and C. C. Lindegren, *Arch. Biochem. Biophys.* **40**, 175 (1952).
[20] M. L. Morse and C. E. Carter, *J. Bacteriol.* **58**, 317 (1949).
[21] P. Mitchell and J. Moyle, *J. Gen. Microbiol.* **5**, 421 (1951).
[22] B. J. Katchman and W. O. Fetty, *J. Bacteriol.* **69**, 607 (1955).
[23] A. N. Belozersky, G. N. Zaitseva, L. P. Gavrilova, and L. V. Mineyeva, *Mikrobiologia* **26**, 409 (1957).
[24] A. S. Spirin, A. G. Skavronskaja, and A. Pretel Martines, *Mikrobiologia* **27**, 273 (1958).

sion, was analyzed, it was found that during this period, in particular at the end of the lag-phase, considerable DNA synthesis occurred in the cells, and thus an increase of DNA content per cell was observed.[17, 20, 23, 25-28] Just prior to cellular division the DNA content in the cells was found to double.[17, 20]

The method of synchronous culture has given particularly valuable data on the dynamics of the DNA content during the cellular division in microorganisms. In *Escherichia coli*, under the conditions of such synchronous cell division, it was shown that the DNA content per cell remains constant almost during the entire period, with the exception of a relatively short period just prior to division[29, 30] or at the very beginning of the latter.[31] During this short interval, the DNA content in the cell doubles, so that the subsequent cellular division again leads to the usual DNA content, constant for the given kind of cells.[29-31] The same regularities were shown, recently, for the synchronous culture of *Corynebacterium diphtheriae*.[32] Much earlier, Ogur *et al.*[33] studied synchronously budding yeasts. They showed that DNA synthesis proceeds, only, either prior to or during the budding. If the buds are not counted separately, the DNA content per cell doubles at the end of the first budding cycle. If the cells are counted in such a manner that each bud is counted as an independent unit, the DNA content proves to be constant. Thus, the constancy of the DNA content per nucleus seems to be completely preserved, whereas the increased DNA content in the budding cell is due to the appearance of an independent nucleus in the bud.

The alga *Chlorella* was also studied by means of the method of synchronous culture.[34] Here, also, it was shown that the DNA content per nucleus was constant in the course of growth. DNA synthesis proceeded only prior to the cellular division, during the stage of "ripening of light cells."

Thus, all the data presented show that the DNA content in the cells of microorganisms is constant, with the exception of the period just prior to cellular division.

There seem to exist some special cases, however, when the DNA content in the cells of microorganisms can alter considerably. Wacker *et al.*[35] re-

[25] P. C. Fitz-James, *Can. J. Microbiol.* **1**, 525 (1955).
[26] K. G. Lark and O. Maaløe, *Biochim. et Biophys. Acta* **21**, 448 (1956).
[27] N. A. Eliasson, E. Hammarsten, H. Palmstierna, S. Aqvist, and L. Reio, *Acta Chem. Scand.* **11**, 1381 (1957).
[28] J. H. Stuy, *J. Bacteriol.* **76**, 179 (1958).
[29] H. D. Barner and S. S. Cohen, *Federation Proc.* **14**, 177 (1955).
[30] H. D. Barner and S. S. Cohen, *J. Bacteriol.* **72**, 115 (1956).
[31] Y. Maruyama, *J. Bacteriol.* **72**, 821 (1956).
[32] T. Sall, S. Mudd, and A. Takagi, *J. Bacteriol.* **76**, 640 (1958).
[33] M. Ogur, S. Minckler, and D. O. McClary, *J. Bacteriol.* **66**, 642 (1953).
[34] T. Iwamura, *J. Biochem. (Japan)* **42**, 575 (1955).
[35] A. Wacker, D. Pfahl, and I. Schröder, *Z. Naturforsch.* **12b**, 510 (1957).

corded that on the addition of vitamin B_{12} to the culture of *Lactobacillus leichmannii* the cells synthesize 3–4 times more DNA than without the vitamin. It should be noted that the authors calculated the DNA content per dry weight and not per cell. It was shown recently that in the presence of β-2-thienylalanine the DNA content in the cells of *E. coli*, strain B, increased twofold during 1 hour.[36] A 3–4 times increase of the DNA content per cell under the influence of penicillin was found for *Proteus vulgaris*.[37] In the latter case, however, the question is complicated by the transition of the microbe to the L-form, i.e., a certain type of variability.

Interesting data based, by necessity, on calculation per dry weight and not per cell, were obtained by Demyanovskaya and Belozersky[38] in 1954. They failed to find DNA at the early stages of growth of *Streptomyces streptomycini*, i.e., during 6–12 hours after the transfer of the inoculum into large fermenters, though the inoculum had the usual DNA content (2–2.5%). These data were confirmed by Guberniyev *et al.*[39] on *Streptomyces aureofaciens*. However, the reinvestigation of this case undertaken in our laboratory in collaboration with N. V. Shugayeva showed DNA to be present, though its amount became very small in the first hours of mycelium growth, namely 0.05–0.2% per dry weight. It was also shown that the DNA of this young mycelium had the same composition as the DNA of the mycelium having a normal DNA content. After the early period of mycelium growth, the DNA content in the actinomycetes returns to the normal level and then remains constant.[38, 39] Apparently, analogous phenomena of a sharp fall of the DNA content during certain periods of the early growth of mycelium may be observed in fungi.[40, 40a]

2. Ribonucleic Acid

Changes in RNA are responsible, mainly, for the general picture of the nucleic acid dynamics revealed in the early studies.[3, 7-11] The decrease of the RNA content in the course of growth during the logarithmic phase and the transition from the logarithmic to the stationary phase was confirmed in a number of investigations carried out on various species and strains of microorganisms.[20-22, 24, 38, 39, 41-48] The RNA content in the cell appears to

[36] A. B. Pardee and L. S. Prestidge, *Biochim. et Biophys. Acta* **27**, 412 (1958).
[37] M. V. Nermut and V. Drášil, *Nature* **181**, 1740 (1958).
[38] N. S. Demyanovskaya and A. N. Belozersky, *Biokhimiya* **19**, 688 (1954).
[39] M. A. Guberniyev, N. A. Ugoleva, and L. I. Torbochkina, *Antibiotiki* **1**, 8 (1956).
[40] E. Hammarsten and H. Palmstierna, *Acta Chem. Scand.* **11**, 1378 (1957).
[40a] H. Venner, *Abstr. 4th Intern. Congr. Biochem., Vienna* No. 3-44, p. 36 (1958).
[41] A. N. Belozersky, V. B. Korchagin, and T. I. Smirnova, *Doklady Akad. Nauk S.S.S.R.* **71**, 89 (1950).
[42] C. A. Fish, I. Asimov, and B. S. Walker, *Proc. Soc. Exptl. Biol. Med.* **75**, 774 (1950).
[43] W. H. Price, *J. Gen. Physiol.* **35**, 741 (1952).
[44] H. E. Wade, *J. Gen. Microbiol.* **7**, 24 (1952).
[45] H. Sato, *Tôhoku J. Exptl. Med.* **60**, 375 (1954).
[46] A. N. Belozersky, I. V. Asseeva, and A. F. Moroz, *Doklady Akad. Nauk S.S.S.R.* **109**, 149 (1956).
[47] S. A. Popienenkova, *Zhur. mikrobiol. epidemiol. immunobiol.* No. 1, 26 (1956).
[48] R. Vaamonde Fernandez and B. Regueiro Varela, *Microbiol. españ.* **10**, 461 (1957).

decrease in accordance with the decreased multiplication rate of the cells in culture, and, correspondingly, with the rate of protein synthesis. The maximal RNA amount is found during the period of most intensive growth or just prior to this period. In the periods during which the multiplication rate of the cells is constant, the RNA content per cell is also approximately constant.

The direct connection between the RNA content per cell and the rate of growth, multiplication, and protein synthesis was shown, also, in studies with cultures in which different rates of synthesis and growth were achieved through changes in the medium,[12, 21, 49-52] by various inhibitors,[12, 21, 51, 53] and also by studying mutants and strains possessing different rates of growth.[12, 43] The RNA amount per cell was shown to be directly proportional to the reciprocal of the average generation time, i.e., to the rate of cell multiplication.[12] It was noted that the RNA content in the cells controls the rate of growth and protein synthesis,[21] and that at the constant rate of multiplication in steady-state systems, there exists an exact correlation between the rate of protein synthesis and the RNA amount which is excessive with respect to the constant basic amount that is always present in the cell.[50] Of recent studies, data on the thermophilic bacteria are of interest, in which the cells contained the maximal RNA amount at temperatures optimal for their multiplication.[54] German authors[54-57] showed that during the inhibition of the growth of microorganisms by metal ions, temperature, and other agents, a decrease of the RNA content and an increase of the DNA/RNA ratio takes place. In this connection, the work of Russian authors[46, 47] should be mentioned in which a study of the RNA content in the original bacteria and in variants produced under the influence of antibiotics was carried out. Here, a lower rate of growth and multiplication of the cells and a more prolonged cycle of growth in modified variants were accompanied by a low level of RNA content.

Thus, the presence of a definite level of RNA content in the microbial cells seems to ensure a corresponding rate of growth and multiplication of cells in the culture.

Valuable material, in this respect, is given by the study of the lag-phase

[49] F. J. DiCarlo and A. S. Shultz, *Arch. Biochem.* **17,** 293 (1948).
[50] R. Jeener, *Arch. Biochem. Biophys.* **43,** 381 (1953).
[51] E. F. Gale, *2nd Intern. Congr. Biochem. Paris* "Symp. sur le mode d'action des antibiotiques," p. 5 (1952).
[52] E. F. Gale and J. P. Folkes, *Biochem. J.* **53,** 483 (1953).
[53] E. F. Gale and J. P. Folkes, *Biochem. J.* **53,** 493 (1953).
[54] R. Wellerson and P. A. Tetrault, *J. Bacteriol.* **69,** 449 (1955).
[55] O. Kandler, J. Müller, and C. Zehender, *Arch. Mikrobiol.* **24,** 250 (1956).
[56] A. Rippel-Baldes, G. Busch, and F. Radler, *Arch. Mikrobiol.* **23,** 423 (1956).
[57] E. Petras, *Arch. Mikrobiol.* **30,** 147 (1958).

in nonsporulating microorganisms,[20, 23, 26, 27, 42, 43, 45, 47, 58] or by the investigation of the germination of spores in sporeforming species.[25, 28, 40, 59] During this early period of development, prior to cellular division, an intensive RNA synthesis takes place in the cells. According to some data, RNA synthesis precedes that of DNA in the cell; with the onset of DNA synthesis, which takes place just prior to the division, RNA synthesis slows down somewhat.[25, 26, 40, 45] Protein synthesis in the cell begins, also, only when a certain level of RNA content is achieved, so that RNA accumulation always precedes protein synthesis in the lag-phase or during the germination of spores. It is typical that during the lag-phase the nucleic acid synthesis takes precedence over protein synthesis, whereas in the absence of nitrogen uptake from the medium the former can proceed at the expense of protein.[23] Moreover, even in the case of nitrogen uptake from the medium, there takes place, during this period, a redistribution of nitrogen in the cells, so that the nucleic acids do not utilize the nitrogen of the medium but that of cellular constituents, presumably, of proteins.[27] In the case of the germination of *Bacillus cereus* spores, the nucleic acid synthesis began only when the germinated spores were placed in a nutritive medium.[28]

The same regularities, namely an intensive RNA accumulation preceding intensive growth of mycelium, were found in early phases of the growth of Actinomycetes when the inoculum was transferred to large fermenters.[39] Here, RNA synthesis also preceded that of DNA.

The regularities revealed in the study of the lag-phase and subsequent cellular division are completely confirmed when synchronous cultures of microorganisms are studied.[30-32, 34] Here, the RNA content of the cells also increases very rapidly attaining its maximum by the onset of intensive protein synthesis; thereafter, RNA synthesis in the cell slows down, and then, with the onset of cellular division, proceeds proportionally to the multiplication of the cells. Unlike DNA, which is synthesized only immediately before the division of the cells, RNA synthesis proceeds in the culture more or less continuously.

Thus, the necessity of a latent period seems to be due to the fact that it is this "starting" period, when an accumulation of RNA occurs in the cell up to a certain level, which ensures a corresponding rate of protein synthesis, growth, and multiplication.

During the subsequent period of logarithmic growth, a direct correlation is observed, as a rule, between the RNA content in the cells and the rate of both growth and protein synthesis. RNA and protein synthesis proceeds more or less in parallel, slowing down gradually.

[58] T. Brechbühler, *Bull. soc. chim. biol.* **32,** 952 (1950).
[59] T. Yanagita, *Arch. Mikrobiol.* **26,** 329 (1957).

However, by exposing cells to the action of some reagents, e.g., chloramphenicol,[51, 53, 60] one succeeds in separating the syntheses of protein and RNA, so that RNA synthesis will proceed in the cells, whereas protein synthesis will be completely inhibited. Another group of data, for example, on the effect of penicillin,[21, 51, 53, 61] shows a complete correlation of the inhibition of RNA and protein synthesis. Gale[51] pointed out that no antibiotic was found which would inhibit RNA synthesis without a corresponding slowing down of protein synthesis. It is possible, apparently, to inhibit protein synthesis without inhibiting that of RNA, but the inhibition of RNA synthesis inevitably brings about the suppression of protein synthesis and of growth in microorganisms. (Compare, however, recent observations on the effect of 5-fluorouracil on a uracil-requiring mutant of *E. coli*.[61a])

Recent investigations showed that the RNA which is synthesized in bacterial cells in the absence of protein synthesis, is physiologically inferior and incapable of a prolonged existence in the cell since it undergoes a rapid and continuous disintegration to products of low molecular weight.[62-65]

It should be noted, however, that several investigations have shown the absence of a direct correlation between the rate of growth and protein synthesis and the RNA content of bacteria. For example, all species with a more rapid speed of multiplication, when compared to more slowly growing strains, can have a lower RNA content in both resting and dividing cells and can be characterized by an even smaller RNA accumulation (absolutely and relatively) when going from the resting to the dividing cell.[66] A series of studies by Beljanski[67-71] on *Staphylococcus aureus* and *E. coli* showed that antibiotic-resistant strains, obtained by means of the cultivation of original strains on media containing the antibiotic, when grown on ordinary media (without antibiotic) accumulate, in all cases, considerably

[61] C. L. Wisseman, J. E. Smadel, F. E. Hahn, and H. E. Hopps, *J. Bacteriol.* **67**, 662 (1954).

[61] C. Lark and K. G. Lark, *J. Bacteriol.* **76**, 666 (1958).

[61a] J. Horowitz, J. J. Saukkonen, and E. Chargaff, *Biochim. et Biophys. Acta* **29**, 222 (1958).

[62] F. C. Neidhardt and F. Gros, *Biochim. et Biophys. Acta* **25**, 513 (1957).

[63] F. E. Hahn, M. Schaechter, W. S. Ceglowski, H. E. Hopps, and J. Ciak, *Biochim. et Biophys. Acta* **26**, 469 (1957).

[64] M. G. Harrington, *J. Gen. Microbiol.* **18**, 767 (1958).

[65] J. Horowitz, A. Lombard, and E. Chargaff, *J. Biol. Chem.* **233**, 1517 (1958).

[66] H. E. Wade and D. M. Morgan, *Biochem. J.* **65**, 321 (1957).

[67] M. Beljanski, *Ann. inst. Pasteur* **83**, 80 (1952).

[68] M. Beljanski, *Compt. rend. acad. sci.* **236**, 1102 (1953).

[69] M. Beljanski, *Ann. inst. Pasteur* **84**, 402 (1953).

[70] M. Beljanski, *Ann. inst. Pasteur* **84**, 756, 760 (1953).

[71] M. Beljanski, *Ann. inst. Pasteur* **85**, 463 (1953).

more RNA in the lag-phase than do the original strains. Moreover, in most cases, resistant strains were distinguished by a more prolonged lag-phase and a slower speed of multiplication during the logarithmic phase. These data were also confirmed with other bacteria.[72, 73]

Hedén *et al.*,[74] applying continuous cultivation techniques, also pointed out the absence of a correlation between the nucleic acid content in steady-state cells and the rate of growth.

Two circumstances should be borne in mind here. First, it has not been ruled out that it is not the total RNA of the cell but rather the "additional RNA" which can be correlated with the rate of growth and protein synthesis, i.e., that portion of the RNA which is responsible for the basophilia of cells.[3, 7, 66, 75] It is just this RNA which accumulates in the lag-phase above the constant amount of the basic RNA which is always present in the cells, and it is this former which gradually disappears with the slowing down of growth and synthetic processes and is lacking in resting cells.[3, 7, 43, 59, 66, 75] Proportions of these two RNA fractions, the "additional" (fluctuating) and the "basic" (constant) one, may be different in different strains and species of microorganisms,[66] and this makes it difficult to draw parallels between the rate of growth and the content of total RNA. Second, even when taking into consideration the "additional" (fluctuating) RNA, one cannot always solve the problem of the direct correspondence of the amount of this RNA to the rate of growth and protein synthesis. One may suppose that not all of this RNA, but only a certain portion of it which varies from species to species, participates in the synthesis of the bulk of the proteins. Besides, the accumulation of the same amount of proteins in the same time may require a different amount of RNA in different species, depending on the quality of these proteins, their half-life, etc. At any rate, even if there often exists a correlation between RNA accumulation and the rate of growth, the comparison of different species and strains of bacteria shows that it does not follow a general rule as is the case in the analysis of the same organism in various states and conditions.

With respect to such kinds of variability as the formation of involutionary forms of bacteria, transition to the L-form, etc., it should be noted that a considerable increase of the DNA/RNA ratio is recorded in the literature. Apparently, this is mainly attributable to the lowered RNA content in such forms,[55, 56, 76-78] but may to a certain degree, at any rate in some cases, also be due to an increased DNA content.[37]

[72] J. Smolens and A. B. Vogt, *J. Bacteriol.* **66**, 140 (1953).

[73] V. D. Tymakov, D. G. Kudlaj, V. G. Petrovskaja, A. M. Korneeva, and L. A. Kodina, *Zhur. mikrobiol. epidemiol. immunobiol.* No. 8, 3 (1957).

[74] C.-G. Hedén, T. Holme, and B. Malmgren, *Acta Pathol. Microbiol. Scand.* **37**, 50 (1955).

[75] H. E. Wade and D. M. Morgan, *Nature* **176**, 310 (1955).

[76] R. Vendrely and R. Tulasne, *Bull. soc. chim. biol.* **34**, 785 (1952).

[77] R. Vendrely and R. Tulasne, *Nature* **171**, 262 (1953).

[78] O. Kandler, C. Zehender, and J. Müller, *Arch. Mikrobiol.* **24**, 219 (1956).

III. Constituents of Nucleic Acids

1. Deoxyribonucleic Acid

a. Sugar

At present, there are no grounds to doubt the universal occurrence of 2-deoxy-D-ribose as the sole sugar DNA constituent in all representatives of the living world, including the microorganisms. This problem has been reviewed earlier.[79, 80] It should be mentioned, only, that special investigations on the identification of 2-deoxyribose were carried out on the DNA of yeast,[81] *Mycobacterium tuberculosis avium*[81] and *Mycobacterium tuberculosis bovis*.[82]

b. Nitrogenous Constituents

Multiple investigations of the composition of very different DNA showed that the overwhelming mass of nitrogenous constituents consists of common purine (guanine and adenine) and pyrimidine (cytosine and thymine) bases.[80] Microorganisms are no exception in this respect. Moreover, in none of the microorganisms studied, including bacteria,[80-86] actinomycetes (our laboratory), yeasts[80, 81, 84] and other fungi (Uryson, our laboratory), algae,[87] and protozoa,[88] could 5-methylcytosine be found as a DNA component. The presence of methylcytosine is a characteristic feature of DNA of higher organisms, both animals and plants.[80, 83-86] In the DNA of all the higher plants examined, methylcytosine occurred in a higher proportion than in that of animals.[89] By this characteristic, the DNA of microorganisms shows quite a distinct qualitative difference from that of higher forms. At the same time, the absence of 5-methylcytosine proved a common feature in the DNA of very different groups of microorganisms.

In 1955, however, Dunn and Smith found a new base, 6-methylamino-

[79] W. G. Overend and M. Stacey, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. 1, p. 9. Academic Press, New York, 1955.

[80] E. Chargaff, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. 1, p. 307. Academic Press, New York, 1955.

[81] E. Vischer, S. Zamenhof, and E. Chargaff, *J. Biol. Chem.* **177**, 429 (1949).

[82] T. Tsumita and E. Chargaff, *Biochim. et Biophys. Acta* **29**, 568 (1958).

[83] G. R. Wyatt, *Nature* **166**, 237 (1950).

[84] G. R. Wyatt, *Biochem. J.* **48**, 581 (1951).

[85] J. D. Smith and G. R. Wyatt, *Biochem. J.* **49**, 144 (1951).

[86] S. G. Laland, W. G. Overend, and M. Webb, *J. Chem. Soc.* p. 3224 (1952).

[87] E. M. Low, *Nature* **182**, 1096 (1958).

[88] O. Scherbaum, *Exptl. Cell. Research* **13**, 24 (1957).

[89] S. O. Uryson and A. N. Belozersky, *Doklady Akad. Nauk S.S.S.R.* **125**, 1144 (1959).

purine, in the DNA of bacteria.[90, 91] At first, it was found in the DNA of an *E. coli* strain which required thymine (15T⁻); with thymine being deficient in the medium, it accumulated in rather considerable amounts in the DNA of this bacterium.[90] Later investigations showed this base to be a normal constituent of DNA in all bacteria studied, namely in *E. coli* (B/r, K12, and 15T⁻), *A. aerogenes*, *Diplococcus pneumoniae*, and *M. tuberculosis bovis*.[91-93] From the DNA of the above mentioned bacteria, not only the base itself was isolated, but also the corresponding deoxyriboside and 5-deoxyribonucleotide.[92] In the DNA of these bacteria, the molar content of 6-methylaminopurine did not exceed 0.7% of the total content of the bases, ranging from 0.4% (in *D. pneumoniae*) to 2.7% (in *A. aerogenes*) as mole % of adenine. This base was not found in the DNA from the tissues of higher plants and animals[91-93]; it seems to be characteristic of microorganisms.

The study of different strains of *E. coli* showed the normal content of 6-methylaminopurine in DNA of all three strains (B/r, K12, and 15T⁻) to be the same (1.7–1.8% mole % of adenine).[92] In contrast, different species differed widely in the content of this base. Thus, one may consider the quantitative distribution of 6-methylaminopurine in DNA as a feature specific for the species. The authors think[92, 93] that under normal conditions this base probably replaces a certain proportion of the adenine in the DNA chain.

The authors showed,[92] however, that a strain, requiring thymine (*E. coli* 15T⁻), when placed under conditions of either thymine deficiency or of the inhibition of its formation by the increase of such thymine antagonists as 5-aminouracil, 2-thiothymine, or 5-chlorouracil, synthesizes DNA with an increased content of 6-methylaminopurine (up to 15% of the adenine) and a decreased content of thymine. Thus, under the conditions mentioned, either 6-methylaminopurine substitutes for a portion of the thymine in the DNA molecule or the DNA synthesized no longer fulfills Chargaff's regularities. At any rate, the DNA formed under these conditions cannot be brought into accord with the Watson-Crick structure, and is supposed[93, 94] to be abnormal.

The data summarized here have a close connection with the problem of the incorporation of unnatural analogs of purine and pyrimidine bases into bacterial DNA. This concerns, mainly, halogenated pyrimidines, such as 5-bromouracil, 5-chloroura-

[90] D. B. Dunn and J. D. Smith, *Nature* **175**, 336 (1955).

[91] D. B. Dunn and J. D. Smith, *Biochem. J.* **60**, Proc. xvii (1955).

[92] D. B. Dunn and J. D. Smith, *Biochem. J.* **68**, 627 (1958).

[93] D. B. Dunn and J. D. Smith, *4th Intern. Congr. Biochem., Vienna* Symposium 7, preprint No 10 (1958).

[94] F. H. C. Crick, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.). Johns Hopkins, Baltimore, 1957.

cil, and 5-iodouracil. When bacteria are grown on a medium containing these pyrimidine analogs, the latter are incorporated into the DNA instead of thymine.[95-105] However, other analogs, e.g., 2-thiothymine,[92, 99, 101] uracil,[101] 5-nitrouracil,[99, 105] 5-methylcytosine,[105] 5-aminouracil,[92, 99] 5-ethyluracil,[99] 2-thiouracil,[99] and others, are not incorporated, as such, into the DNA of bacteria, though they act as growth inhibitors of the strains requiring thymine, presumably by competing with the thymine uptake from the medium. In contrast, the incorporation of 5-bromouracil, 5-chlorouracil, and 5-iodouracil in the place of thymine may proceed with and without inhibition of bacterial growth.[96, 99] It has been shown that, in general, there is no strict correlation between growth inhibition by means of an unnatural base and incorporation of this base into DNA.[101] The extent of thymine replacement in the DNA of bacteria depends upon both the nature of the halogen substituent[101] and the strain under study.[96, 105] For example, in *E. coli*, 5-bromouracil is incorporated best. In strains requiring thymine, up to 48% of the thymine[101, 105] could be substituted by 5-bromouracil, whereas wild strains of *E. coli* incorporated only up to 18% of the thymine.[105] Wacker *et al.* showed that about 70% of the thymine is substituted by 5-bromouracil[96] in *Enterococcus stei*. The study of *E. coli* containing 5-bromouracil in their DNA showed that the DNA content in the cell remains unaltered, thereby, and the cells preserve their ability to reproduce.[103] Moreover, the substitution of a portion of thymine by 5-bromouracil is completely reversible and these mutual substitutions are not accompanied by alterations of the hereditary properties or by typical mutations preserved in the generations after the elimination of 5-bromouracil, though certain modifications and alterations may take place when 5-bromouracil is incorporated into DNA.[103] The occasional appearance of stable mutants of a peculiar type takes place, however, and that may be the result of disturbances in DNA structure.[106] A study of the stability (heat denaturation) and of some physical properties (spectrum, viscosity) of DNA containing 5-bromouracil did not reveal any differences from the usual DNA.[105]

In a number of bacteria, e.g., *E. coli*, *S. aureus*, no incorporation into DNA of growth inhibiting purine analogs, such as 8-azaguanine or other 8-azapurines, was found.[107, 108] In *B. cereus*, however, it has been noticed that the bacterium incorporates some 8-azaguanine into its DNA.[108, 109]

[95] F. Weygand, A. Wacker, and H. Dellweg, *Z. Naturforsch.* **7b**, 19 (1952).
[96] A. Wacker, A. Trebst, D. Jacherts, and F. Weygand, *Z. Naturforsch.* **9b**, 616 (1954).
[97] D. B. Dunn and J. D. Smith, *Nature* **174**, 305 (1954).
[98] S. Zamenhof and G. Griboff, *Nature* **174**, 306 (1954).
[99] S. Zamenhof and G. Griboff, *Nature* **174**, 307 (1954).
[100] F. Weygand, A. Wacker, and K. M. Patil, *Ber.* **89**, 475 (1956).
[101] S. Zamenhof, B. Reiner, R. DeGiovanni, and K. Rich, *J. Biol. Chem.* **219**, 165 (1956).
[102] T. D. Price, P. B. Hudson, H. A. Hinds, R. A. Darmstadt, and S. Zamenhof, *Nature* **178**, 684 (1956).
[103] S. Zamenhof, R. DeGiovanni, and K. Rich, *J. Bacteriol.* **71**, 60 (1956).
[104] D. B. Dunn and J. D. Smith, *Biochem. J.* **67**, 494 (1957).
[105] S. Zamenhof, K. Rich, and R. DeGiovanni, *J. Biol. Chem.* **232**, 651 (1958).
[106] S. Zamenhof, R. DeGiovanni, and S. Greer, *Nature* **181**, 827 (1958).
[107] I. Lasnitzki, R. E. F. Matthews, and J. D. Smith, *Nature* **173**, 346 (1954).
[108] J. D. Smith and R. E. F. Matthews, *Biochem. J.* **66**, 323 (1957).
[109] H. G. Mandel, G. I. Sugarman, and R. A. Apter, *J. Biol. Chem.* **225**, 151 (1957).

2. Ribonucleic Acid

a. Sugar

Long ago it was shown that D-ribose was the sugar constituent of the RNA of yeast.[110] During recent years, D-ribose was identified in different RNA[79, 111] including that of microorganisms *M. tuberculosis avium*,[81] *Clostridium perfringens*,[112] *E. coli*,[113] and *Euglena gracilis*.[114] It was shown in the RNA of yeast[115] as well. The principal pentose constituent of the nucleotides of all ribonucleic acids, regardless of their origin, seems to be D-ribose. It is not ruled out, however, that some other minor sugar constituents can be present in certain cases.[114, 115a]

b. Nitrogenous Constituents

Until recently, only the usual bases, guanine, adenine, cytosine, and uracil, were known as RNA constituents. The first communications on the discovery of a fifth nucleotide in alkaline hydrolyzates of yeast RNA appeared simultaneously from two laboratories in 1957.[116, 117] This new nucleotide yielded, on degradation, a new nucleoside which was shown to be a derivative of uracil. It is most likely that this compound represents 5-ribosyluracil.[118, 119] The designation "pseudouridine" has been proposed for the nucleoside.[119] The isolation of both the 2′- and 3′-phosphates of this nucleoside by alkaline hydrolysis shows it to be a regular constituent of the RNA chain. In addition to yeast RNA, a substance with the properties of the new nucleotide was demonstrated in alkaline hydrolyzates of bacterial RNA, from *A. aerogenes* and *B. cereus*,[93] in the RNA of a flagellate, *E. gracilis*,[114] as well as in higher forms.[93]

That the list of RNA constituents is not restricted to the four common bases and may turn out to be much more complicated owing to the presence of various "minor constituents" was confirmed, in addition to the data mentioned above, by several new findings published in 1958. This group of data deals with the isolation of a whole set of methylated purines and

[110] P. A. Levene and W. A. Jacobs, *Ber.* **42**, 2102, 2469, 2474, 2703 (1909).
[111] B. Magasanik, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. 1, p. 373. Academic Press, New York, 1955.
[112] C. H. Parsons, *Arch. Biochem. Biophys.* **47**, 76 (1953).
[113] A. Lombard and E. Chargaff, *Biochim. et Biophys. Acta* **25**, 549 (1957).
[114] G. Brawerman and E. Chargaff, *Biochim. et Biophys. Acta* **31**, 172 (1959).
[115] E. Vischer and E. Chargaff, *J. Biol. Chem.* **176**, 715 (1948).
[115a] J. D. Smith and D. B. Dunn, *Biochim. et Biophys. Acta* **31**, 573 (1959).
[116] F. F. Davis and F. W. Allen, *J. Biol. Chem.* **227**, 907 (1957).
[117] W. E. Cohn, *Federation Proc.* **16**, 166 (1957).
[118] C. Yu and F. W. Allen, *Biochim. et Biophys. Acta* **32**, 393 (1959).
[119] W. E. Cohn, *Biochim. et Biophys. Acta* **32**, 569 (1959).

pyrimidines from bacterial RNA. The newly described pyrimidine constituents of RNA included thymine[120-122] and 5-methylcytosine,[123] which were previously known only as DNA constituents. Both these pyrimidine bases were shown in the RNA of *E. coli*, whereas thymine was also found in the RNA of other bacteria (*A. aerogenes*, *S. aureus*) and in that of yeast. However, thymine was not found in the RNA from animal tissues,[120-122] though the RNA of wheat germ contained a considerable amount of thymine.[120, 122] The thymine content in the RNA of microorganisms was of the order of 1 mole % of uracil[120-122]; that of 5-methylcytosine in the RNA of *E. coli* is said to attain up to 1–2% of all RNA nucleotides.[123]

Besides methylated pyrimidines, methylated purine bases such as 2-methyladenine,[120-122] 6-methylaminopurine,[120, 122, 124] 6-dimethylaminopurine,[120-122] as well as 1-methylguanine,[93, 124] 2-methylamino-6-hydroxypurine,[93, 124] and 2-dimethylamino-6-hydroxypurine[93] have been found in microbial RNA. The former three bases (adenine derivatives) were found, along with thymine, in the RNA of *E. coli* (B/r and 15T⁻), *A. aerogenes*, yeast, and, with the exception of dimethylaminopurine, in *S. aureus* as well. Each of them comprises from 0.05 to 1 mole % of uracil.[120-122] Two other bases (guanine derivatives) were found in the RNA of yeast and make up, according to the authors' data,[124] about 0.01 % of the total amount of purines.

It should be noted that the above mentioned methylated bases, thymine and the methylated adenine and guanine derivatives, were obtained in the form of their ribosides and ribonucleotides.[93, 122] Ribose was identified as their sugar constituent. The ribosides and ribonucleotides of thymine and the methylated purines were carefully characterized spectrophotometrically, chromatographically, electrophoretically, and compared with synthetic products. The ribonucleotides of these bases were isolated from the alkaline hydrolyzate in the form of the 2′- and 3′-nucleotides, which proves their presence in the RNA chain in the form of the usual 3′- to 5′-phosphoester bonds.[93, 122] Thus, there are no grounds, at present, to doubt that thymine and other methylted bases can be normal (though minor) constituents of RNA, at any rate, of the RNA of microorganisms.

As concerns a related problem with respect to the constituents of microbial RNA, namely that of the incorporation of unnatural analogs of nitrogenous bases, the main interest here centers on a purine analog, 8-azaguanine. This substance, a strong inhibitor of bacterial growth, was shown to be incorporated into the RNA.[107, 109, 125] Thus, 8-azaguanylic acid[107] was isolated, for the first time, from alkaline hydrolyzates of the RNA of *E. coli* and *S. aureus*, which had been treated with 8-azaguanine. This nucleotide amounted to 0.5% and less of the moles of the total nucleotides. It was shown later that up to 40% of guanine in *B. cereus* can be replaced by 8-azaguanine

[120] J. W. Littlefield and D. B. Dunn, *Biochem. J.* **68**, Proc. viii (1958).
[121] J. W. Littlefield and D. B. Dunn, *Nature* **181**, 254 (1958).
[122] J. W. Littlefield and D. B. Dunn, *Biochem. J.* **70**, 642 (1958).
[123] H. Amos and M. Korn, *Biochim. et Biophys. Acta* **29**, 144 (1958).
[124] M. Adler, B. Weissmann, and A. B. Gutman, *J. Biol. Chem.* **230**, 717 (1958).
[125] R. E. F. Matthews and J. D. Smith, *Nature* **177**, 271 (1956).

from the medium.[125] The study of the effect of other 8-azapurines and substituted triazoles on *E. coli* and *B. cereus* showed that these substances also suppress bacterial growth but they [8-azaguanine, 8-azaadenine, 8-azahypoxanthine, and 5(4)-amino-1-H-1,2,3-triazole-4(5)carboxamide] are incorporated into RNA only in the form of 8-azaguanine.[108]

The entire problem relating to the discovery of new purine and pyrimidine nucleotides occurring naturally in DNA and RNA, as well as the incorporation of unnatural analogs of purines and pyrimidines into both these nucleic acids, opens new vistas for the study of the specificity of nucleic acids.

IV. Nucleic Acid Composition and Specificity

In speaking about the quality and qualitative variety of nucleic acids, we have in mind, first of all, their chemical specificity. As it has repeatedly been pointed out in the literature, the principal features by which the specificity of nucleic acids may display itself are as follows: (1) the proportions of various purine and pyrimidine bases, i.e., the nucleic acid composition; (2) nucleotide sequence; (3) macromolecular configuration. These features may, strictly speaking, be regarded merely as the possibilities of specific differences between individual molecules, i.e., in the purely chemical aspect. When dealing with biological units, however, that is cells, organoids, etc., we may encounter, and probably do encounter, the fact that the nucleic acids of any such unit represent not one type of molecule but a whole set of molecules of various structure and specificity.[80] In other words, we may run into the heterogeneity of nucleic acids in biological objects. The study of the specificity of individual molecules of the nucleic acids is not possible however, at present, because of the difficulties not yet overcome in the isolation, and, in particular, in the complete fractionation of the entire set of cellular nucleic acids in the native state. In this connection, when studying the specificity of composition and structure of nucleic acids, the main point refers to the specificity of the total cell DNA or RNA. From the biological viewpoint, this approach often proves of no less importance than the study of the specificity of individual molecules.

At present, the only available and widely used approach to the solution of the problem of nucleic acid specificity is the study of their composition. There is, however, a very important restriction to this approach which must be noted; in the case of an equal composition, one is unable to decide whether there really is no difference between the nucleic acids under study, or whether there do exist specific differences which have to do with differences in the nucleotide sequence or some other structural differences. Unfortunately, hitherto only preliminary attempts have been made with respect to such an important and maybe decisive approach as the determination of the nucleotide sequence.

Another no less important restriction in the study of the nucleic acid composition lies in the fact that the total DNA or RNA of any two objects

(e.g., of closely related species) may widely differ as to the composition of only some nucleic acid molecules, the main mass of the molecules being the same or very similar in their composition. In this case, even drastic differences in the composition of a few of the molecules of two species will not be observed since they will be veiled or become completely imperceptible because of the similarity of the composition of the main mass of DNA or RNA.

Yet, in spite of the above mentioned restrictions, the determination of the nucleic acid composition is the only method which, to the present time, has been systematically and successfully applied to the study of various aspects of the chemical specificity of total nucleic acids. Proceeding from this assumption, when considering further different aspects of nucleic acid specificity, we shall refer almost solely to the specificity of their composition.

1. Age Specificity of Nucleic Acids and Related Problems

This section deals with a group of problems concerning the nucleic acid composition of microorganisms as it depends on various physiological and functional states of the cells. Besides the age aspect, we shall be concerned with the effect of environmental conditions on nucleic acid composition, as well as the effect of one or another change in cell metabolism on the quality of nucleic acids.

The problems to be discussed in this section are of key importance for the elucidation of all other aspects of the nucleic acid specificity of bacteria. If nucleic acids appreciably altered their composition, either in the course of the development of cells or under the effect of certain conditions, then the nucleic acids of various microorganisms would have to be compared in cells of the same physiological and functional states only. This would considerably complicate the study of nucleic acid specificity. But fortunately, according to the data available, nucleic acids seem, by the criteria available at present, to possess a very stable compositional specificity which does not exhibit appreciable alterations either in the course of the growth and development of the cells or as a result of the conditions of cultivation.

a. Nucleic Acid Composition at Different Ages of the Culture

(1) *DNA.* The first data proving the invariability of the DNA composition in the course of growth of a bacterial culture were obtained on *Pseudomonas hydrophila*[126] and, somewhat earlier, on *Haemophilus influenzae*.[127] Abrams' claim of alterations in the DNA in the course of growth of the

[126] K. K. Reddi, *Biochim. et Biophys. Acta* **15**, 585 (1954).

[127] S. Zamenhof, *in* "Phosphorus Metabolism" (W. D. McElroy and B. Glass, eds.), Vol. 2, p. 336. Johns Hopkins, Baltimore, 1952.

yeast culture[128] has been questioned[127] and was not confirmed by later work on microorganisms. Detailed investigations of the DNA composition at different developmental stages of the bacteria showed its invariability with a number of cultures, *E. coli*,[129] *Salmonella enteritidis*,[130] *Azotobacter agile*,[131] *Sarcina lutea*,[130, 132] *Streptococcus pyogenes*,[130] and *B. cereus*.[28] Besides, as was mentioned above (Section II,1), no alteration in DNA composition with the age of the actinomycetes was found in our laboratory. The same was observed by Scherbaum for the cultures of *Tetrahymena pyriformis* (Ciliata).[133]

When noting the invariability of DNA composition in the course of growth, it should be borne in mind that the DNA of the bacterial cell may be heterogeneous, i.e., it may consist of a number of molecules of different composition and properties.[134-136] Since the total DNA composition of a culture does not show any change, this may favor the view, that despite the age the same proportion of the DNA molecules of different composition is kept invariable for a given species.

(2) *RNA*. The study of RNA composition in the course of microbial development was undertaken with the following organisms: *E. coli*,[129] *A. agile*,[131] *S. lutea*,[132] and *B. cereus*,[28] as well as with a representative of the protozoa, namely *Tetrahymena pyriformis*.[133] As in the case of DNA, the invariability of RNA composition was shown at different growth stages. It is much more difficult to account for the invariability of the RNA composition in the course of growth than it is in the case of DNA, because in the former, unlike DNA, considerable variations are found in the RNA content.

This invariability can occur if the composition of all the RNA molecules in the cell is roughly the same, i.e., if there is no considerable heterogeneity of the RNA molecules within the cell with regard to their composition. This is supported, also, by direct experimental evidence obtained by Wade and Morgan,[66] who have shown the identity of composition of functionally differing RNA fractions of *E. coli*, namely the "fluctuating RNA" which is responsible for the basophilia of cells and is found in the light intracellular particles, and the "constant RNA" which is connected with heavier nucleoprotein particles.

[128] R. Abrams, *in* "Phosphorus Metabolism" (W. D. McElroy and B. Glass, eds.), Vol. 2. p. 335. Johns Hopkins, Baltimore, 1952.

[129] A. S. Spirin, A. N. Belozersky, and A. Pretel Martines, *Doklady Akad. Nauk S.S.S.R.* **111**, 1297 (1956).

[130] Ki Yong Lee, R. Wahl, and E. Barbu, *Ann. inst. Pasteur* **91**, 212 (1956).

[131] G. N. Zaitseva and A. N. Belozersky, *Mikrobiologia* **26**, 722 (1957).

[132] S. K. Dutta, A. S. Jones, and M. Stacey, *J. Gen. Microbiol.* **14**, 160 (1956).

[133] O. Scherbaum, *Exptl. Cell. Research* **13**, 24 (1957).

[134] G. L. Brown and M. Watson, *Nature* **172**, 339 (1953).

[135] C. F. Crampton, R. Lipshitz, and E. Chargaff, *J. Biol. Chem.* **211**, 125 (1954).

[136] L. S. Lerman, *Biochim. et Biophys. Acta* **18**, 132 (1955).

This certainly does not in any way rule out heterogeneity of the RNA within the cell, as the differences between the cellular RNA molecules may be due principally, not to the differences in composition, but to other structural aspects, particularly the nucleotide sequence.

b. Nucleic Acid Composition under Different Conditions and in Different Functional States

(1) *DNA.* Investigations of the effect of the conditions of cultivation on the nucleic acid composition of microorganisms have been carried out since nucleic acid specificity was first studied. It was Smith and Wyatt[85] who found that the DNA composition of *E. coli* was not affected by the medium. This was confirmed later with the DNA of other bacteria.[130] Metabolic changes in the bacterial cell do not seem to affect the DNA composition; for example, no differences in the DNA composition were found in *A. agile* under conditions of nitrogen fixation, and in the same culture, in the presence of ammonium nitrogen.[131]

It was recently shown that the DNA of *B. cereus* prior to and after the irradiation of the cells with ultraviolet (from 90 to 540 seconds, 600 watts per 1 cm.^{2}) had an identical composition.[137]

(2) *RNA.* It has also been shown with respect to RNA that, despite very great variations in the RNA content, far-reaching changes in the conditions of cultivation and in the composition of the culture medium do not affect the specificity of nucleic acid composition. An earlier statement on the variability of the yeast RNA composition depending on the nitrogen nutrition[138] proved to be erroneous. In 1952, Thomas presented data which showed that when growing yeast on diverse sources of nitrogen, such as ammonium sulfate, adenine, uracil, and ammonium sulfate in the presence of sodium azide, no variations in the total RNA composition were to be found.[139] An identical RNA composition was found in a number of *Azotobacter vinelandii* samples grown under different aeration conditions.[140] The nitrogen fixing *Azotobacter* culture exhibited no differences in the RNA composition as compared to the same culture grown on ammonium nitrogen.[131] Resting cells of *E. coli*, and dividing cells which were shown to contain 2.5 times more RNA, do not differ in their RNA composition [66]

Pardee and Prestidge have shown on *E. coli* cultures that the RNA composition was the same in the cells (*a*) under normal growth conditions, (*b*) under conditions of an intensive protein and RNA synthesis, and (*c*) in the presence of chloramphenicol, i.e., under conditions of RNA synthesis

[137] J. H. Stuy, *J. Bacteriol.* **76,** 668 (1958).
[138] K. Dimroth and L. Jaenicke, *Z. Naturforsch.* **5b,** 185 (1950).
[139] R. Thomas, *Biochim. et Biophys. Acta* **8,** 71 (1952).
[140] A. Lombard and E. Chargaff, *Biochim. et Biophys. Acta* **20,** 585 (1956).

in the absence of the protein synthesis.[141] Lombard and Chargaff confirmed these observations with vast experimental material.[113] Furthermore, they have shown that the addition of any of the four ribonucleosides to the medium resulted in its intensive incorporation into the microbial RNA without any change in its composition.[113] Also, various irradiation doses did not alter the RNA composition of *B. cereus*.[137]

Recent work from Chargaff's laboratory[65] revealed that the RNA accumulated in the *E. coli* cells in the presence of chloramphenicol (i.e., in the absence of protein synthesis) was unstable and disintegrated rapidly. The composition of the total RNA of the cell remained invariable both during the presence of this unstable RNA and after its degradation.[65]

Thus, here we encounter the phenomenon of composition invariability of RNA analogous to that observed in the studies on the influence of the age of the cultures. In spite of the variability of the RNA content, the total RNA composition does not change in either case.

Thus, all the data available in the publications of recent years confirm unequivocally the invariability of the DNA and RNA composition both in the course of microbial growth and under different cultural conditions.

Recently, Brawerman and Chargaff presented new data on marked differences in the RNA composition in green and etiolated cells of *Euglena gracilis*[114] (see Table XI). This is a peculiar case, presumably connected with the formation of specific RNA molecules in the course of the development of chloroplasts.

2. Species Specificity of Nucleic Acids

a. DNA Composition

The data presented by Chargaff's laboratory on the DNA of some microorganisms belonging to different systematic groups were of particular and decisive importance for the discovery and the formulation of the concept of the chemical specificity of nucleic acids in general.[14, 81, 142] It was shown that yeast, *H. influenzae*, and *D. pneumoniae* possessed the AT-type of DNA; *Serratia marcescens*, *Bacillus schatz*, and *M. tuberculosis* possessed the DNA of the GC-type; and the DNA composition of *E. coli* occupied an intermediate position having equimolar base proportions.[14, 81, 142-145] No

[141] A. B. Pardee and L. S. Prestidge, *J. Bacteriol.* **71**, 677 (1956).

[142] E. Chargaff, *Experientia* **6**, 201 (1950).

[143] E. Chargaff, S. Zamenhof, G. Brawerman, and L. Kerin, *J. Am. Chem. Soc.* **72**, 3825 (1950).

[144] B. Gandelman, S. Zamenhof, and E. Chargaff, *Biochim. et Biophys. Acta* **9**, 399 (1952).

[145] S. Zamenhof, G. Brawerman, and E. Chargaff, *Biochim. et Biophys. Acta* **9**, 402 (1952).

such great variations were found in higher organisms.[80] Thus, the studies carried out in Chargaff's laboratory have, for the first time, opened special prospects for the study of microorganisms from the viewpoint of bringing out all the regularities and ranges of the species specificity of DNA composition. A large number of data on the DNA composition of microorganisms was obtained some time later through the fundamental work of Ki Yong Lee, Wahl, and Barbu,[130] as well as in the work of our laboratory.[129, 131, 146-148] The data on the DNA composition of gram-negative bacilli are shown in Tables I and II, those for gram-positive bacilli in Table III, and those for cocci in Table IV.

Analyzing all the data available, first those for bacteria, it should be mentioned that they are confirmed by vast factual material on the regularities which were established by Chargaff for the DNA composition,[14, 80, 142] i.e., A = T, G = C, Pu = Py, and G + T = A + C. The ratio (A + T)/(G + C) is the index of the composition specificity of DNA which sums up all possible differences in the DNA composition in bacteria.

The data on the DNA composition in different species of bacteria, which are presented in Tables I–IV, allow the following conclusions: (1) the DNA of bacteria possesses a marked specificity of composition; (2) in closely related species the differences in the DNA composition are usually much smaller than in systematically remote species; (3) the DNA composition of bacteria markedly varies from species to species revealing the diversity of all the types possible: from the pronounced AT-type to the extreme GC-type with all the intermediate ratios.

Proceeding to the analysis of the differences in DNA composition between different groups of bacteria, there should be noted the absence of any direct connection between the DNA composition with either the gram-positive or gram-negative features of the bacteria, as both groups include species with the DNA of the AT- and the GC-types.

French authors[130] were particularly interested in the problem as to whether the aerobic or anaerobic character of the bacteria affects the DNA composition in some manner. However, no correlations could be found with respect to this character.

Analysis of the experimental material on the DNA of bacteria suggests that the DNA composition is most closely related to the systematic position of the organisms.[15, 130, 146, 147, 149] Therefore, the investigation of the differ-

[146] A. S. Spirin, A. N. Belozersky, N. V. Shugayeva, and B. F. Vanyushin, *Biokhimiya* **22**, 744 (1957).

[147] A. N. Belozersky, N. V. Shugayeva, and A. S. Spirin, *Doklady Akad. Nauk S.S.S.R.* **119**, 330 (1958).

[148] G. P. Serenkov and M. V. Pakhomova, *Doklady Vyschej Schkoly* No. 4, 156 (1959).

[149] A. N. Belozersky, "Nucleoproteins and Nucleic Acids of Plants and Their Biological Significance." Acad. Sci. U.S.S.R., Moscow, 1959.

TABLE I

DNA COMPOSITION OF BACTERIA* (GRAM-NEGATIVE BACILLI, EXCEPT ENTEROBACTERIACEAE)

Species	Base proportions (moles %)				$\frac{A+T}{G+C}$	Ref.
	G	A	C	T		
Pseudomonas tabaci	33.7	16.2	33.7	16.4	0.48	*a*
Pseudomonas aeruginosa SI	34.2	17.0	31.9	16.9	0.51	*a*
Pseudomonas aeruginosa 9W	34.1	17.5	30.6	17.8	0.55	*a*
Pseudomonas aeruginosa A22	33.1	18.0	30.9	18.0	0.56	*a*
Pseudomonas aeruginosa 22W	33.1	17.8	31.3	17.8	0.56	*a*
Pseudomonas aeruginosa	33.0	16.8	34.0	16.2	0.49	*b*
Pseudomonas fluorescens	33.0	18.2	30.0	18.8	0.59	*a*
Pseudomonas fluorescens B/S_{1000}	33.0	17.3	30.8	19.0	0.57	*c*
Alcaligenes faecalis	33.9	16.5	32.8	16.8	0.50	*b*
Alcaligenes faecalis	33.8	19.3	36.1	10.8	0.43	*d*†
Alcaligenes faecalis	31.8	15.9	36.0	16.2	0.47	*c*
Agrobacterium tumefaciens (nontumorogenie)	30.6	21.2	28.1	20.1	0.70	*a*
Agrobacterium tumefaciens	30.2	21.7	28.0	20.1	0.72	*a*
Fusobacterium polymorphum	29.9	20.7	29.0	20.4	0.70	*a*
Brucella abortus	29.0	21.0	28.9	21.1	0.73	*b*
Azotobacter chroococcum (N_2)	28.8	20.5	28.5	22.2	0.75	*e*
Azotobacter chroococcum (NH_4^+)	29.8	20.3	27.6	22.3	0.74	*e*
Azotobacter chroococcum (slimy)	29.0	20.3	28.5	22.3	0.74	*f*
Azotobacter chroococcum (dull)	28.8	20.5	28.7	21.9	0.74	*f*
Azotobacter vinelandii (N_2)	27.4	22.1	28.9	21.7	0.78	*e*
Azotobacter vinelandii (NH_4^+)	28.0	21.9	28.0	22.1	0.79	*e*
Azotobacter agile (N_2)	28.3	21.4	26.6	23.8	0.82	*e*
Azotobacter agile (NH_4^+)	28.5	21.5	26.6	23.5	0.82	*e*
Vibrio cholerae	20.0	28.8	23.3	27.9	1.31	*a*
Ristella (Bacteroides) insolitus IS9	23.2	28.2	20.0	28.6	1.31	*a*
Ristella (Bacteroides) insolitus NL12	20.7	29.3	20.5	29.5	1.43	*a*
Haemophilus influenzae	18.2	31.9	19.6	30.2	1.64	*g*
Pasteurella boviseptica	18.8	31.0	18.8	31.4	1.66	*a*
Pasteurella aviseptica	18.2	32.0	18.1	31.7	1.75	*a*
Pasteurella tularensis	17.6	32.4	17.1	32.9	1.88	*b*
Fusiformis fusiformis	15.7	34.8	15.8	33.7	2.17	*a*
Ristella (Bacteroides) clostridiformis	15.8	34.4	15.4	34.4	2.20	*a*

* The following abbreviations are used (Tables I–VII, XII): G, guanine; A, adenine, C, cytosine, T, thymine.

† In this case, a marked deficit of thymine is observed.

References

[a] Ki Yong Lee *et al.*, *Ann. inst. Pasteur* **91**, 212 (1956).

[b] A. S. Spirin *et al.*, *Biokhimiya* **22**, 744 (1957).

[c] B. W. Catlin and L. S. Cunningham, *J. Gen. Microbiol.* **19**, 522 (1958).

[d] H. Goto and S. Akashi, *J. Biochem. (Japan)* **44**, 681 (1957).

[e] G. N. Zaitseva and A. N. Belozersky, *Mikrobiologia* **26**, 722 (1957).

[f] A. N. Belozersky *et al.*, *Mikrobiologia* **27**, 150 (1958).

[g] S. Zamenhof *et al.*, *Biochim. et Biophys. Acta* **9**, 402 (1952).

TABLE II

DNA Composition of Bacteria (Enterobacteriaceae)

Species	Base proportions (moles %)				$\frac{A+T}{G+C}$	Ref.
	G	A	C	T		
Serratia marcescens	27.2	20.7	32.0	20.1	0.69	*a*
Serratia marcescens	29.0	21.1	29.0	20.9	0.72	*b*
Aerobacter aerogenes	29.3	20.5	27.8	22.4	0.75	*b*
Aerobacter aerogenes	28.8	21.3	28.0	21.9	0.76	*c*
Aerobacter aerogenes (normal)	28.4	21.2	29.2	21.2	0.74	*d*
Aerobacter aerogenes (streptomycin resistant)	29.2	20.8	29.7	20.3	0.70	*d*
Aerobacter aerogenes	28.1	19.8	27.5	19.2	0.70	*e**
Aerobacter aerogenes	28.2	20.3	27.0	19.1	0.71	*e**
Erwinia carotovora	27.1	23.3	26.9	22.7	0.85	*c*
Salmonella typhimurium	25.4	24.6	24.8	25.2	0.99	*b*
Salmonella typhimurium	27.1	22.9	27.0	23.0	0.85	*c*
Salmonella typhosa	26.7	23.5	26.4	23.4	0.88	*c*
Salmonella gallinarum	26.1	24.8	24.1	25.0	0.99	*b*
Salmonella paratyphi A	24.9	24.8	25.0	25.3	1.00	*b*
Salmonella enteritidis	25.0	24.9	25.0	25.1	1.00	*b*
Salmonella enteritidis (resistant to D_4 phage)	24.7	24.4	25.2	25.7	1.00	*b*
Shigella dysenteriae	26.7	23.5	26.7	23.1	0.87	*c*
Shigella paradysenteriae Flexner Y6 R0	24.8	24.9	24.5	25.8	1.02	*b*
Shigella paradysenteriae Flexner Y6 R4	25.3	25.8	23.9	25.0	1.03	*b*
Proteus morganii	26.3	23.7	26.7	23.3	0.89	*c*
Escherichia coli (B/r)	24.5	22.5	25.8	27.2	0.99	*f*
Escherichia coli K12	24.9	26.0	25.2	23.9	1.00	*g*
Escherichia coli UQ	25.0	25.6	25.5	23.9	0.98	*g*
Escherichia coli (thymineless)	24.1	25.4	25.7	24.8	1.01	*g*
Escherichia coli SM	26.0	23.8	26.4	23.8	0.91	*h*
Escherichia coli I	26.0	23.9	26.2	23.9	0.92	*c*
Escherichia coli W	25.7	25.1	25.9	23.4	0.94	*i*
Escherichia coli W	26.3	24.4	25.5	23.8	0.93	*i*
Escherichia coli (B/r)	26.4	23.1	25.1	25.0	0.94	*e**
Escherichia coli ($15T^-$)	27.5	23.6	26.1	22.4	0.86	*e**
Proteus vulgaris	18.6	32.0	17.9	31.5	1.74	*b*
Proteus vulgaris	19.8	30.1	20.7	29.4	1.47	*c*

* The sum of the four bases given does not equal 100; the missing mole % consist of 6-methylaminopurine.

References

[a] S. Zamenhof *et al.*, *Biochim. et Biophys. Acta* **9**, 402 (1952).
[b] Ki Yong Lee *et al.*, *Ann. inst. Pasteur* **91**, 212 (1956).
[c] A. S. Spirin *et al.*, *Biokhimiya* **22**, 744 (1957).
[d] A. S. Jones *et al.*, *J. Gen. Microbiol.* **17**, 586 (1957).
[e] D. B. Dunn and J. D. Smith, *Biochem. J.* **68**, 627 (1958).
[f] J. D. Smith and G. R. Wyatt, *Biochem. J.* **49**, 144 (1951).
[g] B. Gandelman *et al.*, *Biochim. et Biophys. Acta* **9**, 399 (1952).
[h] A. S. Spirin and A. N. Belozersky, *Biokhimiya* **21**, 768 (1956).
[i] E. Chargaff *et al.*, *Nature* **180**, 851 (1957).

TABLE III

DNA COMPOSITION OF BACTERIA (GRAM-POSITIVE BACILLI)

Species	Base proportions (moles %)				$\frac{A+T}{G+C}$	Ref.
	G	A	C	T		
Corynebacterium parvum	29.8	21.5	28.4	20.3	0.72	*a*
Mycobacterium (Corynebacterium) vadosum Kras.	29.2	20.7	28.5	21.6	0.73	*b*
Corynebacterium diphtheriae	27.2	22.5	27.3	23.0	0.83	*b*
Corynebacterium diphtheriae	24.8	24.2	27.1	23.9	0.92	*a**
Corynebacterium acnes	21.1	26.3	26.8	25.8	1.08	*a*
Lactobacillus bifidus	28.3	21.9	29.3	20.5	0.74	*a*
Bacillus subtilis	21.0	28.9	21.4	28.7	1.36	*a*
Bacillus megaterium	19.1	31.8	18.5	30.6	1.66	*a*
Bacillus thuringiensis amer.	18.1	32.2	17.8	31.9	1.78	*a*
Bacillus cereus A_{25}	17.9	32.3	16.6	33.2	1.90	*a*
Bacillus cereus alesti	17.3	33.5	16.0	33.2	2.00	*a*
Bacillus cereus alesti (mutant)	18.0	31.9	17.7	32.4	1.80	*a*
Bacillus cereus alesti (mutant)	17.6	32.0	18.7	31.7	1.75	*a*
Bacillus cereus p 1	18	32	18	32	1.78	*c*
Bacillus cereus p 2	17	33	17	34	1.97	*c*
Bacillus cereus ATCC 12137	17	32	19	32	1.78	*c*
Bacillus cereus BTCC 7587	18	32	17	33	1.85	*c*
Bacillus cereus (not irradiated)	18	32	17	33	1.85	*d*
Bacillus cereus (irradiated)	19	31	18	32	1.70	*d*
Clostridium bifermentans	17.0	34.0	15.5	33.5	2.08	*a*
Clostridium valerianicum	16.2	35.1	15.6	33.1	2.14	*a*
Clostridium saprogenes	16.0	34.7	14.3	35.0	2.30	*a*
Ramibacterium ramosum	14.9	35.1	15.2	34.8	2.32	*a*
Clostridium perfringens (var. Fred)	14.0	36.9	12.8	36.3	2.70	*a*
Clostridium perfringens	15.8	34.1	15.1	35.0	2.24	*b*

* In this case a strong deficit of guanine is observed.

References

[a] Ki Yong Lee *et al.*, *Ann. inst. Pasteur* **91**, 212 (1956).
[b] A. S. Spirin *et al.*, *Biokhimiya* **22**, 744 (1957).
[c] J. H. Stuy, *J. Bacteriol.* **76**, 179 (1958).
[d] J. H. Stuy, *J. Bacteriol.* **76**, 668 (1958).

ences in the DNA composition in different bacteria from the viewpoint of their systematics and phylogeny seems to be most promising.

As a comparison with the Bergey classification[150] will show, representatives of at least 13 families have been examined so far. The families most widely studied with respect to their specific composition are: Micrococcaceae, Brucellaceae and Bacteroidaceae (Parvobacteriaceae), Enterobacteriaceae, and Bacillaceae.

[150] "Bergey's Manual of Determinative Bacteriology," 7th ed. Williams & Wilkins, Baltimore, 1957.

TABLE IV

DNA Composition of Bacteria (Gram-Positive and Gram-Negative Cocci)

Species	Base proportions (moles %)				$\frac{A+T}{G+C}$	Gram	Ref.
	G	A	C	T			
Sarcina lutea	37.1	13.4	37.1	12.4	0.35	+	*a*
Sarcina lutea	36.4	13.6	35.6	14.4	0.39	+	*b*
Sarcina lutea	32.4	18.7	31.5	17.4	0.56	+	*c*
Micrococcus lysodeikticus	37.3	14.4	34.6	13.7	0.39	+	*e*
Sarcina flava	33.5	15.6	35.1	15.8	0.46	+	*e*
Neisseria meningitidis	25.5	24.6	25.0	24.9	1.00	−	*e*
Neisseria gonorrhoeae	25.2	25.3	24.4	25.1	1.01	−	*c*
Diplococcus pneumoniae type III	20.5	29.8	18.0	31.6	1.59	+	*d*
Veillonella parvula	18.5	31.7	18.0	31.8	1.74	−	*c*
Streptococcus faecalis ("bound DNA")	18.3	29.2	20.3	32.2	1.59	+	*e*
Streptococcus faecalis ("free DNA")	16.5	30.0	21.0	32.5	1.67	+	*e*
Streptococcus faecalis (group D)	16.9	33.4	17.7	32.0	1.89	+	*c*
Streptococcus foetidus	16.8	32.1	16.8	34.3	1.97	+	*c*
Streptococcus pyogenes (group A)	16.6	33.4	17.0	33.0	1.97	+	*c*
Streptococcus zymogenes (group D)	16.2	33.1	17.4	33.3	1.97	+	*c*
Micrococcus asaccharolyticus	18.8	31.2	15.3	34.7	1.91	+	*c*
Micrococcus (*Staphylococcus*) *aureus* SA-B	21.0	30.8	19.0	29.2	1.50	+	*f*
Micrococcus (*Staphylococcus*) *aureus* 209P	18.5	31.0	19.2	31.2	1.65	+	*f*
Micrococcus pyogenes (*Staphylococcus aureus*)	17.3	32.3	17.4	33.0	1.88	+	*b*
Micrococcus pyogenes Oxf.	15.0	34.1	15.7	32.2	2.26	+	*c*
Micrococcus pyogenes 1161	16.0	35.7	14.9	33.4	2.26	+	*e*
Micrococcus pyogenes 1149	16.1	34.2	15.1	34.6	2.20	+	*c*
Micrococcus pyogenes 145	16.2	34.4	15.2	34.2	2.18	+	*c*
Micrococcus pyogenes m 320	15.6	34.2	16.4	33.8	2.13	+	*e*
Staphylococcus epidermidis	17.5	31.0	17.6	33.9	1.85	+	*f*

References

[a] S. K. Dutta *et al.*, *J. Gen. Microbiol.* **14**, 160 (1956).
[b] A. S. Spirin *et al.*, *Biokhimiya* **22**, 144 (1957).
[c] Ki Yong Lee *et al.*, *Ann. inst. Pasteur* **91**, 212 (1956).
[d] M. M. Daly *et al.*, *J. Gen. Physiol.* **33**, 497 (1950).
[e] H. S. Sherratt and A. J. Thomas, *J. Gen. Microbiol.* **8**, 217 (1953).
[f] B. W. Catlin and L. S. Cunningham, *J. Gen. Microbiol.* **19**, 522 (1958).

On inspection of Table I (gram-negative bacilli), it will be noticed that the arrangement of the organisms in the order of increasing values of the ratio (A + T)/(G + C) may not be fortuitous from a systematic point of view. As a matter of fact, with some exceptions, the species which have been placed at the top of the table (species with the marked GC-type of the DNA) represent such families as Pseudomonadaceae, Achromobacteriaceae, and Azotobacteriaceae. The bottom of the table (the marked AT-

type of the DNA) is occupied by the representatives of the families of Brucellaceae and Bacteroidaceae which were united earlier in one family of Parvobacteriaceae.

As to the family of Enterobacteriaceae as a whole (Table II), its representatives generally possess the DNA of more or less intermediate type which nevertheless trends to a slightly expressed GC-type. Within this family the variations of the (A + T)/(G + C) ratio run from 0.7 to 1.0. The exception to this is *P. vulgaris* in which this value is significantly higher than 1 (AT-type of the DNA). Besides, another representative of the *Proteus* genus, *Proteus morganii*, drastically differs in DNA composition from *P. vulgaris* and actually is very similar to the bacteria of the *Shigella-Salmonella* group in respect to this feature. Two questions then arise: (1) Is it actually correct to refer *P. morganii* to the same genus as *P. vulgaris*? (2) How close is *P. vulgaris* to most representatives of the family of Enterobacteriaceae? Thus, if one does not take into consideration the exception mentioned, the family of Enterobacteriaceae may be said to differ from all the other gram-negative bacilli in a slightly expressed GC-type of DNA.

Most species of closely related families of Brucellaceae and Bacteroidaceae (*Pasteurella*, *Bacteroides*, *Fusobacterium*, *Hemophilus*) are, on the other hand, characterized by a marked AT-type of DNA. The value of the (A + T)/(G + C) ratio varies from 1.6 to 2.2. However, *Brucella*, as well as *Fusobacterium polymorphum* are drastically different, possessing a marked GC-type of DNA [(A + T)/(G + C) = 0.7]. This fact must also raise a number of questions concerning the systematics, at any rate, with respect to the genus *Brucella*. In any case, most of the different representatives of the two families in question form a rather close group with respect to DNA composition.

The analysis of gram-positive bacilli (Table III) is also of interest. Here, the representatives studied divide into groups in exact correspondence with their systematic positions. The organisms having the GC-type of DNA belong to the first group. They represent, as a rule, the species belonging to the family of Corynebacteriaceae (the genus *Corynebacterium*). The organisms with the AT-type of DNA in which the (A + T)/(G + C) ratio does not exceed 2 belong to the second group. All belong to the genus *Bacillus*, the family of Bacillaceae. Finally, the third group, following the second, includes the species with the most marked AT-type of DNA. All of them also belong to the family of Bacillaceae, but to another genus, namely, *Clostridium*. Thus, the systematic division into groups coincides with that obtained from their DNA composition and their DNA types.

Turning now to the analysis of the cocci (Table IV), it will be seen that the marked GC-type of DNA in the cocci is typical of the representatives of the family of Micrococcaceae (*Sarcina*, *Micrococcus lysodeikticus*). A radically different DNA composition, of the extreme AT-type, is found

however in the staphylococci. It is not ruled out that these two groups of the Micrococcaceae are, indeed, phylogenetically much more remote from each other than is usually thought. But the streptococci appear to be closely related to the staphylococci in respect to the composition of their DNA which shows a marked AT-type. Gram-negative cocci (family of Neisseriaceae) occupy, by their DNA composition, an intermediate and peculiar position among the cocci. The *Neisseria* genus is characterized by equimolar base proportions.

Thus, the analysis of the DNA composition of the bacteria carried out in respect to their classification in families confirms the viewpoint that the DNA composition is closely related to the systematic position of the organism and its phylogenic origin.

When one now considers the problem of the differences in the DNA composition in species belonging to the same genus, i.e., in closely related species, certain conventionality of the classification of the bacteria should be noted at once. We do not know to what extent the bacterial species correspond to the conception of the species in the systematics of higher organisms. At any rate, biological differences between the bacterial species within a genus may prove to be of a very different order. This may be just the reason for occasionally finding considerable differences in the DNA composition in species belonging to the same genus (for example, the genera of *Pseudomonas*,[130] *Azotobacter*,[131] *Pasteurella*,[130] *Bacteroides*,[130] *Corynebacterium*,[130] and *Bacillus*[130]), whereas in other cases, species of the same genus are indistinguishable by their DNA composition (for example, the genera of *Neisseria*,[130] *Streptococcus*,[130] and *Salmonella*[130]). In any event, it is nevertheless possible to define slight species differences in some cases in which special statistical treatment of the data shows the complete reliability of these differences though they are very small.[146] We succeeded in showing the reliability of such very small differences in *Salmonella typhosa* and *Salmonella typhimurium*, as well as their difference from the DNA of *E. coli*.[146]

The problem of strain differences in the DNA composition is not yet completely solved. In the literature, there is mentioned repeatedly the possibility of the existence of small but statistically significant differences in DNA composition between different strains of the same species. For example, significant differences in the DNA composition are found between the original strain *B. cereus* var. *alesti* and its mutant obtained by means of cultivation in an alkaline medium.[130] Difference of the DNA composition is shown in a streptomycin-resistant strain of *A. aerogenes* when it is compared to the normal strain.[151] In most cases, however, the authors stress the absence of any appreciable strain differences in DNA composition.[28, 85, 130, 131, 132, 144]

It is evident that in connection with the agreement shown here between the DNA composition of the bacteria and their systematic position, the analysis of the data on the DNA composition in other microorganisms is of great interest. In Table V, the findings on the DNA composition in mycobacteria and actinomycetes are summarized. The species shown in the table are grouped in 3 families of the order Actinomycetales. All these representatives are characterized by the extreme GC-type of DNA. The mycobacteria, however, show a considerable difference in their DNA com-

[151] A. S. Jones, G. E. Marsh, and S. B. H. Rizvi, *J. Gen. Microbiol.* **17**, 586 (1957).

TABLE V

DNA COMPOSITION OF MYCOBACTERIA AND ACTINOMYCETES

Species	Base proportions (moles %)				$\frac{A+T}{G+C}$	Ref.
	G	A	C	T		
M. tuberculosis (human)	28.5	18.0	33.5	20.0	0.61	*a*
M. tuberculosis (human)	28.2	19.3	34.9	17.5	0.58	*b*
M. tuberculosis (bovine)	29.3	17.8	33.8	19.0	0.58	*a*
M. tuberculosis (bovine)	34.2	16.5	33.3	16.0	0.48	*c*
M. tuberculosis (bovine)	32.7	18.3	32.3	16.7	0.54	*d*
M. tuberculosis (avian)	34.9	15.1	35.4	14.6	0.42	*e*
M. phlei	31.6	18.0	34.8	15.5	0.50	*b*
M. phlei	34.5	15.8	34.2	15.5	0.46	*f*
Micromonospora coerulea	36.2	14.3	35.6	13.9	0.39	*g*
Nocardia citrea	35.3	14.2	36.7	13.8	0.39	*g*
Streptomyces griseus	35.8	13.8	37.4	13.0	0.37	*g*
Streptomyces streptomycini	36.1	13.4	37.1	13.4	0.37	*c*
Streptomyces globisporus	36.3	13.8	37.2	12.7	0.36	*g*
Streptomyces viridochromogenes	36.6	13.3	37.2	12.9	0.36	*g*

References

[a] J. D. Smith and G. R. Wyatt, *Biochem. J.* **49**, 144 (1951).
[b] S. G. Laland *et al.*, *J. Chem. Soc.* p. 3224 (1952).
[c] A. S. Spirin *et al.*, *Biokhimiya* **22**, 744 (1957).
[d] T. Tsumita and E. Chargaff, *Biochim. et Biophys. Acta* **29**, 568 (1958).
[e] E. Vischer *et al.*, *J. Biol. Chem.* **177**, 429 (1949).
[f] A. S. Jones *et al.*, *J. Chem. Soc.* p. 2454 (1957).
[g] A. N. Belozersky *et al.*, *Doklady Acad. Nauk S.S.S.R.* **119**, 330 (1958).

position from that of the true actinomycetes. This is in accord with the fact that the actinomycetes and acid-resistant mycobacteria represent two different, rather isolated groups of the order Actinomycetales. However, the low value of the (A + T)/(G + C) ratio in mycobacteria may testify to their relationship with the Actinomycetales.

As to the true actinomycetes (the families of Actinomycetaceae and Streptomycetaceae), all analyzed representatives, being characterized by an extreme GC-type of DNA, show a rather considerable similarity in their DNA composition.[147] It seems that we may regard them as rather closely related organisms forming one peculiar group which is perhaps narrow in systematical aspect. All four representatives of the genus *Streptomyces* studied are statistically absolutely indistinguishable by their DNA composition.[147] Thus, in the case studied, no species specificity of DNA composition was found in the species within a genus. At the same time, there can be found some differences in the DNA composition of different actinomycete genera.

The data on the DNA composition of fungi are presented in Table VI. Unfortunately, there exist only few data on this group of microorganisms,

insufficient to permit any conclusions as to the variability of their DNA composition. However, the data presented show a great difference in the DNA composition between the representatives of two orders belonging to the same class of Ascomycetes: *Saccharomyces cerevisiae* and *Aspergillus niger*. Hence, it may seem that this group of microorganisms can also be characterized by a great variety of DNA composition. This seems promising for the investigation of fungi from the viewpoint of the species specificity of DNA composition.

The data obtained by Serenkov[148] on the DNA composition of some algae are shown in Table VII. Unfortunately Low's[87] data could not be included

TABLE VI

DNA Composition of Fungi

Species	Base proportions (moles %)				$\frac{A+T}{G+C}$	Ref.
	G	A	C	T		
Saccharomyces cerevisiae	18.3	31.7	17.4	32.6	1.80	*a*
Saccharomyces cerevisiae	18.7	31.3	17.1	32.9	1.79	*b*
Psalliota campestris	22.6	28.2	21.7	27.5	1.26	*c*
Aspergillus niger	25.1	25.0	25.0	24.9	1.00	*c*

References

[a] E. Vischer *et al.*, *J. Biol. Chem.* **177**, 429 (1949).
[b] S. Zamenhof and E. Chargaff, *J. Biol. Chem.* **187**, 1 (1950).
[c] S. O. Uryson, our laboratory (unpublished).

TABLE VII

DNA Composition of Algae*

Species	Base proportions (moles %)				$\frac{A+T}{G+C}$
	G	A	C	T	
Diatomeae					
Rhabdonema adriaticum	18.6	31.4	18.3	31.7	1.71
Chaetoceras decipiens	19.9	30.8	19.2	30.1	1.55
Thalassiosira nordenscheldii	20.1	29.8	20.2	29.9	1.48
Chlorophyceae					
Hydrodictyon reticulatum	27.3	23.1	26.2	23.4	0.87
Ankistrodesmus sp.	29.7	21.8	29.0	19.5	0.70
Scenedesmus quadricauda	30.8	20.2	30.2	18.8	0.64
Scenedesmus accuminatus	32.9	18.7	30.9	17.5	0.57
Phaeophyceae					
Cystosira barbata	29.5	20.8	29.3	20.4	0.70

* Taken from G. P. Serenkov and M. V. Pakhomova, *Doklady Vyschej Schkoly* No. 4, p. 156 (1959).

in this table as the author did not present figures, restricting himself to the values for the A/T, G/C, and Pu/Py ratios. It should be noted that the findings of two studies[87, 148] show the DNA composition of algae to be in full accord with the regularities stated by Chargaff for the base ratios in DNA. The data presented in Table VII show that the DNA of the group of algae, as well as that of the bacteria, exhibits a considerable variability of its composition. One encounters, here, representatives with a marked AT-type of DNA (diatomic) and, on the other hand, others, of which the DNA belongs to a rather marked GC-type (green). The data, however, not being numerous up to the present, do not allow wider conclusions on the species specificity of the DNA composition of algae.

With respect to the representatives of protozoa, only the data from the paper of Scherbaum on *T. pyriformis* may be quoted.[133] The DNA composition of this organism is as follows (in mole %): guanine, 11.5; adenine, 36.6; cytosine, 15.0; and thymine, 36.9.

In reviewing the data available in the literature, there can be found an extraordinary variability of the DNA composition of microorganisms depending upon the species to which they belong. And indeed, the value of the (A + T)/(G + C) ratio varies from 0.35 in *Actinomyces* and *Sarcina* to 2.7 in *Clostridium*. When comparing these data with those available on the DNA composition in higher organisms, animals[80, 152] and plants,[89] attention is drawn to the fact that the variations in DNA composition are, by far, more restricted in higher organisms. Thus, the value of the (A + T)/(G + C) ratio ranges in the representatives of different types of animals[152] from 1.3 to 2.2, whereas in the representatives of different types and families of plants[89] it fluctuates only within the range of 1.1 to 1.7. Besides, unlike the microorganisms, all the DNA of higher forms belong to the AT-type. Such an exceptional diversity of the DNA composition of microorganisms may be connected with the unusual differentiation of lower forms in functional aspects and with the polyphyletic origin of species in this group of living beings.

All the material analyzed above, shows the taxonomic value of the DNA composition. There are grounds for thinking that the determination of the DNA composition may be useful for the precise definition of the phylogenetic relations of different groups of bacteria and, perhaps, of other microorganisms as well.

b. RNA Composition

In the early stages of the study of RNA composition, the possibility of wide species and even individual variations was not ruled out.[111] This con-

[152] Ki Yong Lee and E. Barbu, *Compt. rend. soc. biol.* **150**, 865 (1956).

TABLE VIII

RNA Composition of Gram-Negative Bacteria*

Species	Base proportions (moles %)				(G + U)/(A + C)	Pu/Py	(G + C)/(A + U)	Ref.
	G	A	C	U				
Pseudomonas aeruginosa	31.6	25.1	23.8	19.5	1.05	1.31	1.24	*a*
Alcaligenes faecalis	30.9	25.7	24.1	19.3	1.01	1.31	1.22	*a*
Alcaligenes faecalis	30.6	24.8	21.8	22.7	1.14	1.24	1.10	*b*
Azotobacter chroococcum (N_2)	30.4	24.7	24.7	20.1	1.02	1.18	1.23	*c*
Azotobacter chroococcum (NH_4^+)	31.2	24.2	25.4	19.3	1.02	1.24	1.30	*c*
Azotobacter chroococcum (slimy)	30.9	23.7	25.1	20.3	1.05	1.20	1.27	*d*
Azotobacter chroococcum (dull)	31.5	24.1	24.6	19.8	1.05	1.25	1.28	*d*
Azotobacter vinelandii	30.6	24.2	25.7	19.5	1.00	1.21	1.29	*e*
Azotobacter vinelandii (N_2)	30.3	24.0	25.6	20.2	1.02	1.19	1.27	*e*
Azotobacter vinelandii (NH_4^+)	29.8	24.4	26.0	19.8	0.98	1.18	1.26	*e*
Azotobacter agile (N_2)	31.0	24.2	26.0	18.7	0.99	1.23	1.33	*c*
Azotobacter agile (NH_4^+)	30.4	24.6	25.7	19.3	0.99	1.22	1.28	*c*
Serratia marcescens	31.2	20.3	24.3	24.1	1.24	1.06	1.25	*f*
Aerobacter aerogenes	29.7	24.5	26.0	19.8	0.98	1.19	1.25	*g*
Aerobacter aerogenes	30.3	26.0	24.1	19.6	1.00	1.29	1.19	*a*
Aerobacter aerogenes (normal)	29.6	26.2	24.4	19.7	0.98	1.26	1.18	*h*
Aerobacter aerogenes (strepomycin resistant)	30.1	25.9	24.4	19.5	0.99	1.27	1.20	*h*
Erwinia carotovora	29.5	26.5	23.7	20.3	0.99	1.27	1.14	*a*
Salmonella typhimurium	31.0	26.1	23.8	19.1	1.00	1.33	1.21	*a*
Salmonella typhosa	30.8	26.1	24.0	19.1	1.00	1.32	1.21	*a*
Shigella dysenteriae	30.4	25.9	24.4	19.3	0.99	1.29	1.21	*a*
Proteus morganii	31.1	26.0	23.7	19.2	1.01	1.31	1.21	*a*
Escherichia coli	28.8	25.3	24.7	21.2	1.00	1.18	1.15	*f*
Escherichia coli SM	31.4	26.6	23.7	18.3	0.99	1.38	1.23	*i*
Escherichia coli I	30.7	26.0	24.1	19.2	1.00	1.31	1.21	*a*
Escherichia coli B	35.0	27.0	21.0	17.0	1.08	1.63	1.27	*j*
Escherichia coli (resting)	31.2	23.2	23.2	22.3	1.15	1.20	1.19	*k*
Escherichia coli (dividing)	31.9	23.9	21.8	22.4	1.19	1.26	1.16	*k*
Escherichia coli B	29.6	26.3	25.1	19.0	0.95	1.27	1.21	*l*
Escherichia coli M26-26	29.3	25.6	24.4	20.8	1.00	1.22	1.16	*m*
Escherichia coli M26-26	30.5	25.2	24.7	19.6	1.00	1.26	1.23	*m*
Proteus vulgaris	31.0	26.3	24.0	18.7	0.99	1.34	1.22	*a*
Proteus vulgaris	29.0	27.0	21.0	23.0	1.08	1.27	1.00	*n*
Proteus vulgaris (L-form)	29.0	26.4	21.4	23.2	1.09	1.24	1.01	*n*
Brucella abortus	30.2	25.4	24.9	19.5	0.99	1.26	1.23	*a*
Pasteurella tularensis	29.8	27.3	21.0	21.9	1.07	1.33	1.03	*a*

* The following abbreviations are used (Tables VIII–XI, XIII): G, guanine; A, adenine; C, cytosine; U, uracil; Pu, purine bases; Py, pyrimidine bases.

cept was favored, together with the analogy to the species specificity of DNA, and also with the data obtained by means of the study of the RNA composition of isolated RNA preparations.

Reliable and trustworthy data with respect to the specificity of the RNA composition became available only when the necessity was realized of studying its composition directly on the biological material without the isolation of the RNA in the form of purified preparations (so as to prevent secondary changes in composition due to fermentative and chemical effects). The work of Thomas, who studied the RNA composition of the yeasts by means of direct hydrolysis of the cells, was one of the first to point to this necessity.[139] Investigations on a wider scale were undertaken in Chargaff's laboratory.[153-155] On the basis of such investigations it became possible to formulate general regularities of the native RNA composition[154, 155]; this was a very important factor in judging the reliability or unreliability of the data obtained. "Chargaff's rule" (G + U = A + C) is thus, at present, the main criterion of the native character of the RNA composition.

The data on the RNA composition in bacteria and actinomycetes are presented in Tables VIII and IX, those for fungi in Table X, and those for algae and protozoa in Table XI.

When reviewing the analytical data in all of these tables it should be noted that the regularity stated from Chargaff's laboratory[154, 155]—(G + U = A + C)—was completely confirmed; at any rate, when the analysis was performed directly on the material and not on isolated preparations.

When analyzing the RNA composition of bacteria (Tables VIII and IX), we are struck by the comparatively small variation range of the RNA composition when passing from species to species. Thus, according to our own data,[146, 156] the molar content in guanylic acid fluctuates in different species of bacteria from 26.7 to 33.0%; that of adenylic acid from 22.6 to 28.1%; cytidylic acid from 21 to 26.1%; and that of uridylic acid from 18.3

[153] D. Elson, I. Gustafson, and E. Chargaff, *J. Biol. Chem.* **209**, 235 (1954).
[154] D. Elson and E. Chargaff, *Nature* **173**, 1037 (1954).
[155] D. Elson and E. Chargaff, *Biochim. et Biophys. Acta* **17**, 367 (1955).

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[b] H. Goto and S. Akashi, *J. Biochem. (Japan)* **44**, 681 (1957).
[c] G. N. Zaitseva and A. N. Belozersky, *Mikrobiologia* **26**, 722 (1957).
[d] A. N. Belozersky *et al.*, *Mikrobiologia* **27**, 150 (1958).
[e] A. Lombard and E. Chargaff, *Biochim. et Biophys. Acta* **20**, 585 (1956).
[f] D. Elson and E. Chargaff, *Biochim. et Biophys. Acta* **17**, 367 (1955).
[g] R. A. Cox *et al.*, *Biochim. et Biophys. Acta* **21**, 576 (1956).
[h] A. S. Jones *et al.*, *J. Gen. Microbiol.* **17**, 586 (1957).
[i] A. S. Spirin and A. N. Belozersky, *Biokhimiya* **21**, 768 (1956).
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[k] H. E. Wade and D. M. Morgan, *Biochem. J.* **65**, 321 (1957).
[l] A. Lombard and E. Chargaff, *Biochim. et Biophys. Acta* **25**, 549 (1957).
[m] J. Horowitz *et al.*, *J. Biol. Chem.* **233**, 1517 (1958).
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TABLE IX

RNA Composition of Gram-Positive Bacteria Including Mycobacteria and Actinomycetes

Species	Base proportions (moles %)				(G + U)/(A + C)	Pu/Py	(G + C)/(A + U)	Ref.
	G	A	C	U				
Bacilli								
Mycobacterium (*Corynebacterium*) *vadosum* Kras.	31.7	23.8	23.5	21.0	1.12	1.25	1.23	*a*
Corynebacterium diphtheriae	31.6	23.1	23.8	21.5	1.13	1.21	1.24	*a*
Bacillus cereus p1	30.7	25.5	21.3	22.5	1.14	1.28	1.08	*b*
Bacillus cereus p2	31.2	24.8	20.1	23.8	1.22	1.27	1.05	*b*
Bacillus cereus ATCC 12137	31.5	25.2	20.4	22.9	1.19	1.31	1.08	*b*
Bacillus cereus BTCC 7587	31.1	25.9	19.4	23.6	1.21	1.32	1.02	*b*
Bacillus cereus (not irradiated)	30.8	25.5	19.5	24.2	1.22	1.29	1.01	*c*
Bacillus cereus (irradiated)	30.8	25.9	19.9	23.4	1.18	1.31	1.03	*c*
Clostridium perfringens	30.3	26.1	24.5	19.2	0.98	1.29	1.20	*d*
Clostridium perfringens	29.5	28.1	22.0	20.4	1.00	1.36	1.06	*a*
Cocci								
Sarcina lutea	32.7	23.2	24.2	19.9	1.11	1.27	1.32	*a*
Sarcina lutea (normal)	28.4	16.7	32.9	22.0	1.01	0.82	1.58	*e**
Sarcina lutea (streptomycin resistant)	29.8	15.7	34.4	20.2	1.00	0.83	1.79	*e**
Streptococcus faecalis	31.3	25.0	15.2	28.5	1.48	1.29	0.87	*f*†
Micrococcus pyogenes (*Staphylococcus aureus*)	28.7	26.9	22.4	22.0	1.03	1.25	1.05	*a*
Mycobacteria and actinomycetes								
Mycobacterium tuberculosis (bovine) (BCG)	33.0	22.6	26.1	28.3	1.05	1.25	1.45	*a*
Mycobacterium tuberculosis (bovine) (BCG)	31.3	20.1	29.4	19.2	1.02	1.05	1.55	*g*
Mycobacterium phlei	30.8	20.9	27.1	21.2	1.08	1.07	1.37	*h*
Streptomyces streptomycini	31.1	23.8	25.2	19.9	1.04	1.22	1.29	*a*

* In these cases, isolated RNA samples were investigated and the possibility of secondary alterations of RNA composition was not excluded.

† Considerable deamination of cytidylic acid appears to have taken place.

References

[a] A. S. Spirin *et al.*, *Biokhimiya* **22**, 744 (1957).
[b] J. H. Stuy, *J. Bacteriol.* **76**, 179 (1958).
[c] J. H. Stuy, *J. Bacteriol.* **76**, 668 (1958).
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[h] D. Elson and E. Chargaff, *Biochim. et Biophys. Acta* **17**, 367 (1955).

TABLE X

RNA COMPOSITION OF FUNGI*

Species	Base proportions (moles %)				(G + U)/(A + C)	Pu/Py	(G + C)/(A + U)	Ref.
	G	A	C	U				
Saccharomyces cerevisiae	24.6	25.4	22.6	27.4	1.08	1.00	0.90	*a*
Saccharomyces cerevisiae	28	25	20	27	1.22	1.13	0.92	*b*
Saccharomyces cerevisiae	27.5	23.5	27.3	21.7	0.97	1.04	1.21	*c*
Saccharomyces cerevisiae, fr. 1	33.0	23.2	20.0	23.7	1.31	1.29	1.13	*d*
Saccharomyces cerevisiae, fr. 2	27.3	24.3	22.5	25.7	1.13	1.07	1.00	*d*
Saccharomyces cerevisiae, fr. 3	33.2	25.6	14.8	26.2	1.47	1.43	0.93	*d*
Penicillium stoloniferum	27.0	24.9	23.6	24.7	1.07	1.07	1.02	*e*
Aspergillus niger	31.1	24.7	25.1	19.1	1.01	1.26	1.28	*f*

* In this table, only the data published since 1955 are included.

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[a] D. Elson and E. Chargaff, *Biochim. et Biophys. Acta* **17**, 367 (1955).
[b] A. M. Crestfield *et al.*, *J. Biol. Chem.* **216**, 185 (1955).
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[d] A. A. Hakim, *J. Biol. Chem.* **225**, 689 (1957).
[e] W. J. Kleinschmidt and J. A. Manthey, *Arch. Biochem. and Biophys.* **73**, 52 (1958).
[f] I. S. Kulaev, our laboratory.

to 22%. Thus, the molar content of each of the nucleotides varies around some mean value for all the species by no more than 10–12%. In all cases of bacterial RNA studied by us, the relationship was in general G > A ≃ C > U. The value of the purine to pyrimidine ratio shows an even greater invariability: the amplitude of its variation in the RNA of the bacteria studied ranged from 1.21 to 1.36, i.e., ± 6%.[146, 156] Another ratio, namely (G + C)/(A + U), changes somewhat more markedly when passing from species to species, with an amplitude of variation between 1.03 and 1.45; this means that (G + C)/(A + U) = 1.25 ± 0.20, i.e., that all the deviations from the value 1.25 keep within ±16%.[146] This ratio brings out best compositional differences between RNA species.

However, analytically demonstrable species specificity in RNA composition seems to be restricted to species remote from each other in systematic aspects. It could not be found in closely related species. For example, according to our data[146] we can definitely speak of the identify (within methodical errors) of the RNA composition in *E. coli*, *P. morganii*, *Shigella dysenteriae*, *S. typhosa*, and *S. typhimurium*. With a rather high probability we

[156] A. S. Spirin and A. N. Belozersky, *Doklady Akad. Nauk S.S.S.R.* **113**, 650 (1957).

TABLE XI

RNA Composition of Algae and Protozoa

Species	Base proportions (moles %)				(G + U)/(A + C)	Pu/Py	(G + C)/(A + U)	Ref.
	G	A	C	U				
Algae								
Rhabdonema adriaticum	28.6	24.0	26.2	21.2	0.99	1.11	1.21	*a*
Chaetocerus decipiens	27.7	25.7	24.4	22.2	0.99	1.15	1.09	*a*
Thalossiosira nordenscheldii	31.3	24.1	25.2	19.4	1.03	1.24	1.30	*a*
Hydrodiction reticulatum	30.1	23.2	26.3	20.4	1.02	1.14	1.29	*a*
Ankistrodesmus sp.	30.5	22.8	25.1	21.6	1.09	1.14	1.25	*a*
Scenedesmus quadricauda	30.1	23.6	26.0	20.3	1.02	1.16	1.28	*a*
Scenedesmus accuminatus	30.1	23.2	25.1	21.6	1.07	1.14	1.23	*a*
Protozoa								
Euglena gracilis (green)	28.5	23.2	26.3	22.0	1.02	1.07	1.21	*b*
Euglena gracilis (colorless)	30.2	21.6	27.5	20.7	1.04	1.07	1.36	*b*
Euglena gracilis (bleached)	29.6	21.9	27.5	21.0	1.03	1.06	1.33	*b*
Tetrahymena pyriformis	19.8	30.9	19.3	30.0	0.99	1.03	0.64	*c*

References

[a] G. P. Serenkov and M. V. Pakhomova, *Doklady Vyschej Schkoly* No. 4, p. 156 (1959).
[b] G. Brawerman and E. Chargaff, *Biochim. et Biophys. Acta* **31**, 172 (1959).
[c] O. Scherbaum, *Exptl. Cell. Research* **13**, 24 (1957).

may consider their RNA composition to be identical with that of *P. vulgaris*, *A. aerogenes*, *Alcaligenes faecalis*, and *Pseudomonas aeruginosa*. Quite a distinct difference from this group of organisms having an identical RNA composition is shown, on the one hand, by *C. perfringens*, *S. aureus*, and *Pasteurella tularensis* [(G + C)/(A + U) = 1.06 and less], and, on the other hand, by *M. tuberculosis*, *S. lutea*, and *Streptomyces streptomycini* [(G + C)/(A + U) = 1.3 and more].[146] If, therefore, RNA also shows some specificity of composition, it is not impossible that this may likewise be useful for the phylogenetic classification of bacteria; this time for larger systematic groups than in the case of DNA.

From the data on the RNA of fungi (Table X) and of algae (Table XI), it follows that their RNA composition, as that of the bacteria, shows little, if any, variations.

The comparison of these data with the results of the RNA analyses in bacteria (Tables VIII and IX), shows that the RNA composition in the whole group of microorganisms does not reveal considerable differences and

belongs, as a rule, to a comparatively slight GC-type. Moreover, when these data are compared with the RNA composition of higher organisms, animals[111, 155, 157] and plants,[89, 158-160] one is struck by the close similarity of the RNA composition in the entire organic kingdom.

As an exception, there may be quoted the data on the RNA composition in a representative of protozoa, namely, *T. pyriformis*, the RNA of which, according to Scherbaum's data,[133] belongs to the high AU-type. It may be that in this group of organisms greater variations in the RNA composition will be found.

c. *Correlation between the Composition of DNA and RNA*

Comparison of the analytical data on DNA and RNA composition in microorganisms will show that the RNA composition does not change as considerably as that of the DNA in relation to the systematic position of the species. From these data, it follows that the RNA composition displays no marked conformity with that of the DNA.

However, when studying in parallel the DNA and RNA composition in 19 species of bacteria, we succeeded in finding a certain positive correlation.[146, 161] This statistically significant correlation was expressed with a tendency toward the increase of the ratio (G + C)/(A + U) in RNA on passing from the species with the smaller value of the ratio (G + C)/(A + T) in their DNA to those with greater values for this ratio.

It should be said that in our case positive correlation was shown with a comparatively small number of species of bacteria, and that further investigations are certainly necessary to prove that this correlation is not fortuitous. Nevertheless, on the basis of the data presented, it was suggested[146, 161] that a certain portion of the cellular RNA, and, apparently, only a small portion of it (this fact being proved by the minute value of the regression of the RNA composition as compared with that of DNA) may fully correlate in its composition with that of DNA. On the other hand, the main bulk of cellular RNA, conceivably, does not exhibit any correlation with DNA, its composition being similar in very different species. It could be thought that the part of RNA correlating with DNA is the connecting link in the transmission of the hereditary information from DNA to other cellular substrates, proteins in particular. The specific synthesis of the larger part of the RNA, on the contrary, may not be under the immediate control of DNA; and may be carried out indirectly, through some other links and substrates of the metabolism.

3. Nucleic Acid Composition and Variability

With respect to bacteria, there is vast microbiological literature available on hereditary resistant forms which are obtained experimentally by dif-

[157] D. Elson, L. W. Trent, and E. Chargaff, *Biochim. et Biophys. Acta* **17**, 362 (1955).
[158] R. Lipshitz and E. Chargaff, *Biochim et Biophys. Acta* **19**, 256 (1956).
[159] K. K. Reddi, *Biochim. et Biophys. Acta* **23**, 208 (1957).
[160] G. A. Medvedev and N. N. Zabolotsky, *Izvest. Timirjazev. Sel'skokhoz. Akad.* No. 3, 207 (1958).
[161] A. N. Belozersky and A. S. Spirin, *Nature* **182**, 111 (1958).

ferent treatments and which differ drastically from the original bacteria in their morphological, cultural, serological, biochemical, and other characters. To this group belong different L-forms,[162, 163] G-forms,[164, 165] and alkali-producing forms.[166, 167] The question naturally arises, whether the nucleic acids change under such conditions of far-reaching variability. The analysis of the specificity of nucleic acid composition must, in its turn, help to answer the question, to what extent the apparent alterations of the bacterial cell correspond to changes in the nucleic acids that are directly associated with the hereditary apparatus of the cell.

In our laboratory, studies were performed on the DNA and RNA composition of some representatives of the intestinal group of bacteria and of their atypical forms produced as a result of experimental variability.[168-170] The material investigated represented hereditary resistant forms, the so-called "saccharolytically inert" forms (which do not ferment sugars) produced from the intestinal bacteria by various treatments.[166, 170] They may be divided into two groups differing widely in their properties. One group is represented by the so-called "neutral forms," which show neutral reaction on peptone media in the presence of sugars, and which seem to be identical with those described in the literature as G-forms. Another group is represented by "alkali-producing forms," showing alkaline reaction on the same media. It was shown that the formation of these forms is accompanied by drastic changes in several properties, such as, morphological, cultural, serological, biochemical characteristics, and resistance to antibiotics. On the other hand, forms of the same type are exceptionally similar in their properties.

Comparing these data with those on the DNA composition presented in Table XII, one finds many regular correlations. Thus, all of the original intestinal bacteria are very similar in a number of biological properties and in their resistance to antibiotics; and all of them have a group antigen in common. Their biological similarity is in accord with the similarity of their DNA composition [a slightly marked GC-type, $(G + C)/(A + T) = 1.2$].

[162] E. Klieneberger-Nobel, *J. Gen. Microbiol.* **3**, 434 (1949).
[163] E. Klieneberger-Nobel, *Bacteriol. Revs.* **15**, 77 (1951).
[164] P. Hadley, *J. Infectious Diseases* **60**, 129 (1937).
[165] P. Hadley, *in* "Pathogenic Microorganisms" (W. H. Park and W. Williams, eds.). London, 1939.
[166] D. G. Kudlaj, "Variability of the Bacteria of the Intestinal Group," Medgiz, Moscow, 1954.
[167] D. G. Kudlaj, *Zhur. mikrobiol. epidemiol. immunobiol.* No. 6, 32 (1954).
[168] A. N. Belozersky, A. S. Spirin, D. G. Kudlaj, and A. G. Skavronskaja, *Biokhimiya* **20**, 686 (1955).
[169] A. S. Spirin and A. N. Belozersky, *Biokhimiya* **21**, 768 (1956).
[170] A. S. Spirin, A. N. Belozersky, D. G. Kudlaj, A. G. Skavronskaja, and V. G. Mitereva, *Biokhimiya* **23**, 154 (1958).

TABLE XII

DNA COMPOSITION OF ORIGINAL INTESTINAL BACTERIA AND THEIR EXPERIMENTALLY PRODUCED FORMS*

Form of bacteria	Base proportions (moles %)				(G + C)/(A + T)
	G	A	C	T	
Escherichia coli					
Original	26.0	23.9	26.2	23.9	1.09
Neutral form	21.0	29.2	20.6	29.2	0.71
Alkali-producing form	33.3	16.7	33.8	16.2	2.04
Salmonella typhosa					
Original	26.7	23.5	26.4	23.4	1.13
Neutral form	21.9	28.2	21.7	28.2	0.77
Alkali-producing form	32.2	17.3	32.4	18.1	1.83
Shigella dysenteriae					
Original	26.7	23.5	26.7	23.1	1.15
Alkali-producing form	32.8	17.0	33.2	17.0	1.94
Alkali-producing form	32.8	16.7	33.9	16.6	2.00

* Taken from A. S. Spirin, A. N. Belozersky *et al.*, *Biokhimiya* **23**, 154 (1958).

The similarity of alkali-producing forms with respect to their properties is accompanied by the similarity of DNA composition in alkali-producing forms produced from different original cultures by very different treatment [strongly marked GC-type, (G + C)/(A + T) = 2]. The same is true for the "neutral G-forms" [AT-type of DNA, (G + C)/(A + T) = 0.7–0.8].

On the other hand, the radical change in properties during the transformation of the intestinal bacterium, for example, into the alkali-producing form is accompanied by a very considerable alteration of the DNA composition. Hence, the changes of biological, serological, and other properties are accompanied by quite definite changes of the DNA composition.

The data on RNA presented in Table XIII show that, in contradistinction, the alterations of the RNA composition in the course of the transformation of intestinal bacteria into "saccharolytically inert" forms are not large. It is possible that only a small fraction of the cellular RNA undergoes a change.

A study of the RNA composition in L-forms produced from *P. vulgaris* was recently carried out by Mandel and Sensenbrenner.[171] As in the case of the RNA of saccharolytically inert forms, no visible differences were found in the RNA composition of the L-forms when compared to the original culture of *P. vulgaris*. Unfortunately, no data are available in the literature on the DNA composition of L-forms.

Lynn and Smith studied the DNA and RNA composition in a pleuropneumonia-

[171] P. Mandel and M. Sensenbrenner, *Biochim. et Biophys. Acta* **29**, 642 (1958).

TABLE XIII

RNA Composition of Original Intestinal Bacteria and Their Experimentally Produced Forms*

Form of bacteria	Base proportions (moles %)				(G + U)/(A + C)	(G + C)/(A + U)
	G	A	C	U		
Escherichia coli						
Original	30.7	26.0	24.1	19.2	1.00	1.21
Neutral form	28.9	27.1	23.2	20.8	0.99	1.09
Alkali-producing form	30.4	25.8	24.1	19.7	1.00	1.20
Salmonella typhosa						
Original	30.8	26.1	24.0	19.1	1.00	1.21
Neutral form	29.8	26.4	22.6	21.2	1.04	1.10
Alkali-producing form	30.7	25.7	24.3	19.3	1.00	1.22
Shigella dysenteriae						
Original	30.4	25.9	24.4	19.3	0.99	1.21
Alkali-producing form	30.5	25.8	23.8	19.9	1.02	1.19
Alkali-producing form	29.9	26.9	23.8	19.4	0.97	1.16

* Taken from A. S. Spirin, A. N. Belozersky *et al.*, *Biokhimiya* **23**, 154 (1958).

like microbe isolated from the human urethra.[172] The DNA of this microbe was of the AT-type and contained 21.1% of guanine, 24.8% of adenine, 25.8% of cytosine, and 28.3% of thymine. The RNA contained 31.5% of guanine, 18.0% of adenine, 30.3% of cytosine, and 20.2% of uracil.

Another group of data on experimental variability concerns less marked deviations from the original forms, i.e., the variability of a single marker and not that of a large complex of characters. These data are intimately connected with those on strain differences in microbes (cf. above). The most illustrative data in this respect are those obtained by means of comparing the nucleic acid composition in forms resistant or sensitive to antibiotics. Dutta, Jones, and Stacey[132] studied the DNA and RNA composition in the original strain of *S. lutea* which is sensitive to streptomycin, and in a resistant strain produced from the original one. It was found that the DNA did not alter in composition, whereas small, but statistically reliable, differences were found for RNA (cf. Table IX). The DNA and RNA composition in *A. aerogenes* —in the original strain and in a streptomycin-resistant one produced from it—were also studied.[151] Here, the DNA composition shows only a small shift (cf. Table II), whereas no considerable alterations were found in the RNA composition (cf. Table VIII).

Studying slimy and dull variants of *Azotobacter chroococcum*, Belozersky, Imšenecki *et al.*[173] found no differences in either DNA (Table I) or RNA (Table VIII).

The data presented in this section on the nucleic acid composition of bacteria in different types of variability show a complicated and unequal picture. It should be recognized that these data are insufficient for any

[172] R. J. Lynn and P. F. Smith, *J. Bacteriol.* **74**, 811 (1957).

[173] A. N. Belozersky, A. A. Imšenecki, G. N. Zaitseva, and K. S. Perova, *Mikrobiologia* **27**, 150 (1958).

definite conclusion on the connection between this or other types of variability and changes in the nucleic acid composition.

4. Conclusion

In the light of the data on the specificity of DNA composition, we cannot but link up this specificity with species specificity in general. Moreover, the comparison of the data on DNA with those on RNA leaves the impression that DNA is much more closely involved in the specific species characters than RNA, whereas RNA seems to be concerned with more general and less specific functions of the cell. We do not rule out, however, the possibility that this connection of DNA with the specific features of the cell is mediated through some small part of the cellular RNA, as may be gathered from certain compositional correlations discussed before.

It may be appropriate to ask a question: can we adhere to the view of a direct genetic connection between DNA and RNA in the cell without any reservation? It is certainly difficult to assume, in view of the substantive differences in DNA composition, that they will determine the synthesis of so identically composed RNA. Nevertheless, it is not excluded that some small portion of RNA, perhaps the functional analog of nuclear RNA, depends on DNA, being determined by its structure; or it may be directly transformed into DNA and vice versa. But still, with respect to the main bulk of cellular RNA, we regard any possibility of its direct transformation into DNA and vice versa as hardly admissible. Even if the nucleic acids are interdependent in the cell, this must be, apparently, by means of a more indirect mechanism.

It should be stressed, in conclusion, that the discussion concerning the specificity of nucleic acids rested entirely on the composition of either the *total* DNA or the *total* RNA. Even in the case of a very similar or identical composition of the nucleic acids of two organisms, there certainly remain unlimited possibilities for a very close specificity, in regard perhaps not only to species but even to strain; a specificity that may express itself structurally through the nucleotide sequence. Therefore, in order to elucidate the chemical mechanisms of heredity and of specific protein synthesis, one must not confine oneself to the study of only the nucleic acid composition but proceed to the solution of the problem of the nucleotide sequence. Unfortunately, up to the present, only preliminary attempts have been made in this respect, which are based on more or less indirect approaches.[174-176] With respect to bacteria in particular, such attempts were made in two different laboratories by different methods, namely, on the DNA from *Mycobacterium phlei*[176] and from *M. tuberculosis*, bovine type.[82]

[174] H. S. Shapiro and E. Chargaff, *Biochim. et Biophys. Acta* **23**, 451 (1957).
[175] H. S. Shapiro and E. Chargaff, *Biochim. et Biophys. Acta* **26**, 608 (1957).
[176] A. S. Jones, M. Stacey, and B. E. Watson, *J. Chem. Soc.* p. 2454 (1957).

CHAPTER 33

The Nucleic Acids of the Bacterial Viruses

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I. Introduction

1. FOREWORD

Because of the variations in experimental techniques employed, it has been convenient to group viruses into three large classes according to their hosts, the animal viruses, the plant viruses, and the bacterial viruses, or bacteriophages. Although the bacterial viruses were the last of the three classes to be discovered,[1, 2] it seems fair to say that it is with this class that the greatest progress has been made in the study of virus-host interactions, of the fate and function of the infecting particle, of the origin of

[1] F. W. Twort, *Lancet* **ii**, 1241 (1915).
[2] F. d'Herelle, *Compt. rend. acad. sci.* **165**, 373 (1917).

the new virus particles, of the genetics of the virus particle, and of the interaction of viral genomes with each other and with the host genome.[3-9]

Several circumstances have combined to favor rapid progress in the bacterial virus field. The assay method is rapid and precise.[9, 10] The ability to grow the host cells on chemically defined media has greatly facilitated the production of virus particles with varied kinds of radioactive, chemical, or density labels. It has been readily possible to obtain purified virus preparations in which a large fraction of the particles could be demonstrated to be infective, thereby insuring that the chemical and physical properties of the bulk virus preparations represented the properties of the infectious entity.

It has been easily possible to obtain essentially simultaneous infection of an entire bacterial culture, thereby permitting the detailed study of the nature and sequence of events in viral infection. At least for the larger bacteriophages, the ratio of the mass of the infecting particle to the mass of the host cell has been several orders of magnitude greater than is the case for the other classes of viruses, so that even the early stages of infection produce significant quantitative and readily observable effects upon the metabolic processes of the host. In addition, one must mention the great ingenuity displayed by a considerable group of investigators in devising means to exploit these intrinsic advantages.

This chapter will attempt to review the information presently available concerning the structures of the nucleic acids of the bacterial viruses, the functions of these nucleic acids in viral infection, and the manner of replication of these nucleic acids. The information to be considered will be primarily that derived from biochemical or biophysical experiments. Evidence from radiobiological or genetic experiments will be used to supplement direct biochemical or biophysical evidence and, when appropriate, to support particular interpretations.

2. Some General Features of Bacteriophage Infection

a. The Process of Infection with T-Even Phages

Bacteriophage replication is a complex process of biosynthesis and molecular organization. For clarity it will be desirable to present a brief sum-

[3] M. Delbrück, *Biol. Revs. Cambridge Phil. Soc.* **21**, 30 (1946).
[4] F. W. Putnam, *Advances in Protein Chem.* **8**, 177 (1953).
[5] A. D. Hershey, *Advances in Virus Research* **4**, 25 (1957).
[6] G. S. Stent, *Advances in Virus Research* **5**, 95 (1958).
[7] G. Bertani, *Advances in Virus Research* **5**, 151 (1958).
[8] S. Brenner, *Advances in Virus Research* **6**, 137 (1959).
[8a] G. S. Stent, *Advances in Biol. Med. Phys.* **7**, in press.
[9] M. H. Adams, "Bacteriophages." Interscience, New York, 1959.
[10] M. H. Adams, *in* "Methods in Medical Research" (J. H. Comroe, ed.), Vol. 2, p. 1. Year Book Publishers, Chicago, 1950.

mary of current ideas concerning the biochemical and biophysical aspects of this process as exemplified in the T-even phages.[11] The evidence from which these ideas have been developed will then be presented in detail. The time scale, for the events to be described, pertains to experiments performed at 37°.

All the bacterial viruses thus far investigated have been found to contain deoxyribonucleic acid (DNA) to the extent of 25–50% by weight.[4] Most of the remainder of the viral mass is protein.[14] In the T-even bacteriophages several proteins are known to be present, including a "head" protein,[15-17] enclosing most of the nucleic acid, "tail" proteins which serve as a mechanism of adsorption to and penetration of the host,[15, 18-22] and certain "internal" proteins of uncertain function.[23-25] In addition, certain dibasic amines[26] have been found which serve as a portion of the neutralizing charge to the nucleic acid.

In an appropriate medium, a T-even phage particle adsorbs to the surface of a susceptible bacterium by means of an adsorption site located on its tail. Following adsorption, an enzymic process of penetration[27, 27a, 28]

[11] The process of bacteriophage infection has been studied largely with a group of seven phages which attack a common host, strain B of *Escherichia coli*. These seven[12] known as the T phages, were independent isolates but were later shown to fall into related classes. One of these classes, which by accident includes T2, T4, and T6 (the "T-even phages"), has been shown to have several uniquely favorable characteristics, and hence has been the object of the great bulk of bacteriophage research. The results of the investigation of the nucleic acids of this subgroup will be considered first (Phage C16 has been shown to be closely related to the T-even phages[13] and may be included with this group).

[12] M. Demerec and U. Fano, *Genetics* **30,** 119 (1945).

[13] M. H. Adams, *J. Bacteriol.* **64,** 387 (1952).

[14] About 1% of the phosphorus of a T2 phage is acid-soluble (Kozloff[21]). A portion of this is known to be present as ATP and dATP which are involved in contraction of the phage tail during injection.

[15] R. C. Williams and D. Fraser, *Virology* **2,** 289 (1956).

[16] H. Van Vunakis and J. L. Barlow, *Federation Proc.* **15,** 620 (1956).

[17] H. Van Vunakis, W. H. Baker, and R. K. Brown, *Virology* **5,** 327 (1958).

[18] E. Kellenberger and W. Arber, *Z. Naturforsch.* **10b,** 698 (1955).

[19] L. M. Kozloff, M. Lute, and K. Henderson, *J. Biol. Chem.* **228,** 511 (1957).

[20] E. Kellenberger and J. Séchaud, *Virology* **3,** 256 (1957).

[21] L. M. Kozloff and M. Lute, *J. Biol. Chem.* **234,** 539 (1959).

[22] S. Brenner, G. Streisinger, R. Horne, S. P. Champe, L. Barnett, S. Benzer, and M. W. Rees, *J. Mol. Biol.*, **1,** 281 (1959).

[23] A. D. Hershey, *Virology* **1,** 108 (1955).

[24] A. D. Hershey, *Virology* **4,** 237 (1957).

[25] L. Levine, J. L. Barlow, and H. Van Vunakis, *Virology* **6,** 702 (1958).

[26] B. N. Ames, D. T. Dubin, and S. M. Rosenthal, *Science* **127,** 814 (1958).

[27] D. D. Brown and L. M. Kozloff, *J. Biol. Chem.* **225,** 1 (1957).

[27a] G. Koch and W. J. Dreyer, *Virology* **6,** 291 (1958).

[28] P. P. Dukes and L. M. Kozloff, *J. Biol. Chem.* **234,** 534 (1959).

of the cell wall takes place after which the DNA of the phage, mostly contained in its "head" is "injected" into the bacterium.[29] The internal protein and dibasic amines also enter, but most of the protein of the phage remains external to the bacterial cell and appears to play no further role in the infective process.[30]

At this stage no infective particle can be found attached to or within the bacterial cell and the phage is said to be in a "vegetative" phase.[34] The period from infection to the first intracellular appearance of infective particles is known as the "eclipse period."[35]

Consequent to the entry into the cell of the viral DNA, which appears to be composed of several molecules, synthesis of host DNA and ribonucleic acid (RNA) largely ceases. An obligate synthesis of certain proteins essential to the phage replication takes place. These are not the phage antigens; some are certainly enzymes required to make specific viral components. Synthesis of a specific RNA is also observed.

At about 6 minutes after initiation of infection the synthesis of new viral DNA can be detected. The rate of synthesis increases until a limiting rate of about 10 phage units per cell per minute is reached at about 10 minutes. Synthesis of phage antigen commences at about 8 minutes.

At 10 to 12 minutes the first mature intact progeny phage particles can be found inside the infected cell. At the time these appear, a pool of about 40–50 units of phage DNA and 10–20 units of phage antigen has been produced. The pool of phage DNA includes a large fraction, if not all, of the parental DNA. The rates of DNA and protein synthesis and of phage maturation appear to be such as to maintain the pools at approximately constant level. DNA and protein appear to be drawn from the pools into mature phage at random, and an individual particle may contain DNA molecules made at different times. Formation of a mature phage particle appears to be an irreversible process.

Accumulation of mature phage particles in the cell continues until terminated by a process which ordinarily disrupts the cell about 21 minutes

[29] A. D. Hershey and M. Chase, *J. Gen Physiol.* **36,** 39 (1952).

[30] However, the process of attachment of the phage to the bacterium has profound effects on the metabolism of the latter, even in the absence of phage DNA. Thus phage "ghosts" obtained by osmotic shock of T2 phage and containing about 3% of the phosphorus and almost all of the protein of T2 phage[31] upon adsorption to bacterial cells bring about a cessation of RNA synthesis and ultimate lysis of the cell.[32, 33] The mechanism of this action and its relation to the events of normal bacteriophage infection is unknown.

[31] R. M. Herriott and J. L. Barlow, *J. Gen. Physiol.* **40,** 809 (1957).

[32] R. M. Herriott and J. L. Barlow, *J. Gen. Physiol.* **41,** 307 (1957).

[33] I. R. Lehman and R. M. Herriott, *J. Gen. Physiol.* **41,** 1067 (1958).

[34] A. H. Doermann, *Cold Spring Harbor Symposia Quant. Biol.* **18,** 3 (1953).

[35] A. H. Doermann, *J. Gen. Physiol.* **35,** 645 (1952).

after infection. This lytic process can be largely inhibited by appropriate means (lysis inhibition)[36] in which case the accumulation of mature phage particles continues for several hours.

b. Phenomena Observed with Ultraviolet Irradiated T-Even Phages

Many of the experiments to be described make use in varied ways of bacteriophage that have been irradiated with ultraviolet light. Usually the radiation employed is the unfiltered output of a germicidal lamp and is presumed to be, in large part, energy of wavelength of the mercury resonance line at 2537 Å. This radiation produces lesions of unknown nature in the bacteriophage. A variety of evidence, all unfortunately indirect, indicates that these lesions are very largely localized in the DNA of the phage particle.

These lesions are not homogeneous. There are at least two classes of lesions which may be differentiated in a physicochemical sense as photoreactivable and nonphotoreactivable.[37-39]

Lesions may also independently be distinguished as to whether they affect DNA function (functional change) or DNA replication (genetic change) or both. It is conceivable that a lesion may prevent the proper function of a segment of DNA but would permit, at the time of replication, formation of a new undamaged, functional DNA. (Symonds and McCloy have presented evidence that the photoreactivable lesions in phage T2 are of the exclusively functional type.[40])

Following this concept, lesions may also independently be distinguished as to whether they affect a "critical" or a "noncritical" function.[41-43] A "critical" function in this sense is one that must be expressed before replication of phage DNA is possible. Exclusively, functional damage to a "noncritical" locus could then be, in effect, repaired at the first replication, while similar damage to a "critical" locus would, in single infection, prevent replication.

The genetic damages might or might not prevent function but would invariably, in single infection, result in either a total block to replication or in an error at replication. The error might result in a mutation or might be lethal.

These lesions are recognized in the first instance by the fact that they

[36] A. H. Doermann, *J. Bacteriol.* **55,** 257 (1948).
[37] R. Dulbecco, *J. Bacteriol.* **59,** 329 (1950).
[38] R. Dulbecco, *in* "Radiation Biology" (A. Hollaender, ed.), Vol. II, p. 455. McGraw-Hill, New York, 1955.
[39] J. Jagger, *Bacteriol. Revs.* **22,** 99 (1958).
[40] N. Symonds and E. W. McCloy, *Virology* **6,** 649 (1958).
[41] N. A. Barricelli, *Acta Biotheoretica* **11,** 107 (1956).
[42] R. H. Epstein, *Virology* **6,** 382 (1958).
[43] D. R. Krieg, *Virology* **8,** 80 (1959).

cause inactivation—failure to initiate a successful infection—of the particle affected when studied in single infection. Such particles can, however, still adsorb to susceptible bacteria and can inject their DNA. Unless very heavily damaged, their infection results in the death of the bacterium.[44]

These inactivated particles can be "reactivated," in whole or in part, in three distinct ways: photoreactivation, multiplicity reactivation, and cross-reactivation.

It has been observed that when bacteria infected with ultraviolet inactivated particles are irradiated with strong near ultraviolet or blue light (3200–4500 Å.)[37, 38] a significant fraction of the complexes are reactivated and go on to produce normal viable phage progeny. The evidence available[39, 45] suggests that a certain fraction (which is variable among different types of phage) of the ultraviolet lesions are directly reparable by an unknown biochemical process initiated by the reactivating light[46] (see Chapter 30).

If the infection with ultraviolet inactivated phages is made at a multiplicity of phage per bacterium of two or more, instead of a single infection, the infection is successful in many more cells than would be expected from occasional infection with a survivor. This phenomenon[47-49] is known as multiplicity reactivation (MR). If the ultraviolet inactivated phage is composed of a mixture of strains with known genetic differences, the progeny are observed to contain a high proportion of genetic recombinants.[42]

Multiplicity reactivation is explained by the hypothesis that the ultraviolet lesions distributed at random will in general inactivate different sites in the individual particles infecting a particular cell. Then by recombination during or after multiplication—very likely during the first replicative act[42]—an undamaged phage genome can be assembled. If all particles have lesions of a type which prevent replication, it may be that replication can only be initiated through a recombinational event. It is believed that recombination cannot take place before replication, but that it can be very efficient in that even if the same functional genetic unit (cistron) were damaged on, say, all infecting particles, an intact functional unit could be reassembled unless the two lesions were by chance at nearly identical sites within the cistron. However, it seems likely that certain cistrons must function before replication. Lesions to all of the representatives of a given cistron would then be fatal as such damage could not be repaired by a recombinational event at replication.

The third type of reactivation—cross-reactivation—is partial. If ultraviolet irradiated phage at low multiplicity infect a cell simultaneously with unirradiated phage particles of a different genotype, genetic traits of the ultraviolet treated phage appear widely among the progeny. These are believed to be the results of recombinations, during replication of the normal phage, with the genome of the irradiated phage, resulting in "rescue" of the genetic traits of the latter.[50]

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[44] S. E. Luria and M. Delbrück, *Arch. Biochem.* **1,** 207 (1942).

[45] E. S. Lennox, S. E. Luria, and S. Benzer, *Biochim. et Biophys. Acta* **15,** 471 (1954).

[46] C. S. Rupert, S. H. Goodgal, and R. M. Herriott, *J. Gen. Physiol.* **41,** 451 (1958).

[47] S. E. Luria, *Proc. Natl. Acad. Sci. U. S.* **33,** 253 (1947).

[48] S. E. Luria and R. Dulbecco, *Genetics* **34,** 93 (1949).

[49] R. Dulbecco, *J. Bacteriol.* **63,** 199 (1952).

[50] A. H. Doermann, M. Chase, and F. W. Stahl, *J. Cellular Comp. Physiol.* **45,** Suppl. 2, 51 (1955).

c. Inactivation by Decay of Phosphorus-32

It was demonstrated by Hershey, Kamen, Kennedy, and Gest,[51] that T2 phage, heavily labeled with P^{32}, is inactivated upon storage at a rate which makes the effect clearly the result of P^{32} disintegration within the phage DNA. An equivalent β-irradiation from an external source would not produce more than 3% of the observed inactivation, so the effect must be a consequence of the transmutation taking place in the DNA chains.

The kinetic and quantitative aspects of this experiment have made it clear that one P^{32} disintegration could inactivate a phage, but that not every one does, and that at 4° any P^{32} disintegration has one chance in ten of inactivating the virus particle.[51, 52] This efficiency factor of $\frac{1}{10}$ has been shown to be valid for a number of phages, excepting those of the minute group.

This phenomenon—known as "P^{32} suicide"—can be used to determine the importance of the integrity of the parental, progeny, or host DNA molecules at various stages during infection. By halting the infective process at a given time, freezing the infected cells for various periods of time to allow P^{32} decay, then thawing the cells and determining by assay the effect of the P^{32} decay upon the fate of the infection, one can obtain suggestive evidence concerning the importance at the given time of various P^{32}-containing components for the success of the infection.

Conversely if the specific activity of the P^{32} is known and an efficiency factor of $\frac{1}{10}$ is assumed to hold, one can make a good estimate from the "suicide" rate of the DNA content of a particular virus. Other ingenious uses of this phenomenon will be developed later.

II. Nucleic Acids of the T-Even Phages

1. Structural Aspects

a. Macromolecular Properties; Bipartite Nature

The DNA content of a T-even phage particle is of the order of 120×10^6 Daltons (Table I). The macromolecular character of this DNA is a matter of controversy, even of paradox, at the present time. Several lines of evidence suggest that this DNA should be considered to be bipartite, consisting of one very large molecule comprising 36% of the DNA (molecular weight approximately 45×10^6) and several smaller molecules of DNA of weight 12×10^6 and less. Conflicting evidence is available however which suggests that this DNA consists of relatively homogeneous molecules of molecular weight 14×10^6.

(1) *Autoradiographic Evidence.* The most direct evidence of the bipartite

[51] A. D. Hershey, M. D. Kamen, J. W. Kennedy, and H. Gest, *J. Gen. Physiol.* **34**, 305 (1951).

[52] G. S. Stent and C. R. Fuerst, *J. Gen. Physiol.* **38**, 441 (1955).

TABLE I

DNA Content and Composition in Various Bacteriophages

Phage	DNA-P (gm. per particle)	Ref.	Nucleotide composition					
			Adenine	Thymine	Guanine	Cytosine	5-Hydroxymethylcytosine	Ref.
T2	2.0×10^{-17}	*a*						
	2.3×10^{-17}	*b*	32.5*	32.5	18.2	0	16.8	*h, j, k, l*
T4	2.5×10^{-17}	*c*	32.3	33.3	18.1	0	16.3	*h*
T6	—†		32.4	33.4	17.7	0	16.5	*h, k*
T5	1.8×10^{-17}	*b*	30.3	30.8	19.5	19.5	0	*h*
T1	0.7×10^{-17}	*b*	27	25	23	25	0	*m*
T3	0.9×10^{-17}	*b*	22.8‡	27.8	23.5	26.1	0	*n, o*
T7	0.9×10^{-17}	*b*						
	0.6×10^{-17}	*d*	26.0	26.0	24.0	24.0	0	*g, p*
λ_{vir}	1.2×10^{-17}	*e*	21.3‡	28.6	22.9	27.1	—	*e*
Salmonella A1	0.6×10^{-17}	*e*	23.4‡	33.3	18.8	24.6	—	*e*
P22	0.7×10^{-17}	*f*	25‡	25	25	25	—	*q*
ϕX174	2.6×10^{-19}	*g*	24.6	32.8	24.1	18.5	0	*g*

* T2 DNA also contains 0.45 moles of 6-methylaminopurine per 100 moles of adenine.[i]

† The DNA content of T6 is usually considered to be very similar to that of T2 and T4.

‡ "Preliminary" or "tentative" data.

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[a] A. D. Hershey and N. E. Melechen, *Virology* **3**, 207 (1957).

[b] G. S. Stent and C. R. Fuerst, *J. Gen. Physiol.* **38**, 441 (1955).

[c] A. D. Hershey, M. D. Kamen, J. W. Kennedy, and H. Gest, *J. Gen. Physiol.* **34**, 305 (1951).

[d] K. D. Lunan and R. L. Sinsheimer, *Virology* **2**, 455 (1956).

[e] "Preliminary data" of J. D. Smith and L. Siminovitch, quoted by A. Lwoff, *Bacteriol. Revs.* **17**, 269 (1953).

[f] A. Garen and N. D. Zinder, *Virology* **1**, 347 (1955).

[g] R. L. Sinsheimer, *J. Mol. Biol.* **1**, 43 (1959).

[h] G. R. Wyatt and S. S. Cohen, *Biochem. J.* **55**, 773 (1953).

[i] D. B. Dunn and J. D. Smith, *Biochem. J.* **68**, 627 (1958).

[j] A. D. Hershey, J. Dixon, and M. Chase, *J. Gen. Physiol.* **36**, 777 (1953).

[k] V. L. Mayers and J. Spizizen, *J. Biol. Chem.* **210**, 877 (1954).

[l] D. B. Dunn and J. D. Smith, *Biochem. J.* **67**, 484 (1957).

[m] E. H. Creaser and A. Taussig, *Virology* **4**, 200 (1957).

[n] "Tentative" data of Fraser and Nakamura, quoted by F. W. Putnam, *Advances in Protein Chem.* **8**, 175 (1953).

[o] "Unpublished" data of Knight and Fraser, quoted by C. A. Knight, *Advances in Virus Research* **2**, 153 (1954).

[p] E. Volkin, L. Astrachan, and J. L. Countryman, *Virology* **6**, 545 (1958).

[q] "Unpublished" data of A. Garen and N. D. Zinder, quoted by N. D. Zinder, *J. Cellular Comp. Physiol.* **45**, Suppl. 2, 23 (1955).

nature of this DNA is found in the radioautographic experiments of Levinthal and Thomas.[53-55]

By growing virus on cells grown in a medium containing a high specific activity of P^{32}, virus particles are prepared which contain as many as 100–200 P^{32} atoms per particle. This P^{32} is almost entirely in DNA molecules. When such particles are embedded in a nuclear emulsion, each beta particle arising from the P^{32} disintegrations will give rise to a developable track in the emulsion, originating at the phage particle. The cluster of tracks originating from a particle after allowing radioactive decay to take place for one or two half-lives is called a "star."

The average number of tracks per star is thus a measure of the average P^{32} content per particle. If, before embedding in the emulsion, the virus particle is disrupted by any of several means,[56] the tracks will arise from the separated DNA macromolecules and the number of tracks arising from each molecule will be a measure of the P^{32} content and hence (assuming uniformity of labeling) of the size of the macromolecule.

When this experiment is performed, the DNA is found to consist of one large component comprising 36% of the DNA of a single particle (molecular weight 45×10^6) and smaller components each containing 10% or less of the DNA of a single viral particle. Quantitative observations indicate that one 36% piece arises per particle and that the size of this component is discrete. The number of smaller DNA components arising per particle is estimated to be between 10 and 20.

The large component or "star" has been shown by partition cell centrifugation[57] to have a sedimentation coefficient of 41 ± 5 *S*. This result is in good agreement with the sedimentation constant to be expected for a DNA molecule of this size from the empirical relationship between sedimentation rate and molecular weight of DNA described by Doty *et al.*[58]

Brown and Martin have fractionated T2 DNA on columns of histone[59] and have reported that the stars are to be found in their fraction A[60] (*vide infra*).

When such heavily labeled phages are used to initiate an infection in unlabeled medium and the DNA of the progeny phage is examined by the autoradiographic technique, stars approximately one-half the size of the

[53] C. Levinthal, *Proc. Natl. Acad. Sci. U. S.* **42,** 394 (1956).
[54] C. Levinthal and C. A. Thomas, Jr., *Biochim. et Biophys. Acta* **23,** 453 (1957).
[55] C. Levinthal and C. A. Thomas, Jr., *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 737. Johns Hopkins, Baltimore, 1957.
[56] C. A. Thomas, Jr., *J. Gen. Physiol.* **42,** 503 (1959).
[57] C. A. Thomas, Jr., and J. H. Knight, *Proc. Natl. Acad. Sci. U. S.* **45,** 332 (1959).
[58] P. Doty, B. B. McGill, and S. A. Rice, *Proc. Natl. Acad. Sci. U. S.* **44,** 432 (1958).
[59] G. L. Brown and A. V. Martin, *Nature* **176,** 971 (1956).
[60] G. L. Brown, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 743. Johns Hopkins, Baltimore, 1957.

parental "stars" are observed.[53] Roughly one such half-size star is found per parent phage particle.

When these progeny are used to produce a second generation of progeny, the half-size stars appear to be preserved intact in the second generation progeny.

(2) *Evidence from Transfer Experiments with P^{32}-Labeled Ultraviolet Irradiated Phage.* A second less direct line of evidence that the DNA of T2 is bipartite, at least in function, is to be found in the experiments of Hershey and Burgi,[61] and of Tomizawa.[62]

When P^{32}-labeled phages are used to initiate an infection in unlabeled medium, their progeny will contain a fraction of the P^{32} from the parental particles. This fraction may vary from 40–60% dependent upon the conditions of the experiment. (The reasons for recovery of less than 100% of the parental phosphorus in the progeny are believed to be matters only of technique and not of intrinsic importance.[61])

Such P^{32}-labeled phage can be inactivated by irradiation with an adequate dose of ultraviolet light. Such irradiated phage attach to their host cells and inject their DNA into these cells, but, presumably because of damage to some critical function, the infection is abortive. In such cases, the transfer of P^{32} to the progeny particles is clearly zero. When, however, a mixed infection is made in which several live unlabeled particles are caused to infect every cell infected by an ultraviolet irradiated P^{32}-labeled phage, it is then found that the P^{32} of the ultraviolet treated particle appears in the progeny to the usual extent (i.e., to the same extent as would be observed had the labeled phage not been irradiated).

This first generation of progeny is now found to contain a small number of noninfective particles which, however, carry approximately 50% of the transferred P^{32}. That the number of noninfective particles is small is indicated by the fact that such phage preparations have, within a few percent, as many infective units per gram of nitrogen or per optical density unit[63] as do normal stocks. That a large portion of the transferred P^{32} is in noninfective particles is shown by a comparison in a second cycle of propagation of the P^{32} transferred from this first generation of progeny to a second generation in single infection when only viable particles will transfer, with the P^{32} transferred in a mixed infection with a high multiplicity of fully viable particles when all of the labeled particles will transfer.

Experimentally, in such a second cycle of propagation, the ratio of P^{32} transferred in single infection to that transferred in multiple infection de-

[61] A. D. Hershey and E. Burgi, *Cold Spring Harbor Symposia Quant. Biol.* **21,** 91 (1956).

[62] J. Tomizawa, *Virology* **6,** 55 (1958).

[63] A. D. Hershey, J. Dixon, and M. Chase, *J. Gen. Physiol.* **36,** 777 (1953).

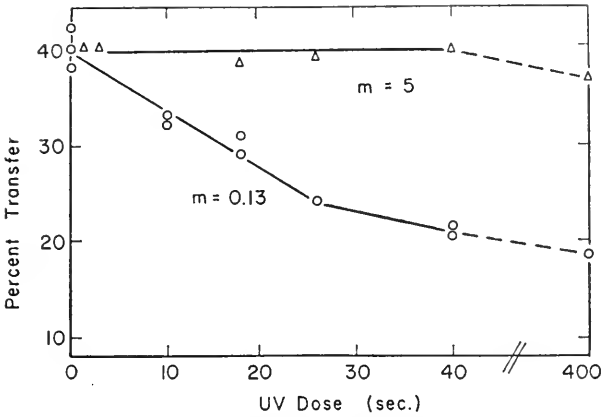

FIG. 1. Second cycle transfer of P^{32} from irradiated parents as a function of ultraviolet dose to the parents. The ordinate indicates the efficiency of transfer of P^{32} from first to second cycle offspring, measured either in single infection (0.13 viable phage per bacterium) or in mixed infection with live phage particles (5 live phages per bacterium). [From A. D. Hershey and E. Burgi, *Cold Spring Harbor Symposia Quant. Biol.* **21**, 91 (1956).]

clined from 1.0 to a limiting value of 0.45 with increasing ultraviolet dose to the progenitor phage used in the first cycle. Thus, in this experiment (Fig. 1) after heavy ultraviolet dosage, a maximum of 55% of the DNA transferred by the initial irradiated phage to its progeny was transferred into a small number of noninfective particles.

This association of a large fraction of the transferred P^{32} with noninfective particles is interpreted to mean that the DNA of a T2 phage can be considered to consist of two functionally independent and physically separable components roughly equal in mass. One component is effectively ultraviolet sensitive and is not widely dispersed upon replication. Once irradiated it bears lesions of such a character that any phage particle carrying this DNA component is noninfective. The second component is effectively ultraviolet resistant. Although it may acquire lesions upon irradiation, these are either reparable or in any case are not lethal to a phage carrying such a component. Studies with mixed infection with varying multiplicities of irradiated and nonirradiated phage suggest that this second component is separable from the ultraviolet sensitive component and is distributed essentially at random among the infective and noninfective progeny of a mixed infection.

Similar experiments by Tomizawa[62] have shown that the DNA of a single phage particle is not necessarily all synthesized in any brief time interval, and specifically that the ultraviolet sensitive and resistant portions as described by Hershey can be synthesized at considerably different times.

These experiments will be described in detail when the evidence concerning viral DNA synthesis is discussed.

(3) *Evidence from P^{32} Transfer Experiments.* A third, indirect line of evidence suggesting a bipartite nature of the DNA of T2 phage is provided by the P^{32} transfer experiments of Stent and Jerne,[64] and Stent, Sato, and Jerne.[65] These experiments, as do the Hershey experiments, attempt to inquire into the distribution among the progeny phage particles of P^{32} atoms from parental particles. As do the experiments of Hershey, these experiments also suggest that a large fraction (roughly half) of the transferred P^{32} is in a few progeny particles, while the remainder is distributed over many particles. The former DNA fraction, which presumably suffers little dispersion through the first generation of propagation, is found to remain essentially intact through a second generation.

In these experiments use was made of the "P^{32} suicide" technique. When heavily P^{32}-labeled phages are prepared and are used immediately in unlabeled medium to produce a first generation of progeny (involving an increase of twentyfold in phage titer), those progeny particles, if any, with an appreciable P^{32} content will be inactivated upon storage. Experimentally it is observed that the decrease in phage titer of the progeny upon storage is so small that although on the average each particle should contain at least 2% of the parental P^{32}, the great majority must contain less than 0.2% of the parental P^{32}. Much of the transferred P^{32} must reside in a few particles.

Such particles, containing a large number of P^{32} atoms, might be expected to die rapidly. Once dead they would not be able to transfer in single infection their remaining P^{32} atoms to further progeny.

An estimate of the P^{32} in the few heavily labeled progeny can be obtained, then, by measurement of the rate of decrease of transfer, after storage, of P^{32} by the first generation of progeny to a second generation. The results of such an experiment are shown in Fig. 2. These results can be analyzed to show that of the P^{32} in the first generation of progeny, approximately 60% is in two particles that die out at such a rate that they must each contain about 15% of the P^{32} of the original particle, while the other 40% is spread over the remaining particles which do not individually contain enough P^{32} to cause them to die off at an appreciable rate (less than 0.3% of the P^{32} of the original particle). These calculations depend upon the assumption that the decay of transferred P^{32} inactivates the heterogeneously labeled phage particles of the first generation of progeny with the same efficiency (0.1) as has been determined for phage particles homogeneously labeled by incorporation of P^{32} from the medium and bacterial constituents.

Experiments measuring the distribution of P^{32} after still another generation indicate that the large fragments of P^{32}-labeled DNA continue to persist without further dispersion.

It is of interest that all of these experiments indicate a transfer of large macromolecular pieces of DNA from parent to progeny without significant molecular disintegration. This evidence that the DNA molecules remain

[64] G. S. Stent and N. K. Jerne, *Proc. Natl. Acad. Sci. U. S.* **41**, 704 (1955).
[65] G. S. Stent, G. Sato, and N. K. Jerne, *J. Mol. Biol.* **1**, 134 (1959).

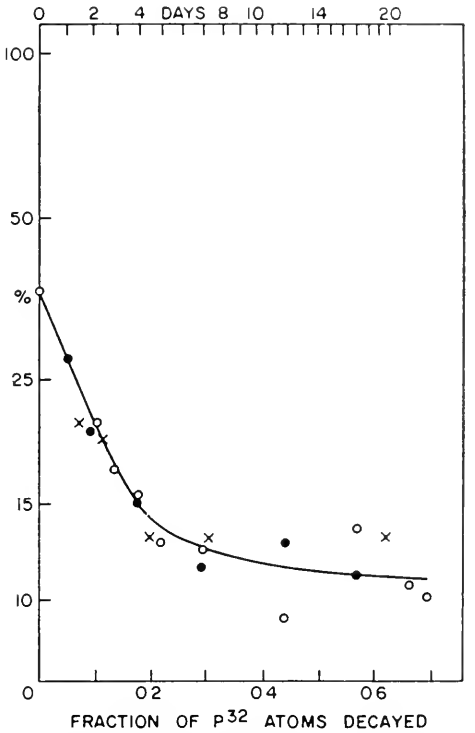

FIG. 2. Percentage of parental P^{32} contained in first generation progeny transferable to the second generation progeny as a function of the fraction of P^{32} atoms decayed. The solid line is the transfer expected if, of the P^{32} in the first generation progeny, 60% is in pieces of 15% each of the original DNA, while the remainder (40%) is in pieces of 0.3% each of the original DNA. [From G. S. Stent, G. H. Sato, and N. K. Jerne, *J. Mol. Biol.* **1**, 134 (1959).]

intact is supported by the fact that supplying infected cells with large amounts of DNA precursors such as thymidine or uridine cannot reduce the transfer of C^{14} from parent to progeny thymidine.[61]

(4) *Possibility of Artifact.* These experiments taken together present a harmonious picture of physically and functionally bipartite DNA in the T-even phages. With some allowance for numerical divergencies they suggest that there is in a T-even phage particle one large molecule of DNA (comprising some 30–50% of the total content) which in normal replication divides into two pieces which in future generations preserve their integrity. If irradiated by ultraviolet light, this piece can be irreparably damaged, but can be, in a mixed infection with live phage, incorporated into a progeny particle.

The remainder of the DNA appears to be very extensively degraded dur-

ing infection and the components are reused in the synthesis of the DNA of many phages. Ultraviolet-changed parts of this DNA fraction may simply be discarded after degradation.

However, it should be pointed out that this bipartite character has always been observed in P^{32}-labeled (often heavily labeled) and, in Hershey's experiments, ultraviolet irradiated particles, and some thought must be given to the possibility that it is an artifact induced by the conditions, including the heavy irradiation during synthesis, necessary to prepare the labeled particles.

That the physical evidence of a bipartite structure, the stars, may be just an artifact is indicated by the following evidence. While the sedimentation rate of the stars has been measured to be 41 *S*, in good agreement with expectation for a DNA of that size, no evidence can be observed of a large 41 *S* component (which should be a 36% component) upon ultracentrifugal analysis of normal T-even phage DNA.[66] When banded in the cesium chloride density gradient, T4 DNA provides a band of almost precisely Gaussian distribution of ultraviolet absorption[67] and a calculated DNA molecular weight of 14×10^6. A mixture of DNA of two components, one of molecular weight 45×10^6 comprising 36% of the total, and a second component of DNA of molecular weight less than 12×10^6, should have resulted in a clearly non-Gaussian band of ultraviolet absorption. Appropriate controls have indicated that the stars are not disrupted by banding in cesium chloride.[56]

These results, together with the previously presented data, comprise at present a paradox. The paradox may be resolved if it is assumed that the stars are an artifact of growth in highly radioactive medium and do not represent a component normally present in T-even phage particles. Such an artifact would also account for the P^{32}-transfer-suicide experiments. The experiments with P^{32}-ultraviolet irradiated phage would, however, then seem to require an *ad hoc* explanation.

Alternatively, the paradox might be resolved if the negative evidence—the failure to obtain any centrifugal indication of a very large DNA molecule—is in error because some stage in the execution of the centrifugal analysis results in the disruption of such large molecules.

b. Composition

The nucleic acids of the T-even phages are unusual in composition; while they contain the customary nucleotides, deoxyadenylic acid (dAMP), deoxyguanylic acid (dGMP), and thymidylic acid (TMP), they do not con-

[66] H. K. Schachman, *J. Cellular Comp. Physiol.* **49** Suppl. 1, 71 (1957).

[67] M. Meselson, F. W. Stahl, and J. Vinograd, *Proc. Natl. Acad. Sci. U. S.* **43**, 581 (1957).

TABLE II

EXTENT OF GLUCOSYLATION OF THE HMC OF T-EVEN AND RELATED PHAGES

Phage	Moles/100 moles P				Ref.
	Glucose	HMC	HMC-gl	HMC-$(gl)_2$	
T2	0.13	0.04	0.13	Small*	*a, b, c, d*
T4	0.17	0	0.17	0	*b, d, e*
T6	0.27	0.06	Small	0.11	*c, d, f*
C16	0.22	—	—	—	*g*

* A small amount of HMC-$(gl)_2$ has been found in hydrolyzates of T2 DNA by I. R. Lehman (personal communication, 1959).

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[a] R. L. Sinsheimer, *Science* **120**, 551 (1954).
[b] R. L. Sinsheimer, *Proc. Natl. Acad. Sci. U. S.* **42**, 502 (1956).
[c] M. A. Jesaitis, *Nature* **178**, 637 (1956).
[d] M. A. Jesaitis, *J. Exptl. Med.* **106**, 233 (1957).
[e] E. Volkin, *J. Am. Chem. Soc.* **76**, 5892 (1954).
[f] M. R. Loeb and S. S. Cohen, *J. Biol. Chem.* **234**, 364 (1959).
[g] M. A. Jesaitis, *Federation Proc.* **17**, 250 (1958).

tain deoxycytidylic acid (dCMP), found in all other DNA investigated.[68] In place of cytosine (C), these nucleic acids contain 5-hydroxymethylcytosine (HMC)[69] often substituted in the 5-hydroxymethyl group with one or two glucose residues.[70-72] The presence of this unusual pyrimidine has proved most useful in studies of the synthesis of the viral nucleic acids (*vide infra*).

As indicated in Tables I and II, the proportions of the nucleotides in the different T-even phages are not significantly different with the exception of the proportion of single or double glycosyl substitution upon the hydroxymethylcytosine. The proportion of glucose substitution is an inherited trait, although in mixed infections of T2 and T4 it does not behave as a simple Mendelian trait. Thus, in a cross of T2 and T4 all the progeny investigated appear to have the glucose content characteristic of T4 regardless of their genetic pattern.[73, 74] Crosses of T2 and T6, however, have yielded progeny with either the glucose content of T2 or of T6 associated with either the host range character of T2 or of T6.[75] Thus, the genetic

[68] E. Chargaff, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. I, p. 307. Academic Press, New York, 1955.
[69] G. R. Wyatt and S. S. Cohen, *Biochem. J.* **55**, 774 (1953).
[70] R. L. Sinsheimer, *Science* **120**, 551 (1954).
[71] E. Volkin, *J. Am. Chem. Soc.* **76**, 5892 (1954).
[72] M. A. Jesaitis, *Nature* **178**, 637 (1956).
[73] R. L. Sinsheimer, *Proc. Natl. Acad. Sci. U. S.* **42**, 502 (1956).
[74] G. Streisinger and J. J. Weigle, *Proc. Natl. Acad. Sci. U. S.* **42**, 504 (1956).
[75] M. A. Jesaitis, *Federation Proc.* **17**, 250 (1958).

trait "glucose content" in these crosses segregates from the host range marker, but not symmetrically, as many more of the recombinant phages are found with the glucose content of T2 and the host range of T6 than vice versa.

While the extent of degradation of T2 DNA by pancreatic deoxyribonuclease is the same as is found with DNA from other sources (approximately 25% of the diester linkages are split) the presence of the glycosyl residues appears to inhibit the action of venom diesterase upon the deoxyribonuclease digest. Thus, it has not been possible to obtain quantitative reduction of DNA from the T-even phage to mononucleotides.[76] Using conditions under which calf thymus DNA would be completely degraded to mononucleotides[78] it is possible with T2 DNA to obtain about 70% of the deoxyguanylic, deoxyadenylic, and thymidylic acids as mononucleotides, 54% of the 5-hydroxymethyldeoxycytidylic acid (dHMP) and only 24% of glucosylated 5-hydroxymethyldeoxycytidylic acid.[73] Jesaitis[79] similarly obtained from T6r$^+$ DNA, 60% of the deoxyguanylic, deoxyadenylic, and thymidylic acids as mononucleotides, about 40% of the 5-hydroxymethyldeoxycytidylic acid and only 20% of the diglucosylated 5-hydroxymethyldeoxycytidylic acid.

There has been a report of a correlation of glucose content with the *r* character of several phages[80] but this was not confirmed with other strains.[73]

Attempts at fractionation of T6r$^+$ DNA from a nucleohistone gel[81] yielded fractions with significant variations of nucleotide composition. While in all cases the fractions preserved the adenine-thymine and guanine-hydroxymethylcytosine equalities, the ratio of adenine to guanine varied from 1.60 to 1.98.

Bendich *et al.* have obtained numerous fractions from T6r and T6r$^+$ phage DNA by column chromatography[82] on Ecteola but no data have been reported as to the nucleotide composition of these fractions.

Brown and Martin obtained two fractions, A and B, on a modified histone column which differed slightly in nucleotide composition[58] and which differed notably in the extent of glucose substitution upon the 5-hydroxymethylcytosine.[60] In fractions A and B, 65% and 100% of the HMC resi-

[76] It has recently been reported that I. R. Lehman has obtained an enzyme fraction from *E. coli* that will accomplish a quantitative degradation of T2 DNA to mononucleotides.[77]

[77] A. Kornberg, S. B. Zimmerman, S. R. Kornberg, and J. Josse, *Proc. Natl. Acad. Sci. U. S.* **45,** 772 (1959).

[78] R. L. Sinsheimer and J. F. Koerner, *J. Biol. Chem.* **198,** 293 (1952).

[79] M. A. Jesaitis, *J. Exptl. Med.* **106,** 233 (1957).

[80] S. S. Cohen, *Science* **123,** 653 (1956).

[81] C. F. Crampton, R. Lipshitz, and E. Chargaff, *J. Biol. Chem.* **211,** 125 (1954).

[82] A. Bendich, H. B. Pahl, and S. M. Beiser, *Cold Spring Harbor Symposia Quant. Biol.* **21,** 31 (1956).

dues were glucosylated, respectively. As mentioned previously, in experiments with DNA from very highly P^{32}-labeled phage, mixed with DNA from unlabeled phage, the stars appeared to migrate on the column with fraction A.

2. Replication

a. Fate of the Infecting Particle

The most careful studies of the fate of the components of the infected particle have been made by Hershey and collaborators,[29, 61] following earlier work by Kozloff and Putnam,[83-85] and French *et al.*[86] In a classic experiment[29] Hershey and Chase demonstrated, using P^{32}- and S^{35}-labeled phage, that upon absorption of the bacteriophage to the host bacterium, the P^{32} representing the DNA, is "injected" into the bacterium, while a major portion of the S^{35} representing the protein, remains external to the bacterium and can be largely stripped from the bacteria by the action of a blender without effect upon the subsequent development of infection.

Of the DNA injected into the host cell a fraction can be recovered in the progeny phage after lysis. This fraction is the same when measured by P^{32} transfer or by C^{14} transfer.[61] All four purine and pyrimidine residues are transferred equally.[61] In early experiments[83] the fraction transferred was of the order of 30%. Successive improvements in technique[61, 87] such as the use of highly active stocks, and of conditions favoring rapid adsorption, a minimum of premature lysis, and a minimum of readsorption, have raised the recovery of parental DNA in the progeny phage to approximately 60%. Most of the remainder can be accounted for and is lost as the result of various "accidents," i.e., failure of about 12% of the phage to inject, 10% loss of DNA by premature lysis before incorporation into mature phage, 12% loss by readsorption of mature phage from premature lysis, etc.

Only a few experiments have been performed that bear upon the state of the DNA after injection. Ultraviolet irradiation experiments[88, 89] suggest that the injected DNA has the same (or slightly less[49]) cross section for inactivation as when it is in the virus for the first minute or so after injection. After this, with T2, the cross section decreases for complex reasons (*vide infra*).

Similarly, gamma-ray inactivation studies[90] indicate that the inactivation cross

[83] F. W. Putnam and L. M. Kozloff, *J. Biol. Chem.* **182,** 243 (1950).
[84] L. M. Kozloff, *J. Biol. Chem.* **194,** 95 (1952).
[85] L. M. Kozloff, *Cold Spring Harbor Symposia Quant. Biol.* **18,** 209 (1953).
[86] R. C. French, A. F. Graham, S. M. Lesley, and C. E. van Rooyen, *J. Bacteriol.* **64,** 597 (1952).
[87] J. D. Watson and O. Maaløe, *Biochim. et Biophys. Acta* **10,** 432 (1953).
[88] S. E. Luria and R. Latarjet, *J. Bacteriol.* **53,** 149 (1947).
[89] S. Benzer, *J. Bacteriol.* **63,** 59 (1952).
[90] W. Harm, *Virology* **5,** 337 (1958).

section for T2, T4 or T6 is the same whether irradiated as extracellular phage particles, or irradiated shortly after injection. If densely ionizing soft X-rays are used, however, the inactivation cross section shortly after injection is 1.6 times that of the phage particles. This has been interpreted[90] as a result of uncoiling of the DNA to a more extended target and consequent lesser waste of lethal hits in multiply hit phage.

A similar decrease of about 20% in sensitivity to incorporated P^{32}-decay, immediately after injection has also been attributed to an uncoiling of the DNA with resultant lesser damage from the emitted β-particle.[40]

Ultracentrifugal studies of the sedimentation properties of the parental DNA (P^{32}-labeled) in cells infected with T2 at low multiplicity have been made by Watanabe *et al.*[91] The cells were disrupted by rapid decompression at various times after infection. Using a partition centrifuge cell, measurements were made of the radioactivity remaining above the partition after various periods of centrifugation. From the data, estimates could be made of the distribution of the radioactive components among five ranges of sedimentation rate.

The results indicated that about 10% of the radioactivity continued to sediment at a rate characteristic of intact virus particles, presumably representing phage which adsorbed but failed to inject. The bulk of the activity (60%) sedimented at a rate characteristic of free DNA (S = 10–20). Approximately 25 to 35% of the activity appeared to have a very low sedimentation rate, less than 0.5 S. A similar proportion of the activity was found to be acid-soluble.

The bulk of the injected parental DNA, then, appears to be present as essentially free DNA in the infected cell. No indication was obtained of any enduring attachments of the parental DNA to bacterial structures, although such structures would have had to be rather large to be evident by this method.

The proportion of parental DNA that has apparently been degraded to the extent of acid solubility seems rather larger in this experiment than in experiments of other workers. Hershey and Burgi[61] found less than 10%. To some extent this undoubtedly represents a degradation of the DNA of superinfecting phages.[92] Within about 2 minutes after infection with T2, the infected cell is "immune" to superinfection by other phage, even if the latter differ by only a single genetic trait. Lesley *et al.*[92] demonstrated that this immunity was in part the result of a mechanism established shortly after infection that results in the extensive degradation of the DNA of any superinfecting particles.

[91] I. Watanabe, G. S. Stent, and H. K. Schachman, *Biochim. et Biophys. Acta* **15,** 38 (1954).

[92] S. M. Lesley, R. C. French, A. F. Graham, and C. E. van Rooyen, *Can. J. Med. Sci.* **29,** 128 (1951).

It would appear then that the bulk of the parental injected DNA persists as large molecules which, as phage DNA synthesis begins, comprise a decreasing portion of the DNA pool from which the DNA of mature particles is drawn at random. This hypothesis is in good accord with the observation that the earliest mature phages contain a larger fraction of parental phage DNA atoms than phage made at a later time.[86, 87, 93] Evidence has already been presented that a portion of the parental DNA transferred to progeny is in large fragments while the remainder is relatively dispersed. The full significance of these observations awaits clarification of the current paradox concerning "stars" in T2 DNA.

Current ideas of DNA replication favor the semiconservative[94] hypothesis originally presented by Watson and Crick[95] whereby the two strands of each DNA molecule are separated at each replication, while each individual strand is effectively immortal.[96] As previously indicated, Levinthal[53] has presented evidence that such a semiconservative replication can be observed with "stars"; the 36% pieces of labeled phage are observed to become approximately 20% pieces in first generation progeny, and to continue as 20% pieces during transfer to a second generation. Unfortunately, there has been difficulty in the repetition of these experiments. Even if this observation can be substantiated, it does not prove that all of the numerous DNA replications taking place during phage multiplication are semiconservative.

Very recently, application of the density labeling method, first applied to the study of DNA replication in *Escherichia coli* by Meselson and Stahl,[96] has been made to the problem of T2 phage DNA replication. It has been reported[97] that when density-labeled parental phage are grown in normal bacteria, DNA molecules of hybrid density are observed among the first generation phage progeny in accordance with the expectation of the Watson-Crick hypothesis.

b. Time Course of Synthesis of New Bacteriophage

(1) *Development of Viral DNA and Protein Pools.* The presence of the unusual pyrimidine component, 5-hydroxymethylcytosine, has made it possible to determine specifically the course of synthesis of the DNA of T-even phages during infection independently of the concomitant changes in amount and synthesis of the DNA of the host bacterium. This possibility

[93] A. D. Hershey, *J. Gen. Physiol.* **37,** 1 (1953).

[94] M. Delbrück and G. S. Stent, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 699. Johns Hopkins, Baltimore, 1957.

[95] J. D. Watson and F. H. C. Crick, *Nature* **171,** 964 (1953).

[96] M. Meselson and F. W. Stahl, *Proc. Natl. Acad. Sci. U. S.* **44,** 671 (1958).

[97] A. W. Kozinski, quoted by W. Szybalski, *Brookhaven Symposia in Biol.* **12,** 33 (1959).

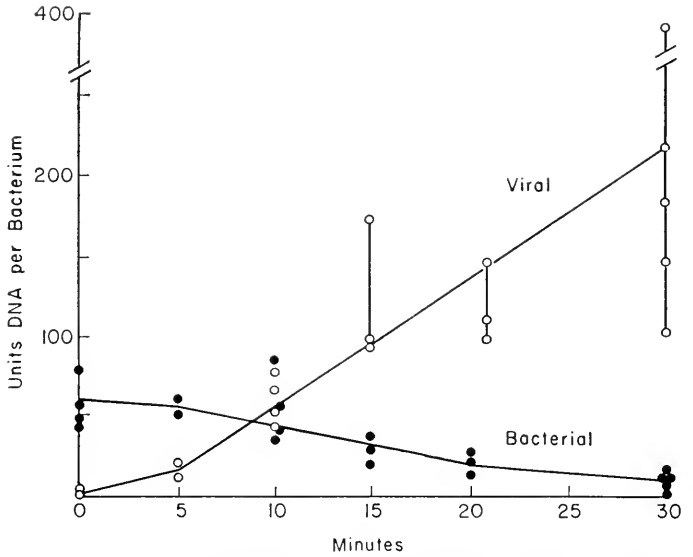

FIG. 3. Amounts of viral (HMC-containing) and bacterial (C-containing) DNA per infected bacterium during growth of phage. (One DNA unit = 8.3×10^{-14} μM HMC for T2 DNA or 1.3×10^{-13} μM C for bacterial DNA.) [From A. D. Hershey, J. Dixon, and M. Chase, *J. Gen. Physiol.* **36**, 777 (1953).]

was first explored by Hershey *et al.*[63] and has since been employed in the study of phage DNA synthesis under a variety of experimental conditions.[98, 99]

Hershey's experiments indicated that in normal infection with T2 phage an increase in acid-precipitable hydroxymethylcytosine-containing DNA can first be observed at about 5 minutes after infection (Fig. 3). More sensitive isotope dilution experiments by Vidaver and Kozloff[98] suggest that little increase over the hydroxymethylcytosine brought in by the infecting particles can be observed until about 6½ to 7 minutes. Following this onset, phage DNA synthesis proceeds at an increasing rate until about 10 minutes at which time the rate reaches a maximum value of about 5 to 10 phage units per minute per cell[63, 98, 99a, 100] (one phage unit of T2 DNA equals 2×10^{-11} μg. P^{100}).[101]

[98] G. A. Vidaver and L. M. Kozloff, *J. Biol. Chem.* **225**, 335 (1957).

[99] R. L. Nutter and R. L. Sinsheimer, *Virology* **7**, 276 (1959).

[99a] S. S. Cohen, *Cold Spring Harbor Symposia Quant. Biol.* **12**, 35 (1947).

[100] A. D. Hershey and N. E. Melechen, *Virology* **3**, 207 (1957).

[101] The maximum rate of phage DNA synthesis is dependent upon the nature of the nutrient medium, being greater in a broth medium than in a synthetic medium. In the latter—glucose-ammonium—the rate of DNA synthesis in the culture increases more than tenfold after infection and is nearly double the combined rate of DNA and RNA synthesis in the uninfected bacterium.[98, 100] In broth-grown

At the same time the bacterial DNA is to a considerable extent degraded and the components used, in part, for the synthesis of phage DNA. (Cytosine of bacterial DNA can serve as a precursor of hydroxymethylcytosine of the phage DNA.[102]) The extent of degradation of the host DNA appears to vary, dependent upon the culture conditions, but as much as two-thirds of the cytosine-containing DNA can disappear from the acid-precipitable fraction in 20 minutes.[63, 99] Phosphorus transfer studies[85, 100] indicate that in 20 minutes an amount of phosphorus equivalent to 15–20 units of phage DNA can be transferred from host to phage DNA. Since in synthetic medium the DNA content of an uninfected cell is only about 30 units of phage DNA, this transfer is highly efficient.

Stent and Maaløe,[103] and Hershey and Melechen[100] have made thorough studies of the origin of the phosphorus found, at various times after infection, in mature phage particles and in phage DNA, respectively. The technique has been to expose the cultures to P^{32} either well before and during infection, or only until infection, or only during infection, or in brief pulses before or during infection. The results are in generally good agreement.

A bacterium, in glucose-ammonium medium, at the time of infection contains phosphorus available for conversion to phage DNA phosphorus to the extent of about 50 phage units. Fifteen to 20 units of this are in bacterial DNA, another 25 units are present as transient intermediates, and some possibly are available from bacterial RNA.[100, 104] This preassimilated phosphorus appears, as might be expected, largely in the first mature particles. The first 25 particles per bacterium receive about 50% of the preassimilated phosphorus although increasing amounts appear until transfer is complete when there are about 110 particles per bacterium.

After phage DNA synthesis begins, a pool of phage DNA develops. This pool amounts to some 40 to 80 phage units before the appearance of intact phage particles inside the infected cell (the size of this pool undoubtedly is a function of nutrient conditions).

In order to form mature phage particles, the phage DNA must be enveloped in a protein "coat." Measurements of the incorporation of S^{35}, administered at various times to the infected cell, into phage progeny indicate that the synthesis of the precursors of phage protein does not begin until shortly after DNA synthesis begins. Antigenic studies of the

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cells the maximum rate of DNA synthesis during infection is rather less than the combined DNA plus RNA rate before infection.[93, 100]

[102] S. S. Cohen and L. L. Weed, *J. Biol. Chem.* **209**, 789 (1954).

[103] G. S. Stent and O. Maaløe, *Biochim. et Biophys. Acta* **10**, 55 (1953).

[104] A. D. Hershey, A. Garen, D. K. Fraser, and J. D. Hudis, *Carnegie Inst. Washington Yearbook* **53**, 210 (1954).

development of T2 serum blocking power in infected cells[105, 106] indicate that the synthesis of the tail antigen of T2 begins at about 9 to 10 minutes after injection—a few minutes after the initiation of phage DNA synthesis, and 2 to 3 minutes before the first appearance of mature phage particles.

Studies of protein synthesis by S^{35}-labeling indicate that virtually no S^{35} assimilated before infection enters into phage particles.[104] During the first 10 minutes of the infection, protein synthesis continues at a rate comparable to that in uninfected cells[99a, 100, 106a] but little of this is precursor to phage protein. By 10 minutes some 3 to 4 units of phage protein (one unit of phage protein = 2×10^{-12} μg. S[100]) have been made, representing about 8% of the total protein synthesis in these 10 minutes.

Once initiated, a pool of some 15 units of phage precursor protein is accumulated and maintained at a nearly constant level, while it is drawn upon for the production of complete phage particles. During this period of constant pool size, about 60% of all protein synthesis in the infected cell is used to maintain the pool.[100]

Once initiated, the production of mature phage particles continues at a rate only slightly less than the rate of synthesis of phage DNA so that the pool of phage DNA continues to grow but at a very much slower rate (in synthetic medium the rate of phage maturation is about 4.4 particles/cell/minute compared to a rate of DNA synthesis of 5 units/cell/minute[100]).

(2) *Effect of Inhibition of Protein Synthesis upon the Development of Phage DNA*. Chloramphenicol at concentrations of 10 μg. per milliliter or greater almost completely blocks protein synthesis in *E. coli*, but permits DNA and RNA synthesis to continue at a constant (linear) rate for at least 40 minutes at 37°.[107]

When chloramphenicol is added to T2 infected cultures of *E. coli* after the eclipse period, phage synthesis is stopped after about a 2-minute lag. If chloramphenicol is added during the eclipse period, phage protein is never produced unless the chloramphenicol is later removed. The effect of chloramphenicol upon the development of phage DNA is dependent upon the time, after infection, of the initial exposure to chloramphenicol. If chloramphenicol is added before infection, at infection, or during the first 4 minutes after infection, there is no subsequent synthesis of phage DNA. If chloramphenicol is added between the fifth and twelfth minute, during the period of normally increasing rate of DNA synthesis, DNA production continues in the presence of chloramphenicol but at just the rate which

[106] R. I. De Mars, *Virology* **1**, 83 (1955).

[106] A. D. Hershey, G. Koch, A. W. Kozinski, J. D. Mandell, R. Thomas, and J. Tomizawa, *Carnegie Inst. Washington Yearbook* **57**, 379 (1958).

[106a] I. Watanabe, *Biochim. et Biophys. Acta* **25**, 665 (1957).

[107] J. Tomizawa and S. Sunakawa, *J. Gen. Physiol.* **39**, 553 (1956).

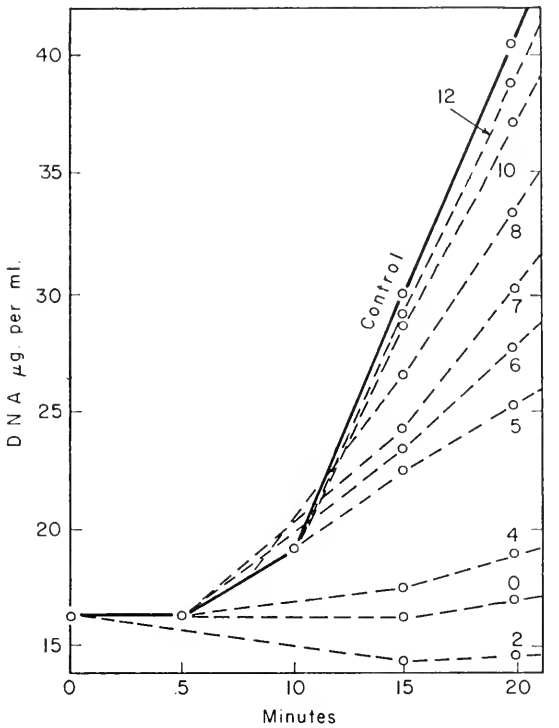

FIG. 4. Effect of chloramphenicol (30 μg./ml.) on the synthesis of DNA in T2-infected bacteria. Chloramphenicol was added to the culture at various times from 0 to 12 minutes after infection and the curves indicate the effect of increasing delay upon subsequent DNA synthesis. [From J. Tomizawa and S. Sunakawa, *J. Gen. Physiol.* **39**, 553 (1956).]

obtained at the time of chloramphenicol addition (Fig. 4). These experiments demonstrate that DNA replication can take place in the absence of concomitant protein synthesis.

Interestingly, although under the latter circumstances DNA accumulates in the infected cells, the progressive decrease in the ultraviolet sensitivity of the phage bacterium complex usually observed during the first 11 to 12 minutes of infection[88, 89, 107] (*vide infra*) is abruptly halted by the addition of chloramphenicol. This decrease can be resumed upon the removal of the chloramphenicol some minutes later. Thus the decline in ultraviolet sensitivity is not simply related to the synthesis of additional DNA but is apparently to be associated with processes involving protein synthesis.

Other modes of inhibition of protein synthesis have yielded similar results. Thus, by the use of analog inhibitors such as 5-methyltryptophan,[99a]

or by the use of mutant bacteria specifically requiring certain amino acids,[108] it has been shown that protein synthesis in the first few minutes of infection is necessary for the initiation of phage DNA synthesis, but that continued protein synthesis is not necessary for continued DNA synthesis. It has also been shown that protein synthesis is a necessary prerequisite to multiplicity reactivation.[99]

(3) *Experiments upon DNA Made in the Presence of Chloramphenicol.* Thus, by adding chloramphenicol at a time just before the first appearance of phage precursor protein (about 8 to 10 minutes after injection), the DNA pool can be caused to accumulate so that in 45 minutes a pool of some 100 to 125 units of phage DNA is produced. If, then, chloramphenicol is removed, appropriate experiments indicate that all of this DNA is available for withdrawal into mature phage.[106, 107] Experiments upon the appearance in mature phage of P^{32} which had been made available to the infected cells for limited periods either during or after the exposure to chloramphenicol indicate that the DNA made during the presence of chloramphenicol is normal and essentially complete, in that it forms a unitary pool with the presumably normal DNA made after the removal of chloramphenicol.[108]

The chloramphenicol experiments provide a means to accumulate phage DNA which can then be treated in various ways before it is permitted to enter mature phage particles. In this way information is gained about the status and properties of the newly synthesized phage DNA in the cell as compared to the properties of phage DNA when in mature particles.

Thus, Tomizawa[109] has shown that when ultraviolet irradiation (doses equivalent to 4 or 11 hits to free phage were used) is applied to phage infected cells which have accumulated DNA in the presence of chloramphenicol, and the chloramphenicol is then removed, many of the final progeny phages are noninfective. These noninfective particles contain ultraviolet-damaged DNA. After 11 hits, they are subject to multiplicity reactivation, photoreactivation, and cross-reactivation to quantitatively just the same extent as phage that have been irradiated as free particles with the same ultraviolet dose.

If the ultraviolet dose to the cell has not been too heavy (4 hits), DNA synthesis continues after irradiation with little decrease in rate. After a greater dose (11 hits) DNA synthesis is totally blocked although mature phage can be produced after removal of chloramphenicol. The number of noninfective particles in the final progeny appears to be directly related to the amount of DNA present at the time of irradiation and is not influenced significantly by the amount of DNA synthesized after irradiation. Thus, it appears that the ultraviolet-damaged DNA is able, upon replication, to give rise to normal DNA and that its lesions are not repaired in the process.

As discussed previously, these experiments performed by adding P^{32} after irradiation indicate that individual phage particles contain both DNA

[108] K. Burton, *Biochem. J.* **61,** 473 (1955).
[109] J. Tomizawa, *Virology* **6,** 55 (1958).

present at the time of irradiation and DNA made subsequently. Both irradiated and nonirradiated DNA can appear in either infective or noninfective particles. Nonirradiated, labeled DNA in noninfective particles can later be separated from irradiated DNA by a second generation experiment in which the noninfective particles are employed in a mixed infection with a large multiplicity of unlabeled live particles. Among the progeny of such a cross, 90% of the transferred P^{32} is found in infective particles. In the reciprocal experiment (with noninfective particles containing labeled, irradiated DNA) 50% of the transferred P^{32} is found in infective particles, while 50% persists in noninfective particles.

These experiments seem to indicate that the DNA irradiated within the cell is incorporated into the mature phage without any process of molecular degradation and loss of identity.

The conservation of ultraviolet lesions per genome after irradiation was only demonstrated in the instance (11 hits) that subsequent DNA synthesis was totally blocked. It would be very interesting to know if this conservation would be observed after a lesser ultraviolet dose which permitted post-irradiation DNA synthesis.

Additional evidence that the phage DNA which accumulates in the presence of chloramphenicol is specific and functional,[106] is provided by experiments with the mutagen 5-bromouracil. If infected cells which require thymine (either as bacterial mutants or by exposure to sulfanilamide) are instead provided with 5-bromouracil, this pyrimidine is extensively incorporated into the phage DNA in the place of thymine.[110] The mutation frequency among such phage may be two orders of magnitude higher than is usually found.[111]

If, specifically during the time of DNA synthesis in the presence of chloramphenicol, thymine-requiring cells are supplied with 5-bromouracil and this is then removed at the time of chloramphenicol removal, the progeny phages are found to contain more than an order of magnitude increase in incidence of mutation.[112, 113] Since, presumably only the DNA made in the presence of chloramphenicol (and its putative progeny) could have been affected by the 5-bromouracil, this DNA must be functional.

3. Biochemistry of T-Even Phage Infection

a. Origin of Phage DNA Components

The biochemistry of the synthesis of phage DNA has not been developed in detail. The phosphorus, as previously indicated, comes to some extent

[110] D. B. Dunn and J. D. Smith, *Nature* **174**, 305 (1954).
[111] R. M. Litman and A. B. Pardee, *Nature* **178**, 529 (1956).
[112] S. Brenner and J. D. Smith, *Virology* **8**, 124 (1959).
[113] R. M. Litman and A. B. Pardee, *Virology* **8**, 125 (1959).

from the host DNA and host intermediates, possibly some from RNA, and largely from inorganic phosphorus assimilated after infection. Pulse P^{32} experiments have shown that, on the average, the transport of a P^{32} atom from medium to phage DNA requires about 8 to 9 minutes. A DNA molecule remains in the phage precursor pool an average of an additional 7 to 8 minutes.[100, 103]

Studies of the acid-soluble fraction of infected cells[114] have demonstrated the presence of compounds related to DNA precursors such as deoxyadenosine triphosphate (dATP), deoxyguanosine monophosphate (dGMP), deoxyuridine monophosphate (dUMP), and thymidine diphosphate (TDP), none of which were present in detectable amounts in uninfected cells. Curiously, no nucleotide containing 5-hydroxymethylcytosine could be found in the acid-soluble fraction.

The contribution of parental DNA to progeny cannot be reduced by addition of external guanine, adenine, or thymine,[104] which suggests that this transfer does not involve degradation to a pool of small intermediates. The contribution of thymine to progeny DNA from host DNA can, however, be reduced to a moderate extent by supplying the cell with thymidine after infection.[104]

Lanning and Cohen[115] have shown that the formation of deoxyribose in T6-infected cells appears to involve either a new pathway or a change in the relative use of existing pathways. When glucose-1-C^{14} is used as the sole carbon source during infection, the deoxyribose of the phage DNA has a specific activity 40–60% of that of the glucose of the medium. In the same medium the deoxyribose of the DNA of normal bacteria has only 20–30% of the specific activity of the glucose.

Loeb and Cohen,[116] again using glucose-1-C^{14} as the carbon source during infection, demonstrated that the deoxyribose of all the nucleotides of the T6 produced, had the same specific activity, indicating a common path of formation for all the deoxyribose. These authors suggest that the precursors of the viral deoxyribose are the acid-soluble ribonucleotides.

The glucose coupled to the HMC of the T6 DNA appears to be derived directly from the glucose of the medium[116] through uridine diphosphate glucose.[77]

b. Enzymes Produced during Infection

The development of several new enzymes during the first minutes of infection with the T-even phages has recently been demonstrated. Flaks and Cohen[117-119] have demonstrated an enzyme which in the presence of

[114] J. F. O'Donnell, R. P. Mackal, and E. A. Evans, Jr., *J. Biol. Chem.* **233,** 1523 (1958).
[115] M. C. Lanning and S. S. Cohen, *J. Biol. Chem.* **216,** 413 (1955).
[116] M. R. Loeb and S. S. Cohen, *J. Biol. Chem.* **234,** 364 (1959).
[117] J. G. Flaks and S. S. Cohen, *Biochim. et Biophys. Acta* **25,** 667 (1957).

formaldehyde plus tetrahydrofolic acid converts dCMP to dHMP (deoxycytidylate hydroxymethylase). Cohen had previously demonstrated that the hydroxymethyl group could be derived from the β-carbon of serine,[102] but not from methionine.[119a] This deoxycytidylate hydroxymethylase could not be demonstrated in extracts of normal cells or of bacteriophage, nor could it be inhibited by mixtures of extracts of normal and infected cells. If the phage infection was made in the presence of the inhibitor 5-methyltryptophan, no enzyme was made until the inhibition was released by the addition of tryptophan.

Kornberg *et al.*,[77] and Somerville and Greenberg[120] have demonstrated the appearance of a 5-hydroxymethyldeoxycytidylic acid kinase which converts the monophosphate (dHMP) to the triphosphate (dHTP), which is the apparent DNA precursor. Studies of the levels of the other deoxyribonucleotide kinases during infection indicate that the levels of deoxyguanylic kinase and thymidylic kinase rise markedly (twenty- to fortyfold), the level of deoxyadenylic acid kinase which is peculiarly high in uninfected cells remains high, and the level of deoxycytidylic acid kinase remains low.

A particularly interesting enzyme which appears during T2 infection is a deoxycytidylic triphosphatase,[77, 121] which converts deoxycytidylic triphosphate (dCTP) to the monophosphate by hydrolysis of the pyrophosphate link. The presence of this enzyme prevents the accumulation of any dCTP and thereby explains the complete substitution of cytosine by 5-hydroxymethylcytosine in phage DNA. The DNA polymerase[122] which increases some twelvefold in activity during infection, does not itself discriminate between dCTP and dHTP.

Another interesting enzyme, described by Kornberg *et al.* in T2 infected cells, glucosylates a fraction of the hydroxymethyl groups of 5-hydroxymethylcytosine after the incorporation of this nucleotide into macromolecular DNA. The glucose is transferred from uridine diphosphate glucose (UDPG).

In T6 phage, most of the hydroxymethyl groups are coupled to a diglucose and an additional enzyme has been isolated from T6 infected cells which adds a second glucose, again from UDPG (Kornberg, personal communication).

These new enzymes, or the increases in existing enzymes, begin to appear about 3 to 4 minutes after infection with the phage.

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[118] J. G. Flaks and S. S. Cohen, *J. Biol. Chem.* **234,** 1501 (1959).

[119] J. G. Flaks, J. Lichtenstein, and S. S. Cohen, *J. Biol. Chem.* **234,** 1507 (1959).

[119a] M. Green and S. S. Cohen, *J. Biol. Chem.* **225,** 387 (1957).

[120] R. Somerville and G. R. Greenberg, *Federation Proc.* **18,** 327 (1959).

[121] J. F. Koerner and M. S. Smith, *Federation Proc.* **18,** 264 (1959).

[122] I. R. Lehman, M. J. Bessman, E. S. Simms, and A. Kornberg, *J. Biol. Chem.* **233,** 163 (1958).

It is also pertinent to point out that during T2 phage infection thymidylic acid can be synthesized in normally thymine-requiring cells.[123] Cohen has shown that the activity of the enzyme system, which in the presence of formaldehyde and tetrahydrofolic acid converts dUMP to TMP (thymidylic synthetase), is increased seven- to eightfold in T2-infected, normal coli cells. In thymine-requiring cells, this enzyme system is not detectable until after phage infection.[124]

Keck, Mahler, and Fraser[125] have described a dCMP deaminase which begins to appear 3 to 5 minutes after infection with T2 phage. By converting dCMP to dUMP the enzyme provides a precursor of thymidylic acid, and may be involved in the phage-directed synthesis. Thus, dCMP may be converted to either dHMP or to TMP via dUMP.

c. RNA Metabolism During Phage Infection

Net RNA synthesis ceases very shortly after the initiation of phage infection.[99a, 126-128] However, P^{32} experiments have indicated that there is a significant turnover of RNA after infection.[5, 129-132] The amount, rate, and duration of this turnover appear to be strongly dependent upon the composition of the external medium. Upon centrifugal fractionation of the cells, the phosphate assimilated into RNA was shown to be present in both of two particulate fractions and in a "soluble" fraction, although with varying specific activity.[130]

Pulse P^{32} experiments indicate that this P^{32} can pass from RNA into phage DNA. This process is accelerated if the cells are washed to deplete their acid-soluble pools. However in T2 infection the rate of turnover of the RNA is not sufficient at any time to account for the rate of production of phage DNA; this is particularly true in the later stages of infection. Thus if RNA is a precursor of the phage DNA, it could not be the only precursor. However, the experiments do demonstrate that the transfer of material from RNA to DNA does not involve degradation to any level lower than that of nucleotides.

In contrast, in T7 infection, the incorporation and turnover of P^{32} into

[123] H. D. Barner and S. S. Cohen, *J. Bacteriol.* **68,** 80 (1954).
[124] S. S. Cohen, *Abstr. 134th Meeting Am. Chem. Soc.* p. 22c (1958).
[125] K. Keck, H. R. Mahler, and D. Fraser, *Arch. Biochem. Biophys.*, **86,** 85 (1960).
[126] S. S. Cohen, *J. Biol. Chem.* **174,** 281 (1948).
[127] L. M. Kozloff, K. Knowlton, F. W. Putnam, and E. A. Evans, Jr., *J. Biol. Chem.* **188,** 101 (1951).
[128] L. A. Manson, *J. Bacteriol.* **66,** 703 (1953).
[129] E. Volkin and L. Astrachan, *Virology* **2,** 149 (1956).
[129a] I. Watanabe, Y. Kiho, and K. Muira, *Nature* **181,** 1127 (1958).
[130] E. Volkin and L. Astrachan, *Virology* **2,** 433 (1956).
[131] E. Volkin, L. Astrachan, and J. L. Countryman, *Virology* **6,** 545 (1958).
[132] L. Astrachan and E. Volkin, *Biochim. et Biophys. Acta* **29,** 536 (1958).

TABLE III

COMPOSITION OF VARIOUS NUCLEIC ACIDS

Nucleic acid	A	C	G	U or T	Ref.
Normal *E. coli* RNA	23	23	32	22	*a*
T2 DNA	32	17	18	32	*b*
RNA made during T2 infection	29	17	25	29	*a*
T7 DNA	26	27	23	24	*c*
RNA made during T7 infection	27	24	22	28	*c*

References

[a] E. Volkin and L. Astrachan, *Virology* **2**, 149 (1956).
[b] G. R. Wyatt and S. S. Cohen, *Biochem. J.* **55**, 773 (1953).
[c] E. Volkin, L. Astrachan, and J. L. Countryman, *Virology* **6**, 545 (1958).

RNA proceed at a faster rate than *de novo* synthesis of DNA (much of the T7 DNA is derived from preinfection host nucleic acids). Thus, in this instance conversion of RNA polymer to DNA polymer could occur, but this could not be the only manner of turnover of RNA.

The most unusual aspect of the RNA metabolism of phage-infected cells is the close relationship between the nucleotide composition of the new RNA and the nucleotide composition of the DNA of the infecting phage.[129, 129a, 131] If it is assumed that only wholly new RNA molecules are made after infection, then, by alkaline degradation to 2′- and 3′-mononucleotides, fractionation, and measurement of the total radioactivity associated with each nucleotide, the relative nucleotide composition of the new RNA can be determined. Since the bulk RNA nucleotide composition of the cell does not change during infection, and since the nucleotides are present in nearly equal proportion, measurement of the relative specific activities of the labeled nucleotides is sufficient to approximate the relative composition of the new RNA. As shown in Table III, this composition remarkably parallels the composition of the phage DNA.

This interesting correlation of RNA and DNA compositions might simply reflect the availability of precursors for RNA and DNA. Alternatively there is the more significant possibility that the RNA made after infection is patterned on the phage DNA and is an intermediary in the synthesis of phage proteins and specific enzymes related to the infective process.

The addition of chloramphenicol before infection prevents this shift of the nucleotide composition of RNA synthesis during infection.[133] Upon infection in the presence of chloramphenicol, RNA synthesis is observed at a greater rate than during normal infection, but the composition of the synthesized RNA remains that of normal coli RNA,[133a] and this RNA does not turn over.

[133] L. Astrachan and E. Volkin, *Biochim. et Biophys. Acta* **32**, 449 (1959).

[133a] Contrary results have been reported by Watanabe *et al.*[129a] This contradiction

Delay in the addition of chloramphenicol to 3 minutes after infection, however, permits the shift of the pattern of RNA synthesis. RNA made under these conditions has the nucleotide composition mimicking that of the phage DNA; no phage DNA is made, however, and the RNA turnover is not observed unless the chloramphenicol is removed. After removal of the chloramphenicol, phage DNA is made and the RNA turnover begins.

There is evidently a requirement for prior or comcomitant protein synthesis during the very early stages of phage infection for the transition of the pattern of RNA synthesis to be achieved.

If chloramphenicol is added after 9 minutes of infection, DNA synthesis, as previously described, continues; RNA synthesis is apparently accelerated by the addition of the chloramphenicol and RNA turnover continues.

d. Infection with Ultraviolet Irradiated Bacteriophage

Ultraviolet irradiation destroys the ability of bacteriophage to reproduce in single infection. If, however, the same irradiated phage preparation is used to infect bacteria at a high multiplicity, so that almost all cells are infected by two or more particles, it is found that a large fraction give rise to progeny (multiplicity reactivation; see p. 192).

Investigations of DNA synthesis in cells multiply infected with ultraviolet irradiated phage[98, 99] have shown that the onset of phage DNA production is delayed by some 5 to 10 minutes beyond the time observed in normal infection. Once initiated, however, the rate and amount of DNA synthesis increase to levels comparable to those observed in normal infection, but always delayed in time.

After heavy ultraviolet irradiation there is little multiplicity reactivation and correspondingly there is very little synthesis of phage DNA in the infected culture.[98]

Studies of the rates of synthesis of the enzymes of phage infection under these conditions would be most instructive. Flaks *et al.*[119] have reported that the synthesis of deoxycytidylate hydroxymethylase is observed at a normal rate upon infection with T6r so heavily irradiated that it was incapable of multiplication or of appreciable multiplicity reactivation. Keck *et al.*[125] have made similar observations upon the appearance of the dCMP deaminase.

4. The Relationship between DNA and the Phage Genome

a. The Size of a Genetic Locus

(1) *Fine Structure Genetics.* The results of many experiments of diverse type find a common explanation in the assumption that the DNA of T-even

might be the result of small differences in the conduct of the experiment if the time of addition of chloramphenicol is critical.

phages is the genetic determinant of the phage. Hershey's experiments demonstrate that DNA is the major substance to enter the host upon infection. Genetic experiments with mixed infection indicate that during the infection a pool of phage genomes is accumulated which undergoes several rounds of recombination before lysis.[134] The physical correlate to this genetic pool appears to be the phage DNA, for recombination is observed to take place in the presence or absence of chloramphenicol which inhibits protein synthesis.[106, 135]

The most direct approach to the determination of the physical size of a genetic determinant of function in T4 phage has come from the genetic fine structure experiments of Benzer.[136, 137]

All of the mutant characters known in a T-even phage can be mapped in a linear order—a genetic map—on which the characters are separated by distances proportional to the recombination frequencies observed when crosses are made.[9] The total length of this map—the sum of the recombination frequencies between adjacent markers—is estimated to be 200–800 units and is assumed to correspond to the DNA content, 200,000 nucleotide pairs, of the phage (or to a portion of the DNA content if some of the DNA is assumed to be nongenetic).

A particular mutant locus—*r*II—can be recognized by the property that a phage bearing this mutant locus produces unusually large plaques when plated on *E. coli* B and produces effectively no plaques when plated on *E. coli* K12 (λ). Many mutants, both natural and induced, have been located in the *r*II region. Since it is possible to select for recombinants from crosses of *r*II mutants by plating the progeny on K12 (λ)—only the wild-type recombinants will plate—it is possible to map this class of mutants very precisely, i.e., to very low recombination frequencies. It has been shown that the extremes of the *r*II region encompass at least eight map units.[138, 138a]

An independent feature of the *r*II region is that it can be subdivided into two, approximately equal, loci known as cistrons (A and B) which have distinct functions; when a K12 (λ) cell is infected with two *r*II mutant phages one of which is mutant in the A cistron and one in the B cistron the infection will be successful and progeny (mostly of the parental types) will be produced.

[134] N. Visconti and M. Delbrück, *Genetics* **38**, 5 (1953).

[135] A. D. Hershey, E. Burgi, and G. Streisinger, *Virology* **6**, 287 (1958).

[136] S. Benzer, *Proc. Natl. Acad. Sci. U. S.* **41**, 344 (1955).

[137] S. Benzer, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 70. Johns Hopkins, Baltimore, 1957.

[138] R. S. Edgar, *Virology* **6**, 215 (1958).

[138a] Two genetic loci are said to be one "map unit" apart when the frequency of recombinant types among the progeny of a cross, involving the two loci, is 1%.

Benzer selected groups of mutants in each cistron and using the property of selective plating of recombinants on K12 (λ), precisely mapped these mutants. The smallest, nonzero, recombination frequency observed was 2×10^{-2} map units. Several mutants showed no recombinants when 10^{-6} map units would have been detectable and presumably are recurrent mutations at the same site. Other mutants, which were presumably deletions of a significant portion of the map, produced no recombinants at all with all mutants known to be located within a certain map range, but recombined with mutants outside this region.

If it is assumed that the DNA complement of T4, 200,000 nucleotide pairs, corresponds to 400 map units, then each *r*II B cistron, about 3 units, would contain 1,500 nucleotide pairs (molecular weight around 2×10^6). The smallest nonzero map distance, 2×10^{-2} map units, is then about 10 nucleotide pairs.

The size of a mutation, then, can be estimated in various ways, such as deviations from the additivity of map distances between very close mutants. From such necessarily uncertain calculations, the maximum size of any mutant which is observed to revert (thus excluding large deletions) is estimated to be 0.05 map units (25 nucleotide pairs or less). The large deletions can amount to several thousand nucleotide pairs. These values of the size of a map unit or a mutation are probably to be regarded as upper limits.

A similar detailed analysis of the "*h*" region[139] has indicated that it is a single cistron encompassing about 2 recombination units.

(2) *Ultraviolet Irradiation Studies.* Much evidence indicates that ultraviolet damage to phage is localized in the DNA. The cross section for phage inactivation is nearly the same immediately after adsorption and injection of DNA into the host cell as it is for free phage particles.[40, 89] The action spectrum for inactivation of T2 parallels the ultraviolet absorption curve of DNA.[140] Phage inactivation is subject to photoreactivation which has been shown to be a property of free nucleic acid[46] (see Chapter 30).

Therefore, it is pertinent that ultraviolet irradiation can destroy genetic loci in the sense that after irradiation they will not appear in the progeny of a mixed infection with unirradiated phage (i.e., no cross-reactivation).[50] Unlinked or weakly linked markers (separation greater than 12–20 recombination units) are inactivated independently. Linked markers are inactivated simultaneously more frequently than would be accountable by chance. These results accord with the hypothesis that genetic loci consist of

[139] G. Streisinger and N. C. Franklin, *Cold Spring Harbor Symposia Quant. Biol.* **21**, 103 (1956).

[140] M. R. Zelle and A. Hollaender, *J. Bacteriol.* **68**, 210 (1954).

discrete regions of the DNA structure and that at least certain types of ultraviolet damage can prevent not only the function of a DNA segment but also its replication per se or in a recombination event.

The significance of the cross section for inactivation of a locus (relative to the cross section for inactivation of an entire particle) is dependent in part upon the physical size of an individual ultraviolet-induced lesion and the influence of such lesions upon genetic recombination at nearby locations along the genetic map. Baricelli[41] has computed, from the multiplicity reactivation data of Luria and Dulbecco,[48, 49] that an ultraviolet lesion of a noncritical locus effectively destroys a length of chromosome equivalent to 0.3 to 0.4% of the total chromosome map. By destruction is meant that this entire segment of chromosome is effectively a damaged area for the process of multiplicity reactivation which presumably involves a recombination of undamaged units of two or more phages to provide an intact genome. For multiplicity reactivation to be possible an equivalent length of undamaged genetic material at an equivalent site on another chromosome must be available.

From the initial rate at which genetic markers are made unavailable by ultraviolet irradiation to cross-reactivation, one could naively compute a cross section for this process of about 4% of the cross section of phage inactivation.[50] However, any attempt to use these data to estimate the fraction of the genome occupied by a specific locus involves complex assumptions about the nature of the cross-reactivation process, the fraction of the locus that is essential for cross-reactivation, and so forth, and such attempts have not produced generally accepted results.

It would seem possible, however, to arrive at a better estimate of the size of a functional locus by experiments designed to measure the rate of inactivation of critical loci, loci that must function before replication and recombination.

The A and B cistrons of the *r*II locus appear to be critical in this sense for infection by *r* mutants in *E. coli* K12 (λ) (they are not critical in this sense for infection by *r* mutants in *E. coli* B).[42, 43, 136] Krieg[43] has determined that the cross section of ultraviolet inactivation of the function of the A locus (measured by the failure of a mixed infection in K12 (λ) of an irradiated *r*II$^+$ phage and an *r*II A mutant) is 0.10, of the cross section for free phage inactivation, while the corresponding cross section for the B locus is 0.05. The two loci are inactivated together more frequently than would be anticipated by chance. If Baricelli's estimate of the size of ultraviolet damage is even approximately correct, this latter result could not be the result of a single lesion overlapping into both cistrons, but seems more likely to be the result of a fraction of less specific ultraviolet damages that may, for instance, completely prevent the entire genome from participation in infection. If an attempt is made to subtract this putative class of lesions, the total cross section remaining for the A and B cistron is still about 0.08 of that of the entire phage. This may be compared with the estimate that the A and B loci comprise about 0.02 of the genetic map. The corrected cross section for inactivation of the A

cistron is two or three times that of the B cistron, although the two cistrons comprise approximately equal portions of the genetic map.

Another genetic locus which in a special circumstance has a critical function is the *u* locus of T4. Streisinger[141] has shown that the *u* locus of T4 is responsible for the nearly 50% reduction in ultraviolet sensitivity of T4 phage as compared to T2 (despite their identical DNA content). Harm[142] demonstrated that this locus appeared to act by repairing a certain fraction of the ultraviolet lesions inflicted upon T4 in a manner very similar to the action of photoreactivation in T2 (T4 is only very slightly subject to photoreactivation). Harm demonstrated that a single heavily irradiated T4 particle in mixed infection with ultraviolet irradiated T2 particles could raise the level of survival of the T2 particles to that of T4 particles at the same ultraviolet dose, as long as the T4 *u* allele was effective. With very heavy doses of ultraviolet the T4 *u* allele itself itself could be destroyed and the effect lost.

The cross section for inactivation of the *u* allele—which is certainly critical in this situation—as neither the T2 phage nor the T4 phage can replicate until the action of the *u* gene repairs lesions—is about 1% of the cross section for inactivation of T4. It is thus about $^1/_5$ the cross section of an *r*II A cistron.

A recently discovered locus which, in mutant form, confers resistance to proflavin inactivation, has been shown to have a cross section for ultraviolet inactivation about 3% of the cross section for inactivation of T4. (E. S. Edgar, personal communication).

The apparent discrepancy between the estimates of the size of the *r*II locus from its proportion of the genetic map and from its large relative ultraviolet cross section could be reconciled in several ways. One of the most appealing is to suggest that two types of ultraviolet lesions are produced, both of which affect function but only one of which influences replication. Lesions of the first type, which are presumed to be the more numerous, prevent function of a locus but permit replication to form an undamaged locus. Such lesions are of no consequence if they occur in a noncritical locus for which the function can be postponed until after replication. Lesions of either kind, however, are fatal to a critical locus. If the number of critical loci is few, one will then expect such loci to be several times as sensitive per unit size as the bulk of the phage genome, and hence to account for an extraordinarily large fraction of the ultraviolet sensitivity.[43]

The difference between the cross sections of different critical loci, however, cannot be explained in such a simple way and may reflect actual differences in the size or ultraviolet sensitivity of the loci.

[141] G. Streisinger, *Virology* **2**, 1 (1956).
[142] W. Harm, *Naturwissenschaften* **45**, 391 (1958).

Similarly the decay of incorporated P^{32} atoms in the DNA of a T-even particle can prevent the appearance of individual genetic loci in progeny particles from mixed infections with allelic nonradioactive particles. In this case, the effect of the destructive event appears to be less discrete, in that unlinked markers are not eliminated entirely independently. However, there is a definite correlation between linkage and joint elimination of marker; the closer the linkage between two markers the greater is the likelihood of dual elimination.[143, 144]

It has been proposed that inactivation by P^{32} is a consequence of scission of the DNA molecule.[52] On this hypothesis the recovery of genetic markers from a P^{32} damaged phage particle in a mixed infection may depend upon incorporation of a fragment of its DNA into the DNA structure of one of the replicating DNA molecules of the carrier phage. In this case the loss of a particular genetic component will bear no simple relationship to its DNA, and hence its P^{32} content.

The pattern of inactivation of the phage genetic markers by X-rays administered to free phage appears to be very similar to that observed with P^{32} inactivation, and hence has not been useful in the estimation of the size of such markers.[144] However, if the X-irradiation is administered to the phage-bacterium complex after injection, multiplicity reactivation and cross-reactivation can be demonstrated with efficiencies comparable to those observed after ultraviolet irradiation. This recent discovery[90, 144a] may permit the use of X-ray inactivation to obtain estimates of the size of genetic loci.

b. Association of Genetic Loci with Parental DNA

Bacteria simultaneously infected with two or more related phages (of the T-even type) will support the growth of each type. Up to 30 phage particles can, at least, contribute genetic markers to progeny.[145, 145a, 146] During such a mixed infection, genetic interactions occur and genetic recombinants appear. Whether such interactions involve actual recombinations of parental DNA molecules (or of their progeny) or are produced during replication by a "copy choice" method[147-149] whereby a new DNA

[143] G. S. Stent, *Proc. Natl. Acad. Sci. U. S.* **39,** 1234 (1953).
[144] F. W. Stahl, *Virology* **2,** 206 (1956).
[144a] J. J. Weigle and G. Bertani, *Virology* **2,** 344 (1956).
[145] R. Dulbecco, *Genetics* **34,** 126 (1949).
[145a] R. S. Edgar and C. M. Steinberg, *Virology* **6,** 115 (1958).
[146] However, it appears that no more than two phage particles can participate in early stages of infection such as the complementary action of *r*II⁺A and *r*II⁺B cistrons to permit mixed growth of T4 mutants on K12 (λ).[42]
[147] A. D. Hershey, *Intern. Rev. Cytol.* **1,** 119 (1952).
[148] C. Levinthal, *Genetics* **39,** 169 (1954).
[149] J. Lederberg, *J. Cellular Comp. Physiol.* **45** Suppl. 2, 75 (1955).

molecule is made, patterned in part after one parent and in part after another, remains a moot question. In attempts to answer this question several studies have been made of the association of a physical label with a genotype in a mixed infection.

If bacteria are mixedly infected with P^{32}-labeled T4 and unlabeled T2 and the progeny put through a second cycle at low multiplicity to eliminate phenotypic mixing,[149a] the distribution of P^{32} among the T2 and T4 type phages can be measured by specifically adsorbing the phage to specifically susceptible bacteria. About two-fifths of the transferred P^{32} appears in T2 type progeny while three-fifths is found in the T4 type.[104]

Similar experiments have been carried out using phage of the same type (T2), differing only as to the host range locus, *h*. If bacteria are mixedly infected with equal numbers of P^{32}-labeled *h* and unlabeled h^+ phage, again two-thirds of the transferred label is found in particles of the originally labeled (*h*) parental genotype; (h^+ and *h* may be separated by the use of bacteria with specific adsorptive ability). However, this transferred label does not appear to be indissolubly linked to the *h* locus, for if this progeny is used in a second generation experiment, only 59% of the phosphorus transferred from the first generation *h* progeny is contained in the second generation progeny of the *h* genotype.[152]

Levinthal has reported[55] that if heavily P^{32}-labeled T2 h^+ phages are crossed with T2 *h* phage, at least 90% of the "stars" among the progeny phage are associated with phage (after a second generation to eliminate phenotypic mixing) of the h^+ genotype. This evidence supports his conclusion that the large DNA piece, or star-forming particle, carries at least the *h* locus and may indeed carry all of the known genetic loci of T2. In this case the P^{32} which appears in the progeny of the originally unlabeled genotype may come from the smaller, nongenetic DNA components.

An alternative experiment to test the hypothesis that a single large piece of the DNA of T2 carries all of the genetic loci was performed by Sato and Stent.[153] Heavily P^{32}-labeled phage bearing 4 markers were crossed with an excess of a nonradioactive phage bearing, at each of the 4 loci, a distinctive allele. Under these conditions very few progeny phage are formed which have all 4 of the markers associated with the

[149a] The progeny of a mixed infection of T2 and T4 will contain both genotypes and both phenotypes. A small fraction of the progeny will be genotypically T2 but phenotypically T4 and vice versa.[150, 151] Presumably this is a consequence of inclusion of a T2 genome in a T4 protein coat. This phenotypic mixing can be eliminated by a second generation of growth under conditions of single infection, so that all of the phage from a given cell will have both the phenotype and genotype corresponding to the genotype of the infecting particle.

[150] M. Delbrück and W. T. Bailey, Jr., *Cold Spring Harbor Symposia Quant. Biol.* **11**, 33 (1946).

[151] A. Novick and L. Szilard, *Science* **113**, 34 (1951).

[152] A. D. Hershey, E. Burgi, J. D. Mandell, and N. E. Melechen, *Carnegie Inst. Washington Yearbook* **55**, 297 (1956).

[153] G. Sato and G. S. Stent, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 724. Johns Hopkins, Baltimore, 1957.

P^{32}-labeled parent, and one would expect that the actual labeled DNA chains of the parent would be present in an appreciable fraction of such quadruply marked progeny. If this were so, decay of these P^{32} atoms upon storage of the over-all progeny would specifically, and markedly, reduce the number of such quadruply marked progeny. This result was not, however, obtained; if correct, these experiments would suggest that these 4 loci are not located on one large DNA molecule which retains its integrity (on the single-strand level). Stent's experiments have been criticized as to technical details and Levinthal and Baylor made in 1956[154] a preliminary report of a contradictory result in a 4 factor cross.

While all of these experiments make it clear that a considerable amount of parental DNA remains associated with the parental genotype, no quantitative explanation can be made at present.

c. Chemical Mutagenesis

The use of the technique of fine structure genetic mapping as developed by Benzer[136, 137] permits detailed analysis of the effects of various chemical mutagens upon the phage genome. These mutagens are believed to act by causing mistakes during the replication of the DNA, thereby resulting in changes in the nucleotide sequence.

Among the most effective mutagens studied are: 5-bromouracil,[155] 2-aminopurine,[156, 157] nitrous acid,[158, 159] pH 5 at 45°,[159] and proflavine.[160, 161]

5-Bromouracil and 2-aminopurine are believed to act as tautomeric mutagens[162] in the manner originally suggested by Watson and Crick.[163] Ordinarily, in its keto form, 5-bromouracil would be expected to replace thymine and pair with adenine. However, in its enolic form, 5-bromouracil will pair as does cytosine, so that it may enter a replicating DNA opposite a guanine; at a later replication it may revert to its keto form and pair with an adenine, thus, resulting in replacement of a guanine with an adenine (mistake in incorporation). Or, if incorporated into DNA in place of thymine (paired with adenine), a 5-bromouracil may during a later replication, enolize, and pair with a guanine, thus, causing a replacement of an adenine with a guanine (mistake in replication). The presence of the bro-

[154] C. Levinthal and M. Baylor, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 745. Johns Hopkins, Baltimore. 1957.
[155] S. Benzer and E. Freese, *Proc. Natl. Acad. Sci. U. S.* **44,** 112 (1958).
[156] E. Freese, *Proc. Natl. Acad. Sci. U. S.* **45,** 622 (1959).
[157] E. Freese, *J. Mol. Biol.* **1,** 87 (1959).
[158] W. Vielmetter and C. M. Wieder, *Z. Naturforsch.* **14b,** 312 (1959).
[159] E. Freese, Brookhaven *Symposia in Biol.* **12,** 63 (1959).
[160] R. I. De Mars, *Nature* **172,** 964 (1953).
[161] S. Brenner, S. Benzer, and L. Barnett, *Nature* **182,** 983 (1958).
[162] 2-Aminopurine could form a single hydrogen bond to 5-hydroxymethylcytosine in its normal state without tautomerization.[157]
[163] J. D. Watson and F. H. C. Crick, *Cold Spring Harbor Symposia Quant. Biol.* **18,** 123 (1953).

mine might be expected to favor enolization of 5-bromouracil as compared to thymine; 2-aminopurine can act similarly.

Nitrous acid is believed to act by oxidative deamination of cytosine or adenine residues. Such deamination will cause erroneous pairing at the next DNA replication.

All three of these agents, then, are expected to cause replacement of adenine-thymine by guanine-5-hydroxymethylcytosine pairs and vice versa. Hence, each of these mutagens should be very effective agents to produce reversions of mutations caused by any of this mutagenic group, and this is found to be the case. 5-Bromouracil can induce reversion of 98% of the *r*II mutants induced by 2-aminopurine, 95% of its own mutations, and 87% of those induced by nitrous acid[159] (as these agents increase the mutation rate by two or more orders of magnitude, the background of spontaneous mutations can be largely ignored). In a few cases, the mutations are apparently more complex. It is of interest that mutations induced by 5-bromouracil tend to be more easily induced to revert by 2-aminopurine, and vice versa, although exceptions are known.[156]

The action of pH 5 at 45° is believed to result in occasional loss of a purine ring from the DNA chain. It might be expected that, during replication, this gap in the template could be paired by any of the four nucleotides available. However, 77% of such pH 5 mutants are reversible by 5-bromouracil or 2-aminopurine.[159] As mentioned, these agents are believed to be able to interchange adenine-thymine and guanine-5-hydroxymethylcytosine pairs but presumably could not interchange a purine-pyrimidine pair for a pyrimidine-purine pair. Hence, it would appear that on most occasions the gap in the template, left by the loss of a purine, is paired by a pyrimidine nucleotide.

The nature of proflavine mutagenesis is obscure. Proflavine increases the mutation rate at the *r*II locus some thirtyfold. The mutants produced are relatively homogeneous as to spontaneous reversion rate. It is of considerable interest that most proflavine-induced mutants, and spontaneous mutants, are almost entirely nonreversible by 5-bromouracil or 2-aminopurine and hence involve a different kind of modification of nucleic acid than those induced by the latter agents. Similarly, 5-bromouracil mutations are not reversible by proflavine although proflavine mutants are self-reversible.[156, 164] No coincidences were observed between the sites of 55 proflavine-induced mutants and 67 5-bromouracil-induced mutants.[161] Proflavine and most spontaneous mutations may then represent replacement of a purine-pyrimidine pair by a pyrimidine-purine pair.

It is of interest that Astrachan and Volkin[165] have demonstrated chro-

[164] S. Brenner, *Brookhaven Symposia in Biol.* **12,** 74 (1959).
[165] L. Astrachan and E. Volkin, *J. Am. Chem. Soc.* **79,** 136 (1957).

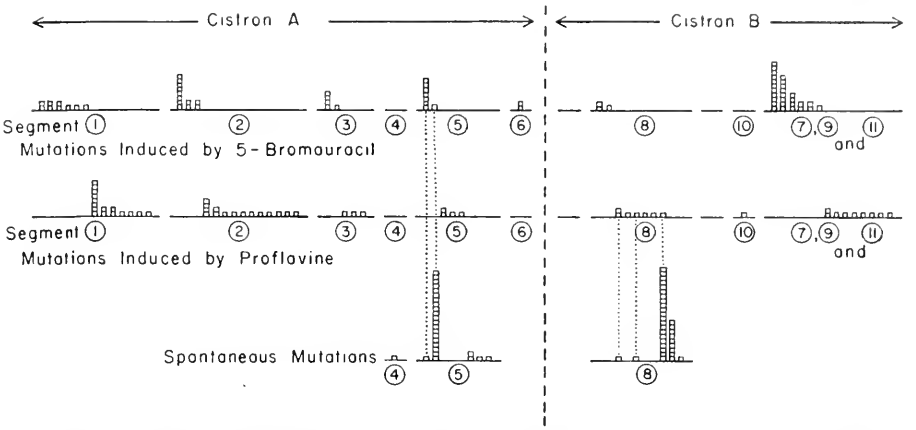

FIG. 5. Genetic map showing the location of mutations in the *r*II region of phage T4. Each mutation is represented by a box placed in the proper segment of the map. (The over-all mutation rate is different in each case. The occurrence at a given location of one mutation in the bromouracil set corresponds to roughly 10^3 times higher mutability at that spot than one occurring in the spontaneous set. In the case of proflavine this factor is of the order of 10^2). [From S. Brenner, S. Benzer, and L. Barnett, *Nature* **182**, 983 (1958).]

matographic differences between heat-degraded DNAs from phage grown in the presence or absence of proflavine.

If the hypotheses presented concerning the action of 5-bromouracil, 2-aminopurine, nitrous acid, and pH 5 are correct—and they form a self-consistent pattern—then it seems evident that changes of a single nucleotide pair can result in demonstrable mutation. This view is supported by the relative homogeneity of spontaneous reversion rates of 5-bromouracil-induced mutants, and by the great rarity of nonreversible mutants of this type.[155]

Fine structure genetic maps have been made of the location in the *r*II region of mutants induced by these agents. The striking result has been obtained that particular sites in the genetic map appear to be unusually susceptible to the action of particular mutagens, e.g., 5-bromouracil will induce mutants at a particular site many times more frequently than at another nearby locus.[155] By genetic analysis all the mutants induced at the particularly sensitive locus failed to recombine with each other.

Such sites of frequent occurrence of mutation are observed upon analysis of spontaneous mutants and of the mutants induced by 5-bromouracil,[155] 2-aminopurine,[156] and proflavine.[161] The sensitive sites are, however, different for each mutagen (Fig. 5). Treatment with nitrous acid, or by pH 5 at 45°, produced a much more uniform distribution of mutants.

The existence of such distinct mutagenically susceptible sites suggests

that the likelihood of mutagenesis must be strongly dependent, not only upon the particular mutagen, but upon the nature of the nucleotide sequence at neighboring sites along the DNA molecule. The influence of neighboring nucleotides must extend for more than one nucleotide pair.

d. Host-Controlled Variation of Bacteriophage

In general the progeny of bacterial virus infection, excepting the relatively rare mutants, has the same phenotypic and genotypic properties as its parents, regardless of the species of host bacterium employed. However, it has been observed that in certain instances, growth on a particular bacterial host will result in progeny with at least one different property from those of the parents. Repeated culture of the phage on the particular host preserves this property among the progeny, but passage through another host will cause a reversion of the phage culture to its original character. Thus, the altered characteristic of the modified phage does not appear to be the result of a permanent mutation of the genotype but is a variation induced by passage through a particular host and only maintained by passage through that host.[166]

The only host-controlled character which has been studied in this way is that of host range, although there is no reason to believe other properties cannot be modified in this manner. Passage through a particular host may enlarge or restrict the host range of the progeny phage.

Thus phage T2 normally infects with an equal efficiency, *E. coli* B, *Shigella dysenteriae*, and a mutant of *E. coli* B, *E. coli* $B/4_0$. However, the progeny of growth on *E. coli* $B/4_0$ infect with full efficiency only the *S. dysenteriae*. With *E. coli* B or *E. coli* $B/4_0$, only one cell in a thousand can be infected by this progeny.[167] A variety of experiments have indicated that the phage population is homogeneous and that it is only an exceptional cell that can be infected.[166] However, passage of the progeny from *E. coli* $B/4_0$ through *S. dysenteriae* or through the small fraction of *E. coli* B cells that will support their growth, results in a second generation progeny with the original ability to infect all three hosts. Only by repeated culture on the small fraction of *E. coli* $B/4_0$ cells that will support their growth can the restricted host range be maintained.

The modified phage particles adsorb to the resistant cells and kill them but no infective phage are ever found. The nature of the block to development is unknown but as it does not appear to involve the external features of the phage, the DNA or the internal protein appear to be implicated. Similar phenomena, more often involving an extension of host range, have been observed with phage T1 and with the temperate phages λ, P2, and P22.[7]

[166] S. E. Luria, *Cold Spring Harbor Symposia Quant. Biol.* **18**, 237 (1953).
[167] S. E. Luria and M. L. Human, *J. Bacteriol.* **64**, 557 (1952).

e. Phage Heterozygotes

Among the progeny of a phage cross are a small number (a few per cent) of particles which appear to contain, in a small region of the genetic map, the genomes of both parents.[167a] Such particles are known as "heterozygotes." Upon further culture such heterozygotes do not usually propagate as such but give rise to particles of both genotypes.

Recently evidence has been obtained that chemical mutagens such as nitrous acid[158] (I. Tessman, personal communication, 1959) and 5-bromouracil (D. Pratt and G. Stent, personal communication, 1959) produce considerable numbers of heterozygotes. Since these mutagens are expected, as an initial act, to cause the formation of a mismatched base pair in a DNA molecule, this evidence strongly suggests that in naturally occurring heterozygotes, in a small portion of the DNA, one strand of the double helix represents one parental genome while the other strand is partially mismatched and represents the other parental genome.

This idea is in accord with the evidence from the minute bacteriophages (*vide infra*) that a single DNA strand is adequate to convey genetic information.

5. Radiobiological Studies of the Process of Phage Infection

Varied attempts have been made to obtain some insight into the process of bacteriophage infection by studies of the changes, during infection, of the sensitivity of the phage-bacterium complex to inactivation, either by ultraviolet radiation,[40, 88, 89, 168] or by X- or γ-radiation,[90, 169] or by decay of P^{32} incorporated within the parental phage DNA.[6, 170]

These studies have indicated that the sensitivity of the phage-bacterium complex to any of these modes of inactivation is, immediately after infection, effectively the same as the sensitivity of the free phage. Since, upon infection the principal phage material introduced into the bacterium is the phage DNA, this result implies that the major action of these agents when applied to the free phage is upon the DNA.

With phage T4, there is, during the first 4 or 5 minutes of infection, no change in the sensitivity of the complex to inactivation by ultraviolet radiation, X-radiation, or P^{32} decay.[40] Following this period, there is a decline in sensitivity until about 10 to 15 minutes after infection. At this time, by which a pool of some 40 to 50 phage DNA complements has been made, the complexes are completely insensitive to the

[167a] A. D. Hershey and M. Chase, *Cold Spring Harbor Symposia Quant. Biol.* **16,** 471 (1951).

[168] N. Symonds, *Virology* **3,** 485 (1957).

[169] R. Latarjet, *J. Gen. Physiol.* **31,** 529 (1948).

[170] G. S. Stent, *J. Gen. Physiol.* **38,** 853 (1955).

decay of P^{32} in parental DNA, they are 20 times less sensitive to ultraviolet radiation than is free phage, and the sensitivity to X-radiation has assumed the form of a multiple target curve, of multiplicity about 10, and with an asymptotic slope similar to that of free phage.[6]

With phage T2 a decline in sensitivity of the complex to ultraviolet inactivation, of a factor of two, is observed during the first 4 or 5 minutes. No similar decline is observed in sensitivity to X-radiation or parental P^{32} decay. Following this period the changes observed in the 5 to 15 minute period of infection are similar to those observed with T4. If the ultraviolet inactivation of the T2 complexes is measured after maximal photoreactivation, however, it is found that there is no change in sensitivity during the first 4 to 5 minutes. Symonds has interpreted these results to mean that phage T2, which is as a free phage twice as sensitive to ultraviolet radiation as T4, is subject to a specifically functional and photoreactivable type of damage to critical cistrons which must function before replication.[40] As these cistrons are brought into function during the first 4 minutes, sensitivity to this damage is lost and the over-all sensitivity of the complex declines. Similar damage presumably occurs to T4 complexes but is rapidly repaired by the action of the u^+ locus described by Streisinger.[141, 142]

Two general patterns of explanation have been developed to account for the decline in sensitivity of the complexes to these various modes of inactivation.[6] One hypothesis relates this decreased sensitivity to completion of certain necessary phage functions such as enzyme production and to the production of multiple copies of the phage DNA, thus, permitting the repair of damages by such processes as multiplicity reactivation. The other hypothesis postulates a transfer of the essential information from DNA to an inherently less ultraviolet-sensitive and P^{32} decay-sensitive structure such as a protein or conceivably a ribonucleoprotein.

The most cogent argument in favor of the latter hypothesis appears to be the remarkable observation by Stent,[6, 170] that even when he used parental phage heavily labeled with P^{32}, with bacteria equally heavily labeled by growth in P^{32} medium, and allowed the infection to take place in heavily P^{32}-labeled medium, the complexes still became insensitive to P^{32} decay during the later stages of the eclipse period. Under these conditions all daughter replicas of the parental phage DNA should be heavily P^{32}-labeled and therefore also sensitive to P^{32} decay. The possibility of reconstitution of an active phage after P^{32} decay by multiplicity reactivation, appears to have been partially excluded by the observation that multiplicity reactivation is not observed after P^{32} decay in labeled phage in multiply phage infected bacteria, whether the decay is allowed to take place before or after injection. It is still conceivable that multiplicity reactivation may be possible if the P^{32} decay takes place after certain essential functions have been performed by the intact DNA in the early minutes of infection. Stent's suggestion that the essential information is trans-

TABLE IV

MORPHOLOGY OF VARIOUS BACTERIOPHAGES

Phage	Head dimensions	Tail dimensions	Ref.
T1	50 mμ (hexagon)	150 × 10 mμ	*a*, *b*
T2, T4, T6	95 × 65 mμ (elongated hexagon)	100 × 25 mμ	*a*, *b*
T3, T7	47 mμ (hexagon)	15 × 10 mμ	*a*, *b*
T5	65 mμ (hexagon)	170 × 10 mμ	*a*, *b*
λ	55 mμ diameter	140 × 10 mμ	*c*
P1	86 mμ diameter	210 × 20 mμ	*d*
P2	66 mμ diameter	125 × 20 mμ	*d*
P22	50–60 mμ hexagon	"short, stubby"	*e*
φX174	25 mμ, pentagonal dodecahedron	None	*f*

References

[a] R. C. Williams, *Cold Spring Harbor Symposia Quant. Biol.* **18**, 185 (1953).
[b] R. C. Williams, *Advances in Virus Research* **2**, 183 (1954).
[c] E. Kellenberger, Thesis, Université de Geneva (1954).
[d] L. W. Labaw and V. M. Mosley, *J. Bacteriol.* **67**, 576 (1954).
[e] N. D. Zinder, *J. Cellular Comp. Physiol.* **45**, Suppl. 2, 23 (1955).
[f] C. E. Hall, E. C. Maclean, and I. Tessman, *J. Mol. Biol.* **1**, 192 (1959).

ferred to some structure insensitive to P^{32} decay provides a plausible, if controversial, explanation of this important observation.

III. Nucleic Acids of Other T Bacteriophages

1. Infection with Bacteriophage T5

Of the other T phages, T5 appears to be most similar in many respects to the T-even phages, while T1, T3, and T7 appear to comprise a distinct group.

T5 is similar in size and DNA content to the T-even phages (Tables I and IV), but differs from the latter most notably in that its DNA contains the more common pyrimidine, cytosine, instead of the unusual 5-hydroxymethylcytosine. Other differences include host range, antigenic composition,[171, 171a] and a requirement for calcium for injection.[172]

Despite the important difference in nucleotide composition, several features of the biochemistry[173] and radiobiology[6] of T5 infection have led to suggestions that T5 be grouped with the T-even phages as a distinctive type of bacterial virus. As with the T-even phages, infection with T5 brings about an extensive degradation of the host DNA.[173, 174] The

[171] Y. T. Lanni, *J. Bacteriol.* **67**, 640 (1954).
[171a] F. Lanni, *Science* **128**, 839 (1958).
[172] S. E. Luria and D. L. Steiner, *J. Bacteriol.* **67**, 635 (1954).
[173] L. V. Crawford, *Virology* **7**, 359 (1959).
[174] E. Pfefferkorn and H. Amos, *Virology* **6**, 299 (1958).

degradation appears to be more rapid than with the T-even phages and a marked decline in total cellular DNA is observed (Fig. 6). Fifty per cent of the host DNA is degraded in the first 4 minutes, and 70–80% by 10 minutes. During this time the T5 DNA is not degraded, as evidenced by retention of an adenine-C^{14} label for at least 20 minutes after infection. Curiously, this breakdown of host DNA does not seem to require the injection of the phage DNA as it proceeds in the presence of external citrate which blocks injection, and it is well under way within 4 minutes, while injection is not normally completed until 10 minutes after infection.

This degradation of the host DNA appears to require prior protein synthesis. It is inhibited or completely blocked by the addition at the time of infection of chloramphenicol, or tetracycline, or amino acid analogs, or by withholding the essential metabolite to infected auxotrophic coli strains requiring proline, adenine, or uracil. This requirement for protein synthesis can be rapidly satisfied, as a delay in the addition of chloramphenicol of only 1 minute after infection permits extensive, if retarded DNA degradation.

Following degradation of the host DNA, a rapid synthesis of presumably viral DNA is initiated at about 10 minutes after infection which results in a fivefold increase in total cellular DNA by the time of lysis. At its most rapid period the rate of DNA synthesis is 5 times that of the bacterial host.

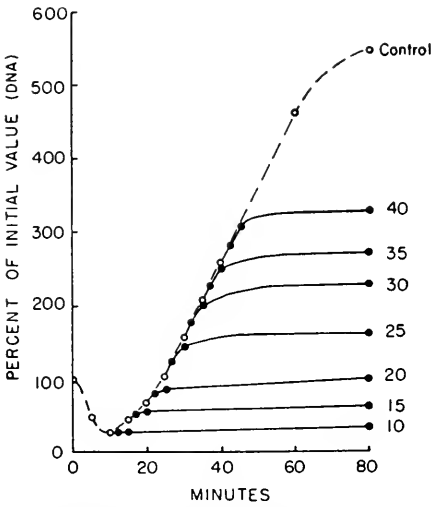

FIG. 6. The effect of the addition of chloramphenicol at various times (indicated on the right) during infection with bacteriophage T5 upon DNA synthesis in the phage-bacterium complex. [From L. V. Crawford, *Virology* **7**, 359 (1959).]

Unlike the observations made with the T-even phages, the DNA synthesis during T5 infection appears to require a continuing protein synthesis (Fig. 6). Addition of chloramphenicol after 10 minutes will completely block the development of DNA synthesis. Addition of chloramphenicol at any time during the stage of DNA synthesis will cause a complete halt after a lag of about 5 minutes.

Also, as with the T-even phages, infection with T5 appears to confer upon thymine-requiring cells the ability to synthesize thymine.[173, 175] This was demonstrated by an increase in DNA and specifically in thymine, in T5 infected cells, in a host strain normally requiring thymine, and upon infection in a thymine-free medium. Additional evidence was provided by the similarity of the burst size of T5 in such cells in media with or without thymine, by incorporation of label from aspartic acid-C^{14} or from serine-3-C^{14} into thymine in such cells during T5 infection (and the absence of such incorporation in uninfected cells), and by a marked decrease in incorporation of exogenous 2-thymine-C^{14} into the DNA of such cells upon T5 infection.

By the use of a reverted thymine-requiring mutant in which the synthesis of DNA in the absence (but not in the presence) of exogenous thymine was strongly temperature dependent in the region 37°–42°, Crawford[173] has demonstrated that the thymine-synthetic ability acquired upon infection appears to be distinct from that of the host in that it does not in this case have a temperature dependence in the 37°–42° range.

Kornberg *et al.*[77] have shown that the levels of thymidylic, deoxycytidylic, and deoxyguanylic acid kinase increase some ten- to fortyfold during T5 infection, while the initially high level of deoxyadenylic acid kinase increases twofold. These increases in activity begin to appear about 10 minutes after infection. Similar increases of thymidylic and deoxyguanylic acid kinases are observed during infection with T-even phages.

As with the T-even phages[176] a rise in deoxyribonuclease activity of bacterial homogenates is observed shortly after T5 infection. However, this may have little to do with the breakdown of host DNA as addition of citrate is observed to inhibit the rise in deoxyribonuclease activity without influencing the course of DNA degradation.

2. Infection with Bacteriophages T1, T3, and T7

Bacteriophages T1, T3, and T7 have a similar content of DNA (approximately one-third the DNA content of T2) which is, in each case, composed of similar proportions of the usual purines and pyrimidines (Table I). They have similar radiobiological properties (*vide infra*) and,

[175] L. V. Crawford, *Biochim. et Biophys. Acta* **30**, 428 (1958).
[176] R. E. Kunkee and A. B. Pardee, *Biochim. et Biophys. Acta* **19**, 236 (1956).

to the rather limited extent of available knowledge, a similar biochemistry of infection. Bacteriophages T3 and T7 are serologically related and and have a similar morphology (Table IV). T1 is serologically different and has a distinctive tail. Because of their common properties it has been suggested[6, 173] that they be considered as members of a common viral class.

The DNA of phage T7 has a molecular weight of about 14 or 15 $\times$ 10^6 (Meselson, unpublished data) by both density gradient analysis[67] and calculation from sedimentation velocity.[58] This result suggests the presence of three DNA molecules in each phage particle.

Net DNA synthesis during the process of infection with these phages is small. Putnam *et al.*[177] have shown with T7 that 60–90% of the phosphorus and the nitrogen of the DNA of the viral progeny is derived from bacterial nucleic acids formed before infection. A smaller proportion (40%) of the nitrogen of the phage protein is derived from preassimilated bacterial nitrogen.

If heavily P^{32}-labeled T1 or T7 phages are used for infection, a progressive stabilization of the complex to P^{32} decay is observed in the early stages of the phage development just as with the T-even phages.[8a]

According to Crawford,[178, 179] the small increase in DNA content during infection with T1, T3, or T7 is not blocked by the presence of chloramphenicol, even when administered shortly before infection. However, Stent[8a] believes that the increase in DNA observed during T1 infection in the presence of chloramphenicol to be a residual increment of host DNA as under these circumstances there is no stabilization of the complex to decay of P^{32} incorporated into the parental DNA until the chloramphenicol is removed.

Crawford has also suggested that, unlike the T-even, T5 group of phages, T1, T3, and T7 do not appear to confer upon the phage-bacterium complexes the ability to synthesize thymine.[175] However, the only evidence for this suggestion is that the small increase in DNA during infection with these latter phages is not observed if thymine-requiring bacteria are employed and thymine is not supplied. In view of the small amount (about 10%) of the DNA increase under normal conditions, this observation cannot be regarded as conclusive.

Amos and Magasanik[179a] have shown that uridine in the culture medium can serve as a major precursor of the thymidine of T1 without cleavage of the glycosidic link.

Crawford[173] has suggested that there is some continuation of net RNA synthesis after infection with the T1, T3, T7 group. No data of this nature has, however, been

[177] F. W. Putnam, D. Miller, L. Palm, and E. A. Evans, Jr., *J. Biol. Chem.* **199,** 177 (1952).

[178] L. V. Crawford, *Biochem. J.* **65,** 178 (1957).

[179] L. V. Crawford, *Biochim. et Biophys. Acta* **28,** 208 (1958).

[179a] H. Amos and B. Magasanik, *J. Biol. Chem.* **229,** 653 (1957).

presented as yet, while the results of Volkin *et al.*[131] indicate an absence of net RNA synthesis during T7 infection.

Watson and Maaløe[87] demonstrated that 30–40% of the phosphorus of parental T3 phage was transmitted to progeny phage. Mackal and Kozloff[180] similarly found a transfer of 20–30% of parental DNA phosphorus to progeny DNA phosphorus and a somewhat lesser (0.6–0.8×) transfer of parental DNA nitrogen to progeny DNA nitrogen. However, they observed that at any multiplicity of infection about 6–12% of the parental phosphorus appeared as nonsedimentable phosphorus in the medium, and that in mixed infection with live T7 and ultraviolet killed N^{15}-labeled T7, as much nitrogen was transferred to progeny as with live labeled T7. Because of these results, Mackal and Kozloff concluded that the transfer to progeny was largely a nonspecific transfer involving reincorporation into progeny DNA of degradation products of parental DNA.

3. Radiobiological Distinctions between the T1, T3, andT7 Group and the T5 Plus T-Even Group

Several lines of evidence[6] have led to the hypothesis that the T1, T3, T7 group is radiobiologically distinct from the T2, T4, T6, T5 group, and it has been suggested that this distinction may be the result of a partial genetic homology between the former group and the host genome. The possibility of such a homology is favored by the similarity of nucleotide composition of the DNA of this phage group and the DNA of the host cell.

Differences between these two groups exist with respect to such properties as ultraviolet sensitivity per milligram of DNA[6] (somewhat lower with the T1, T3, T7 group), multiplicity reactivation of ultraviolet damaged phage (very limited with the T1, T3, T7 group; very frequent with the T2, T4, T6, and T5 group),[47, 181] and the character of the changes of the ultraviolet sensitivity of the phage-bacterium complex during the early stages of infection[6, 88, 89] (with the T2, T4, T6, and T5 group the survival curves of the complex remain exponential, but with rapidly diminishing slope as infection proceeds; with the T1, T3, T7 group the survival curves quickly become multi-hit, with increasing multiplicity as the infection proceeds, but with a constant ultimate slope at high dose).

The two groups may also be distinguished with respect to the effect of ultraviolet irradiation on the capacity of the host cell to support their multiplication.[182, 183] The capacity of *E. coli* B to support the growth of

[180] R. P. Mackal and L. M. Kozloff, *J. Biol. Chem.* **209**, 83 (1954).
[181] I. Tessman and T. Ozaki, *Virology* **4**, 315 (1957).
[182] T. F. Anderson, *J. Bacteriol.* **56**, 403 (1948).
[183] E. S. Tessman, *Virology* **2**, 679 (1956).

members of the T2, T4, T6, and T5 group is extremely resistant to irradiation; it is very much more resistant than the capacity of the irradiated cells to form colonies. The capacity of *E. coli* B cells to support the growth of T1 or T3 is much more sensitive to ultraviolet irradiation, although still not as sensitive as the colony forming ability. Apparently phage of the latter group require a considerably greater contribution to the function of the phage-bacterium complex from the host genome than do phages of the T2, T4, T6, and T5 group.

The idea of a partial genetic homology between phages of the T1, T3, and T7 group and the host bacterium has been introduced to explain the relatively low ultraviolet sensitivity of members of this class (explained by a reconstitution of active phage during infection by recombination bebetween an ultraviolet damaged phage genome and an undamaged host genome), and to interpret the effect of ultraviolet irradiation of the host cell prior to infection, upon the apparent survival of phage irradiated with ultraviolet light prior to infection.[6, 183, 184]

With members of the T1, T3, T7 group the apparent survival of phage after a given ultraviolet dose is strongly dependent on the exposure of the host cells to ultraviolet irradiation prior to infection (Fig. 7). With a light dose of ultraviolet irradiation to the cells, the apparent phage survival is markedly increased. This is interpreted as a result of a "stimulus to recombination" by ultraviolet irradiation of the host, giving rise to an increased number of phage reactivations by recombination. This phenomenon of "ultraviolet reactivation" is not observed with members of the T2, T4, T6, and T5 group.

With increasing ultraviolet dose to the bacterial cell, however, the apparent phage survival, after a given ultraviolet dose, falls off extremely rapidly. Indeed, if the survival of ultraviolet-treated phage of this group is measured on heavily ultraviolet irradiated bacteria, their apparent sensitivity per milligram of DNA becomes comparable to that of members of the other phage group, T2, T4, T6, and T5.

These data are interpreted to mean that the apparent low sensitivity of members of this group to ultraviolet irradiation when tested upon normal bacteria is due to a considerable amount of phage reactivation by recombination with homologous regions of the bacterial genome. With low doses of ultraviolet light to the host cell, recombination and thus reactivation are facilitated. With increasing dose the region of the bacterial genome necessary for reactivation becomes damaged, and the ultraviolet sensitivity of the phage increases to the level it would have had in the absence of a reactivation process.

[184] A. Garen and N. D. Zinder, *Virology* **1**, 347 (1955).

IV. Nucleic Acids of Temperate Bacteriophages

1. The Lysogenic State

The viruses of the T series are all members of the "virulent" class of bacteriophage. These bacteriophages—and many others which prey upon a wide range of bacterial species—kill the host cells which support their replication. An entirely different type of parasitic relationship can be established by the members of another large class of bacteriophage, the "temperate viruses."

Upon infection of a susceptible bacterial culture by a temperate phage stock, a portion of the infected cells will be invaded and lysed as by the virulent phage. However, in another portion, which may under appropriate circumstances be much the larger portion, a different relationship is established. At some time after invasion, a genetic element carrying at least the hereditary characteristics of the parental phage particle is added to the genetic complement of the infected bacterial cell (or one of its progeny).

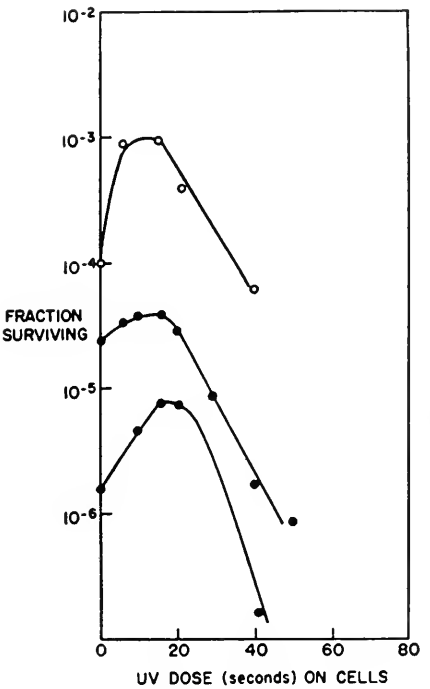

FIG. 7. Apparent survival of ultraviolet irradiated T1 (●) and T3 (○) phage upon ultraviolet irradiated host cells as a function of the dose administered to the cells. [From E. S. Tessman, *Virology* **2**, 679 (1956).]

This accessory element, to which has been given the name "prophage," once incorporated into the bacterial genome is then reproduced each time the bacterial genome is reproduced, and in this way is passed on to all the descendants of the modified cell. Such a bacterial culture, carrying a prophage, is said to be "lysogenic."[7, 185]

Analysis of bacterial matings involving cells carrying prophage indicates that these distinctive accessories are located at a specific site on the bacterial chromosome. Prophages from different species of temperate viruses are in general found at different sites on the chromosome. Some species of temperate phage (P_2) have apparently more than one possible site of incorporation. One cell may carry two or more prophages. If two are present they may be at the same site or at different sites.

The presence of the prophage invariably confers new properties upon the lysogenic cells. The most striking of these is the potential of the prophage to break free of metabolic control, to assume the status of vegetative phage, and to initiate an unrestricted virus synthesis eulminating in lysis and the release of many mature phage particles This process—the expression of the inherent phage potential—occurs spontaneously in lysogenic cell cultures at a low rate of the order of 10^{-4} per cell generation. Hence, such cultures always contain a significant amount of free phage particles.

Some temperate phages when carried in the lysogenic condition are "inducible"; the application of appropriate agents to a lysogenic culture of such phage will cause a transformation from prophage to vegetative phage in a large proportion (up to 90%) of the cells, resulting in a subsequent mass lysis.

It is of interest that the most effective agents for induction are also well known mutagens, including ultraviolet irradiation, X- or γ-irradiation, nitrogen mustards, and hydrogen peroxide.[185] Many species of temperate phage are, however, as prophage, resistant to all known modes of induction and can only be obtained as a consequence of spontaneous liberation.

The presence of prophage confers other properties upon the lysogenic cell. Such cells are almost invariably immune to attack by other phages of the same species. Immunity may also be produced to some unrelated phages. This immunity appears to be a physiological condition which prevents either the prophage or superinfecting phage from multiplying vegetatively.

In some instances the presence of prophage confers upon the cell an ability to make toxin,[186] or a change of colony form from smooth to rough,[187] or a change of somatic antigen.[188]

[185] A. Lwoff, *Bacteriol. Revs.* **17,** 269 (1953).
[186] N. B. Groman, *J. Bacteriol.* **69,** 9 (1955).

Simple mutations of many temperate phages are known which result in an extreme reduction of the ability to enter the prophage state. Cells infected by such mutants are almost invariably lysed. Such mutants are called "clear," as lambda$_{clear}$ (λ_c).

Another more complex class of mutants of temperate phages is known, the members of which invariably lyse the infected cells. These mutants can also overcome the immunity conferred upon a cell by the presence of the prophage from which they were derived. Such mutants are called "virulent," as lambda$_{vir}$ (λ_{vir}).

Relatively little is known as yet of the biochemistry of lysogenization or of induction and its consequent events. Even the nature of prophage, which is almost invariably assumed to be DNA, can scarcely be regarded as convincingly demonstrated.

2. Structural Aspects of Some Temperate Phages

Only "preliminary" analyses are available of the DNA of only three species of temperate bacteriophage (Table I). Since there are reasons to believe that the DNA of such phages bears some genetic homology to the DNA of their host cells,[6, 7] it is perhaps to be expected that these phage contain the usual deoxyribonucleotides found in the host. Whether the obvious deviations of the data available from the usual adenine-thymine and guanine-cytosine complementarity are to be attributed to the preliminary character of the data or represent a more fundamental feature remains to be decided.

The most detailed studies of the properties of temperate phage and the lysogenic state have been made with four species which attack various strains of *E. coli* and *Shigella*: lambda (λ), P1, P2, and P22.[7] Data concerning the composition and morphology of these phages are included in Tables I and IV.

3. Biochemistry of Lysogenization

The fraction of cells lysed as compared to the fraction made lysogenic by a particular temperate phage depends strongly upon the culture conditions including the medium, the age of the cells, the temperature, etc. In general, processes which lead to an inhibition of protein synthesis favor the establishment of the lysogenic state.[189, 190] Such inhibiting agents (chloramphenicol, 5-hydroxyuridine, amino acid analogs) are more effective if given some minutes after infection. It is believed that this delay permits vegetative replication of the phage; inhibition of protein synthesis then

[187] H. Ionescu, *Compt. rend. acad. sci.* **237**, 1794 (1953).
[188] N. D. Zinder, *Virology* **5**, 291 (1958).
[189] J. R. Christiansen, *Virology* **4**, 184 (1957).
[190] L. E. Bertani, *Virology* **4**, 53 (1957).

prevents an unknown but apparently irrevocable step toward lysis. If, at this time of inhibition, there are many vegetative phage components present in the cell, the chance of a successful lysogenization is apparently enhanced.[191]

A final decision between a lytic or lysogenic development is apparently not made until some time after infection. Thus with P1 the frequency of lysogenization is 0.8 if the cells are kept at 20° and 0.2 if the cells are kept at 37°.[7] If the cells are infected at 37°, however, they may be transferred to 20° at any time up to 20 minutes after infection and the 80% lysogenization will be obtained.

It is generally assumed, in the absence of direct evidence, that the DNA of temperate phage is injected into the host cell as with T2. Goodgal[192] has shown that the phosphorus of labeled temperate phage P1 is released in phage-free form if infected cells are lysed shortly after adsorption. He has also shown using phosphorus-labeled P1, that under conditions providing 80 to 90% lysogenization, over 75% of the phosphorus taken into the cells in the first few minutes remains in the cells for up to 8 generations of growth. He states that this phosphorus is present in the form of DNA.

Presuming that the DNA of the infecting phage enters the cell, various lines of evidence suggest that replication of the DNA occurs before a definite transformation to prophage is made. Levine[193] has presented evidence that genetic recombination may occur before formation of a definite prophage. Luria *et al.*[191] have shown by careful pedigree studies that a stable lysogenic state may not be achieved for several cell generations during which replication of the phage genome must occur. These studies indicate that after the "decision not to lyse"[188, 191] has been made, a loose association is established between one or two phage genomes and the bacterial genome. Then this replicates, occasionally throwing off either stable lysogenic cells, or nonlysogenic cells, or persisting for a protracted period as an unstable clone.

Stent and Fuerst[194] have used heavily P^{32}-labeled lambda phage and studied the sensitivity to phosphorus decay of phage-bacterium complexes at various times after infection. They demonstrated that both lytic and lysogenic centers are equally sensitive, indicating that comparable amounts of phage DNA are necessary to produce either response. They also demonstrated that by 15 to 25 minutes after infection both types of complex were completely resistant to decay of the P^{32} of the parental phage. This

[191] S. E. Luria, D. K. Fraser, J. H. Adams, and J. W. Buras, *Cold Spring Harbor Symposia Quant. Biol.* **23**, 71 (1958).
[192] S. H. Goodgal, *Biochim. et Biophys. Acta* **19**, 333 (1956).
[193] M. Levine, *Virology* **3**, 22 (1957).
[194] G. S. Stent and C. R. Fuerst, *Virology* **2**, 737 (1956).

result appears to demonstrate that the initial prophage structure, if it is DNA, is not composed of the DNA molecules of the parental phage. Siminovich[185] has indicated that DNA synthesis continues unaltered upon infection with lambda phage.

That the phage particle must carry out certain functions before lysogenization has been shown by the studies of temperate phage cooperation by Levine[193] and by Kaiser.[195] Mutant phages of P22 or of λ were obtained with reduced frequencies of lysogenization (clear mutants). These could be grouped into phenotypic classes according to the frequency of lysogenization. If mixed infections with phages from different phenotypic classes were carried out, a high frequency of lysogenization, characteristic of the wild-type phage, could be obtained, even though in many cases the prophage which became established was not a recombinant but one of the parental types. This result indicates a cooperative type of action preceding lysogenization analogous to the cooperation between phages mutant in the A and B cistrons of the *r*II region of T2 in infection of K12 (λ), as demonstrated by Benzer.[136]

Also, the new somatic antigen, which develops in *Salmonella typhimurium* infected by phage P22, can be detected within 6 to 8 minutes after infection.[188] At this time the cells become immune to superinfection with phage P22. It is of interest that even though, through segregation, many of the originally infected cells throw off nonlysogenic progeny, this immunity persists among such progeny for 8–10 generations. This result indicates that the direct presence of the prophage is not essential for immunity.[191]

Studies of the changes in the ultraviolet sensitivity of the phage-bacterium complex during both the lytic and the lysogenic responses following infection by a temperate phage have been made by L. E. Bertani.[196] In either case, ultraviolet sensitivity, which is initially similar to that of the free phage, decreases during the first 15–20 minutes by a factor of 5–10. In the lytic complexes the sensitivity then remains at this low level until lysis. In the lysogenic complexes, however, the sensitivity increases after 15 minutes of infection, reaching a maximum which is greater than that of free phage, or of established lysogenic cells, at about 60 minutes after infection. Not until 2 hours after infection do the lysogenized cells acquire the ultraviolet resistance characteristic of an established culture.

4. Nature of the Prophage

The most nearly definitive data upon the nature and size of the prophage may be derived from experiments of Stent *et al.*[197] When a bac-

[195] A. D. Kaiser, *Virology* **3**, 42 (1957).
[196] L. E. Bertani, *Virology* **7**, 92 (1959).
[197] G. S. Stent, C. R. Fuerst, and F. Jacob, *Compt. rend. acad. sci.* **244**, 1840 (1957).

terium carrying an inducible prophage (λ) is employed as the donor in a bacterial mating with a nonlysogenic recipient, the prophage will in most instances be induced upon passage into the recipient cell ("zygotic induction"[198]). If the donor cells have been heavily P^{32}-labeled by growth in strongly radioactive media, the prophage will, if it contains phosphorus, also be heavily labeled. The time of entry of the prophage into the recipient cell is known and aliquots of zygotes can be taken at various times after initiation of prophage entry—i.e., at various times during the induction process—and then stored to permit P^{32} decay. The effect of such decay in the zygote upon the induction process and the ultimate production of free temperate phage can then be measured. In this way, it is found that the prophage apparently does contain phosphorus and can be inactivated by P^{32} decay. When the prophage first enters the recipient cell it is inactivated at the same rate at which the free λ phage of the same specific P^{32} activity is inactivated. This result suggests that if the prophage contains DNA, it contains the same amount as the free phage, assuming an equal sensitivity to P^{32} decay in these dissimilar circumstances. During the zygotic induction process the sensitivity to P^{32} decay declines to complete insensitivity by 60 minutes after the start of the bacterial conjugation.

5. Biochemical Events Following Induction

Following induction of a lysogenic culture (by ultraviolet irradiation) RNA synthesis and protein synthesis continue and respiration increases, although at decreasing rates until lysis.[185, 199] In the 90 minutes preceding lysis at 27° (*Bacillus megaterium*) the RNA content and over all turbidity of the culture more than double. Adaptive enzymes [e.g., β-galactosidase in K12 (λ)[200]] can be formed at any time during the latent period. These observations are in distinct contrast to the parallel observations, during T-even phage infection, of the cessation of RNA and host enzyme synthesis,[99a] and the loss of capacity for enzyme induction.[201] However, cell division does not take place during the post-lysogenic induction period despite the "residual growth."

DNA synthesis is abruptly blocked by the ultraviolet dose employed for induction. The duration of the block depends upon the temperature, being less at higher temperature, and upon the ultraviolet dose employed, increasing with increasing dose. At 27° a dose of 1000 ergs/sq. mm. blocks DNA synthesis for 30 minutes of a 90 minute latent period (Fig. 8).

[198] F. Jacob and E. Wollman, *Compt. rend. acad. sci.* **239,** 317 (1954).
[199] L. Siminovitch and S. Rapkine, *Biochim. et Biophys. Acta* **9,** 478 (1952).
[200] L. Siminovitch and F. Jacob, *Ann. inst. Pasteur* **83,** 745 (1952).
[201] J. Monod and E. L. Wollman, *Ann. inst. Pasteur* **73,** 937 (1947).

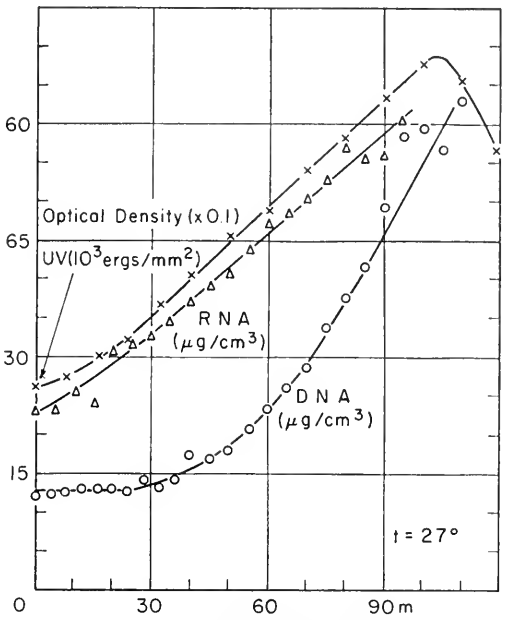

FIG. 8. Growth (optical density) and RNA and DNA synthesis at 27° after ultraviolet light induction of *B. megaterium*. [From L. Siminovitch and S. Rapkine, *Biochim. et Biophys. Acta* **9**, 478 (1952).]

Following this block, DNA synthesis resumes and increases exponentially achieving a rate greater than is observed in normal cells at the same temperature.

Since ultraviolet light of comparable dosage is known to block DNA synthesis in some bacteria,[202, 203] it is questionable whether this block represents a specific consequence of induction. Siminovitch and Rapkine report that a comparable dose to a related nonlysogenic bacterial strain produced no block of DNA synthesis, but the data presented are unconvincing.

It is of interest that exposure of induced lysogenic cells (*B. megaterium*) to ribonuclease specifically inhibits the synthesis of phage coat protein relative to total protein synthesis.[204] If ribonuclease is added immediately after induction, no phage antigens are ever made although cell growth (turbidity) continues at about one-third the normal rate. If the treatment with ribonuclease is delayed for increasing times after induction,

[202] A. Kelner, *J. Bacteriol.* **65**, 252 (1953).

[203] D. Kanazir and M. Errera, *Cold Spring Harbor Symposia Quant. Biol.* **21**, 19 (1956).

[204] R. Jeener, *Biochim. et Biophys. Acta* **32**, 106 (1959).

increasing amounts of phage protein and of intact phage are made by the time of lysis. Phage antigen does not begin to appear until 50 minutes after induction, but addition of ribonuclease at 25 or 40 minutes after induction cannot prevent production, at the later time, of some antigen.

An analysis of the total amount of phage antigen and total number of infective phage particles made by the time of lysis, as compared to the amount of antigen made by the time of addition of the ribonuclease, suggests that infective phage particles can be formed only from the phage protein made before the addition of the enzyme. Further analysis using P^{32}-labeling of the DNA suggests that phage antigen which is made at a reduced rate after ribonuclease addition is apparently defective; it can be assembled into phagelike particles but these cannot adsorb to the susceptible bacteria. This result may indicate an action of ribonuclease on the centers producing the phage proteins.

Measurements of the changes in the ultraviolet sensitivity of the phage-bacterium complex during the course of phage development following ultraviolet induction of *E. coli* C (λ) have been reported by Benzer and Jacob.[205] Immediately following completion of the inducing dose of ultraviolet radiation, the complex is more sensitive to additional ultraviolet radiation than is the free λ phage.

In contrast, if *E. coli* C is infected with λ phage, the ultraviolet sensitivity immediately after infection is the same as that of the free phage. With increasing time after induction, the ultraviolet sensitivity decreases but more slowly than is observed following direct infection of *E. coli* C with λ phage.

V. Nucleic Acids of Minute Bacteriophages

While the existence of a class of minute bacteriophages has been known for several decades, it is only within the past few years that any member of this class has been seriously studied. It is now clear that these phages comprise a group with quite distinct properties from those previously described.

The best known members of this group are S13[206, 207] and φX174.[208, 209] These phages are serologically related, have similar radiobiological properties, and appear from the early ultrafiltration and centrifuge studies to be of similar size. As yet only φX174 has been purified and subjected to physical and chemical study.[209]

Phage φX174 is but one-fortieth the size of the T2 phage and has about

[205] S. Benzer and F. Jacob, *Ann. inst. Pasteur* **84,** 186 (1953).
[206] D. E. Lea and M. H. Salaman, *Proc. Roy. Soc.* **B133,** 434 (1946).
[207] S. A. Zahler, *J. Bacteriol.* **75,** 310 (1958).
[208] V. Sertic and N. Bulgakov, *Compt. rend. soc. biol.* **119,** 1270 (1935).
[209] R. L. Sinsheimer, *J. Mol. Biol.* **1,** 37 (1959).

one-sixtieth the DNA content of T2 (Tables I and IV). The DNA of this phage does not have a complementary purine and pyrimidine composition, and a variety of lines of evidence have led to the conclusion that it is a single-stranded form of DNA.[210] This observation poses most interesting problems concerning its mode of replication.

As yet little is known concerning the biochemistry of infection of phages of this type. Kozinski and Syzbalski[211] using both P^{32} and density labeling (by extensive substitution of thymine with 5-bromouracil, phages of higher density are obtained) have demonstrated an extensive transfer (50%) of phosphorus from parental to progeny phage, with, however, apparent dispersal of the material of the parental phage among the progeny. No progeny phage showing any of a variety of distinctive properties of the parents could be found.[212]

The results seem to preclude the presence of intact parental DNA molecules among the progeny. However, the original experiments concerning the transfer of P^{32} from parent to progeny are subject to criticism as the multiplicity of phage particles (as contrasted to the multiplicity of plaque-formers) was unknown and thus the apparent transfer of P^{32} may have been entirely from defective phages in mixed infection with viable phages.

The radiobiological properties of ϕX174 differ considerably from those of phages previously described. The sensitivity of this phage to decay of incorporated P^{32} at 4° is 10 times that of other phages; every P^{32} decay inactivates.[209, 213]

The capacity of host cells to support the growth of the related phage S13 and the colony-forming ability of the cells are, under certain growth conditions, quite close in their sensitivity to ultraviolet irradiation (E. S. Tessman, personal communication, 1959), indicating that replication of the phage requires the function of a major portion of the host genome. Ultraviolet reactivation, an increase in the apparent survival of ultraviolet irradiated phage if they are plated on lightly ultraviolet irradiated host cells, is also observed with S13.

[210] R. L. Sinsheimer, *J. Mol. Biol.* **1**, 43 (1959).

[211] A. W. Kozinski and W. Szybalski, *Virology* **9**, 260 (1959).

[212] Nonradioactive phage were grown on heavily P^{32}-labeled bacteria in heavily P^{32}-labeled media. No progeny resistant to P^{32} decay, as would be progeny incorporating the intact nonradioactive DNA of the parent, were found. Dense, 5-bromouracil labeled phage were grown in cells in normal media lacking 5-bromouracil. No progeny with the density of the parental phage were found. Normal phage were grown in 5-bromouracil cells in media containing 5-bromouracil. No progeny with the relatively low sensitivity to ultraviolet light of the parental phage (substitution with 5-bromouracil increases the ultraviolet sensitivity of this phage) were found.

[213] I. Tessman, *Virology* **7**, 263 (1959).

VI. Conclusion

It is clear that the bacterial viruses include particles of a wide range of size and morphological complexity, with a corresponding diversity of biochemical, genetic, and radiobiological properties. Four major groups of bacteriophage have been described: the T-even group plus T5, the T1, T3, plus T7 group, the temperate phages, and the minute phages, and each group can be readily subdivided.

Our knowledge of the biochemistry of infection and the associated genetic and radiobiological phenomena is most advanced with respect to the first group. Despite the complex nature of the T-even plus T5 group, as it has been progressively revealed, it seems certain that much further progress will be made with these phages toward the understanding of virus infection and the correlation of genetic and biochemical phenomena. It must be said, however, that much of our knowledge of the biochemistry of infection with this group of phages is concerned, for obvious reasons, with the unique and highly specialized feature of the unusual pyrimidine of the T-even phages.

Since all of these groups are viruses, it seems plausible that there is a basic sequence of biochemical events common to infection with all of them. As yet knowledge of such a basic pattern is meager. Such problems as the manner in which the invading DNA assumes direction of the cellular metabolism, how it displaces the host DNA as the pattern for DNA synthesis, and how it is preserved from degradation in those instances in which the host DNA is extensively disrupted, are as yet quite obscure. Add to these problems such special features as the manner of the creation and preservation of the lysogenic state by the temperate phages, and the manner of replication of the single-stranded DNA by the minute phage, and it is clear that the bacterial virus systems still present remarkable opportunities for biochemical exploration. The morphogenetic problems implicit in the complex structure of such phage as the T-even group have yet even to be approached on the molecular level.

Considering the significance of the problems mentioned, it is apparent that the study of the processes of bacterial virus infection offers a particularly favorable route to analysis of the most basic problems of cellular and genetic biochemistry.

CHAPTER 34

The Ribonucleic Acids of Viruses

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I. Introduction

In 1944 Avery, MacLeod, and McCarty[1] demonstrated, for the first time, that deoxyribonucleic acid (DNA) isolated from bacteria was capable of transmitting a specific biological property: DNA obtained by extraction from an encapsulated strain of *Streptococcus pneumoniae* was able to transform noncapsulated cells into capsulated ones. Several years later, Hershey and Chase[2] demonstrated that bacteriophage DNA penetrates into the host cell after adsorption of the virus to the cell and that this DNA can be transmitted to the daughter virus particles.

The studies of these two groups of workers have resulted in an important experimental confirmation of the thesis that DNA is the genetic material

[1] O. T. Avery, C. M. MacLeod, M. McCarty, *J. Exptl. Med.* **79**, 137 (1944).
[2] A. D. Hershey and M. Chase, *J. Gen. Physiol.* **36**, 39 (1953).

of a cell or virus. One might suspect that ribonucleic acid (RNA) might also serve as genetic material since many types of viruses are known which contain RNA as the sole nucleic acid component. This assumption was experimentally verified by the isolation of biologically active RNA from tobacco mosaic virus (TMV) by Gierer and Schramm,[3] and by Fraenkel-Conrat.[4]

Virus RNA can be biologically distinguished from cellular RNA through the infectivity of the former. Both cellular and virus RNA are concerned with protein synthesis. The relationship between RNA and protein synthesis has already been treated in detail by Brachet (Chapter 28). At that time, however, our knowledge concerning the size and structure of biologically active RNA was still rather slight. Only very recent studies on viral RNA have given us some idea of the properties which an RNA molecule must possess in order to function as a biologically active agent in a living cell. Therefore, in this presentation, the author will emphasize the relationship between constitution and biological activity of RNA.

II. Viruses Which Contain Ribonucleic Acid; General Remarks

Viruses can be distinguished from microorganisms chiefly by their smaller size and the absence of metabolic activity outside the host cell. These properties are reflected in the relatively simple structure of viruses as compared with cells. The smallest viruses contain only nucleic acid and protein, but the composition becomes increasingly complex for larger viruses where carbohydrates and lipids may also be present.

Both types of nucleic acid, DNA and RNA, have been found in viruses although it appears that any one virus type contains only one sort. Early reports on the coexistence of both types of nucleic acid in the same virus have not been confirmed in more recent work employing greatly improved techniques of viral purification. This is especially true for animal viruses. For example, recent analyses of highly purified influenza virus have shown that only RNA is present in this virus,[5, 6, 7] whereas earlier studies report the presence of both DNA and RNA.[8, 9]

[3] A. Gierer and G. Schramm, *Nature* **177**, 702 (1956); *Z. Naturforsch.* **11b**, 138 (1956).
[4] H. Fraenkel-Conrat, B. Singer, and R. C. Williams, *Biochim. et Biophys. Acta* **25**, 87 (1957).
[5] L. Hoyle, *4th Intern. Congr. Biochem., Vienna 1958* Symposium No. VII (1958).
[6] G. L. Ada, *in* "The Nature of Viruses" (G. E. W. Wolstenholme and E. C. P. Miller, eds.), p. 104. Churchill, London, 1957.
[7] W. Schäfer, *in* "The Nature of Viruses" (G. E. W. Wolstenholme and E. C. P. Miller, eds.), p. 104. Churchill, London, 1957.
[8] H. K. Miller, *Virology* **2**, 312 (1956).
[9] D. C. Burke, A. Isaacs, and J. Walker, *Biochim. et Biophys. Acta* **26**, 576 (1957).

TABLE I

RNA-CONTAINING PLANT VIRUSES

Virus	Particle weight ($\times 10^{-6}$)	RNA content (% of the particle weight)	RNA "molecular weight"[a]
Spherical			
Turnip yellow mosaic	5	34	1.7×10^6
Tomato bushy stunt	10.65	16.5	1.65×10^6
Southern bean mosaic	6.6	21	1.4×10^6
Tobacco necrosis	8.0	18	1.45×10^6
Tobacco ringspot	3.4	44	1.5×10^6
Rod-shaped			
Tobacco mosaic virus	40	5–6	2.2×10^6
Potato X	36–39	5–6	2.0×10^6

[a] The RNA content of one virus particle expressed as "molecular weight."

1. PLANT VIRUSES

All plant viruses which have been subjected to a detailed chemical analysis have proved to contain only RNA. The form and chemical composition of these viruses is relatively simple since they can be classified as either spherical or rod-shaped. All are composed of only RNA and protein, and may be considered to be nucleoproteins. Many of these viruses have been crystallized so that it is possible to subject such viruses to structure analysis by X-ray crystallography.

In Table I, the sizes and RNA content of some familiar plant viruses are presented. It will be noted that the individual viruses have a highly variable percentage of RNA. However, if the RNA content is compared with the particle weight of the individual viruses, it may be shown that the total RNA per particle corresponds to an RNA of "molecular weight" $1.5–2 \times 10^6$.[10] It will be shown that this quantity represents the biologically active unit of RNA for the case of tobacco mosaic virus (TMV).[11]

The structure of the virus nucleoprotein has been deduced chiefly on the basis of X-ray analysis.[12-15] The RNA of the spherical viruses is centrally located within a spherical shell of protein. In rod-shaped viruses, the RNA

[10] W. Frisch-Niggemeyer, *Nature* **178**, 307 (1956).
[11] A. Gierer, *Nature* **179**, 1297 (1957); *Z. Naturforsch.* **13b**, 485 (1958).
[12] R. E. Franklin, A. Klug, J. T. Finch, and K. C. Holmes, *4th Intern. Congr. Biochem., Vienna 1958* Symposium No. VII (1958).
[13] A. Klug, J. T. Finch, and R. E. Franklin, *Biochim. et Biophys. Acta* **25**, 242 (1957).
[14] F. H. C. Crick and J. D. Watson, *Nature* **177**, 473 (1956).
[15] D. L. D. Caspar, *Nature* **177**, 475 (1956).

TABLE II

BASE COMPOSITION OF THE RIBONUCLEIC ACID OF VARIOUS PLANT VIRUSES

Source of nucleic acid	Bases of nucleic acid (mole % nucleotide)				Reference
	Adenine	Guanine	Cytosine	Uracil	
Turnip yellow mosaic	22.6	17.2	38.1	22.1	*a*
Tobacco ringspot	23.9	24.7	23.2	28.2	*b*
Tomato bushy stunt					
BS 3	25.7	27.9	20.8	25.7	*c*
BS 9	25.7	28.2	20.5	25.5	*c*
BS 10	25.9	28.1	20.4	25.6	*c*
Southern bean mosaic	25.8	26.0	23.0	25.3	*d*
TMV normal	29.8	25.3	18.5	26.3	*e*
Aucuba mosaic	29.7	25.4	18.5	26.4	*e*
Rib grass	29.3	25.8	18.0	27.0	*e*
Potato X	34.2	21.8	22.8	21.3	*d*

[a] R. Markham and J. D. Smith, *Biochem. J.* **46**, 513 (1950).
[b] J. M. Kaper and R. L. Steere, *Virology* **7**, 127 (1959).
[c] D. de Fremery and C. A. Knight, *J. Biol. Chem.* **214**, 559 (1955).
[d] R. W. Dorner and C. A. Knight, *J. Biol. Chem.* **205**, 959 (1953).
[e] C. A. Knight, *J. Biol. Chem.* **197**, 241 (1952).

is centrally located and is embedded in a cylindrical protein shell. The internal location of RNA in rod-shaped viruses is in accord with the fact that the virus particle is not inactivated by ribonuclease, whereas isolated infectious RNA is rapidly destroyed. Since the spherical plant viruses are also resistant to ribonuclease, the argument must hold in this case also.

Although different viruses have different percentages of nucleic acid, the various strains of one type of virus have the same percentage of RNA. This has been shown to be true for TMV[16, 17] and tomato bushy stunt virus,[18] for example. The base ratios of RNA from different strains of the same virus are also approximately the same, whereas the base ratios are quite different for RNA from different viruses (Table II).

In a comparison of RNA from 5 strains of TMV, Reddi and Knight[19] found a ribonuclease-resistant residue after 36 hours of treatment with ribonuclease. After precipitation of this residue with 6% trichloroacetic acid, it was possible to show that the residue had the same base composition in all 5 cases. The residues were rich in purine and deficient in pyrimidine and had an average length of 6 nucleotides. It

[16] C. A. Knight, *J. Biol. Chem.* **197**, 241 (1952).
[17] W. D. Cooper and H. S. Loring, *J. Biol. Chem.* **211**, 505 (1954).
[18] D. de Fremery and C. A. Knight, *J. Biol. Chem.* **214**, 559 (1955).
[19] K. K. Reddi and C. A. Knight, *J. Biol. Chem.* **221**, 629 (1956).

may be concluded that the nucleic acids of different strains possess certain oligonucleotides of identical composition. The identical composition of the different ribonuclease-resistant cores does not necessarily imply a structural identity, meaning identical nucleotide sequences. Residues of identical composition may have quite different nucleotide sequences. Further, the linkage of these oligonucleotides with each other in the original nucleic acid could be quite different in the different species of RNA; some may be joined together by uracil or cytosine mononucleotides and others by smaller pyrimidine oligonucleotides. Thus, Reddi[20] observed that the amount of pyrimidine mononucleotides released upon ribonuclease digestion was approximately identical for 3 strains of TMV, whereas the amount released by a fourth strain was significantly larger by some 40%. It may be concluded that the nucleic acid of the latter strain (strain M) can be distinguished from the other 3 strains with respect to the intramolecular distribution of pyrimidine mononucleotides.

The mononucleotide arrangement in the nucleic acids of some TMV strains must be something more complex than the mere repetition of one simple pattern involving only a few residues as was made clear by comparing the numbers and types of split products obtained by Reddi and Knight[19] with those expected from the degradation of a hypothetical "random" RNA under the same conditions. This hypothetical RNA has the same relative number of adenylic, cytidylic, guanylic, and uridylic residues as do the RNA's of the viral strains studied. The sequence of these residues along the hypothetical nucleotide polymer is assumed to be completely random. Hart[21] calculated that the core composition for the viral nucleic acid digest did not differ from that expected for a random polymer.

In contrast to the studies which have been discussed so far, Commoner and Basler[22] found different RNA contents and different base ratios for more than 50 separate preparations of one strain of TMV. These authors claimed that the values were influenced by the duration of infection and the nature of the tissue from which the virus was isolated. However, the analytical method could not completely exclude impurities, such as foreign protein and a maximum of 5% nonviral RNA. Since RNA from tobacco leaves has a markedly different adenine and uridine content than TMV-RNA,[23] a small amount of cellular RNA could be responsible for the reported variable base ratios of TMV-RNA.

2. Animal Viruses

There is also a variable percentage of RNA in those animal viruses that contain the ribose type of nucleic acid. Several typical RNA animal viruses are listed in Table III. A simple calculation shows that the absolute amount of RNA in all these viruses, with one exception, corresponds to a "molecular weight" of 2×10^6.[10] The exception is Newcastle disease virus which also seems to have a much higher particle weight than the other viruses listed in Table III. On the other hand, the purity of the Newcastle disease virus preparations which have been analyzed is questionable.[23a]

[20] K. K. Reddi, *Biochim. et Biophys. Acta* **25**, 528 (1957).
[21] R. G. Hart, *Proc. Natl. Acad. Sci. U.S.* **43**, 457 (1957).
[22] B. Commoner and E. Basler, *Virology* **2**, 477 (1956).
[23] K. K. Reddi, *Biochim. et Biophys. Acta* **23**, 208 (1957).
[23a] W. Schäfer, *in* "The Viruses" (F. M. Burnet and W. M. Stanley, eds.), Vol. I, p. 475. Academic Press, New York, 1959.

TABLE III

THE PARTICLE WEIGHT AND RNA CONTENT OF SOME ANIMAL VIRUSES

Virus	Particle weight	RNA content (in % of the particle weight)	RNA "molecular weight"[a]
Poliomyelitis	6.7×10^6	22–30	$\sim 2 \times 10^6$
Equine encephalitis	50×10^6	4.4	$\sim 2 \times 10^6$
Myxoviruses			
Influenza type A	280×10^6	0.7–1.0	$\sim 2 \times 10^6$
Fowl plague	150×10^6	1.8	$\sim 2 \times 10^6$
Newcastle disease	800×10^6	4	$\sim 32 \times 10^6$

[a] Compare footnote in Table I.

TABLE IV

BASE COMPOSITION OF THE RNA OF VARIOUS ANIMAL VIRUSES[a]

Source of nucleic acid	Bases of nucleic acid (mole % nucleotide)			
	Adenine	Guanine	Cytosine	Uracil
Poliomyelitis	30.4	25.4	19.5	24.7
Influenza A				
PR 8	23.1	20.1	24.0	32.8
MEL	23.0	19.8	25.3	32.0
WSE	22.5	20.1	24.1	33.3
Swine	22.8	20.4	24.5	32.4
CAM	22.8	19.3	24.5	33.5
Influenza B				
LEE	23.0	18.3	23.1	35.6
MIL	22.8	17.5	23.8	36.0
ROB	22.5	18.5	23.5	35.5

[a] Taken from W. Schäfer, in "The Viruses" (F. M. Burnet and W. M. Stanley, eds.), Vol. I, p. 475. Academic Press, New York, 1959.

Until recently, it was most difficult to prepare sufficient quantities of purified animal viruses. Improvements in methods of cultivation and purification have made it possible to determine the RNA content and base composition of several animal viruses. The pertinent data are presented in Table IV for poliovirus and influenza virus. The RNA of unrelated serotypes, influenza A and B, seems to have different base compositions; B strains have less guanine and more uracil than A strains. It is not clear whether these differences are highly significant and, therefore, Ada[6] com-

pared the ratio adenine + uracil/guanine + cytosine for RNA[24] from A and B influenza. The average values were 1.25 for type A and 1.40 for type B. This ratio is 0.6 for the RNA in an alkaline digest of uninfected host cells. This would mean that even a minute contamination of the virus preparation with host cell RNA would result in a significant alteration of the base ratio of viral RNA. The base ratios of various strains of one serotype are, however, approximately the same in all cases studied so far.

In summary: there are no qualitative differences between viral and host cell RNA. Rare bases, such as the methylated bases found in cellular RNA,[25] have not been observed in viral RNA. Considering the accuracy of the analytical methods used, it seems safe to state that no significant differences have been found, to date, in the base compositions of RNA from related virus strains. In contrast, large differences have been found in the base ratios of RNA from different types of viruses. The fact that the absolute amount of RNA contained in a single virus particle corresponds to a "molecular weight" of 2×10^6 or perhaps a multiple of this value (in the case of Newcastle disease virus, for example) suggests that a certain minimal "amount" of RNA is necessary in order to be biologically active.

III. The Ribonucleic Acids of Plant Viruses

Since it is much easier to purify plant viruses than animal viruses and since the virus yield of the former is much greater than the latter, plant viruses present certain advantages as objects for chemical studies. For example, a liter of sap from infected plants can contain up to 2 gm. of virus,[26] whereas the amount of purified poliovirus which can be harvested from a liter of infected tissue culture fluid is less than 1 mg.[27] Thus, it is clear that it is quite possible to obtain larger amounts of virus RNA from plant viruses.

TMV is the virus of choice for most studies on plant viruses. It was the first virus to be obtained in pure form as paracrystalline needles,[28] and further, in comparison to other plant viruses, it is relatively simple to obtain in rather large quantities. Using TMV, it was also possible, for the first time, to separate the two components, RNA and protein, in such a way that the isolated RNA was still biologically active. This observation led to a series of studies on the structure, physical, chemical, and biological

[24] D. Elson and E. Chargaff, *Nature* **173**, 1037 (1954).
[25] J. W. Littlefield and D. B. Dunn, *Biochem. J.* **70**, 642 (1958).
[26] G. Schramm, *in* "Die Biochemie der Viren" (H. Bredereck and E. Müller, eds.), p. 135. Springer, Berlin, 1954.
[27] C. E. Schwerdt and F. L. Schaffer, *Virology* **2**, 665 (1958).
[28] W. M. Stanley, *Science* **81**, 644 (1935).

properties of TMV-RNA and, therefore, it is worthwhile to consider this compound in some detail.

1. Tobacco Mosaic Virus Ribonucleic Acid

a. Arrangement of the Ribonucleic Acid in Tobacco Mosaic Virus

TMV has also been studied in great detail with respect to structure, both of the virus particle and of the RNA and protein components. The virus particles are rods about 3000 Å.[29, 30] long, with a packing diameter of about 150 Å.[31] Essentially uniform particles may be best obtained by differential centrifugation.[32, 33, 34] The RNA content per particle may be determined by ultraviolet (UV) absorption after removal of the protein[35] or, more exactly, by determining the phosphorus content. A recent determination of the P content made by Knight and Woody[36] gave a value of 0.45% as an average of 19 samples, leading to a value of 5.1% RNA. The remainder of the particle is protein.

The structure of the virus particle has been revealed in great detail by X-ray crystallographic analysis. Figures 1 and 2 show a schematic representation of the structure of the virus protein and RNA. In order to obtain the structure of the intact virus particle, one must conceive of the two figures superimposed so that the particle axes are the same. It was shown that the virus protein is in the form of a helical array of structural equivalent subunits, the pitch of the helix being 23 Å.[37] The whole particle has a rather deep helical groove following the line of the main protein helix[38] (packing diameter = 150 Å., maximum diameter ~180 Å.). The radial electron density distribution, which is approximately proportional to the mass density for biological substances consisting mainly of light atoms, has its strongest maximum at a radial distance of 40 Å. for intact virus particles,[39] whereas RNA-free repolymerized *A* protein[40] has a pronounced minimum at just that position. This strong density maximum at 40 Å. in TMV must

[29] R. C. Williams and R. L. Steere, *J. Am. Chem. Soc.* **73**, 2057 (1951).
[30] C. E. Hall, *J. Am. Chem. Soc.* **80**, 2556 (1958).
[31] J. D. Bernal and I. Fankuchen, *J. Gen. Physiol.* **25**, 111 (1941).
[32] H. K. Schachman, *J. Am. Chem. Soc.* **73**, 4808 (1951).
[33] I. Watanabe and Y. Kawade, *Bull. Chem. Soc. Japan* **26**, 294 (1953).
[34] H. Boedtker and N. S. Simmons, *J. Am. Chem. Soc.* **80**, 2550 (1958).
[35] G. Schramm, H. Dannenberg, and H. Flammersfeld, *Z. Naturforsch.* **3b**, 241 (1948).
[36] C. A. Knight and B. R. Woody, *Arch. Biochem. Biophys.* **78**, 460 (1958).
[37] J. D. Watson, *Biochim. et Biophys. Acta* **13**, 10 (1954).
[38] R. E. Franklin and A. Klug, *Biochim. et Biophys. Acta* **19**, 403 (1956).
[39] D. L. D. Caspar, *Nature* **177**, 928 (1956).
[40] G. Schramm, *Z. Naturforsch.* **2b**, 112, 249 (1947).

be due, therefore, to the presence of RNA at this radius.[41] The density minimum at 40 Å. in RNA-free virus protein is no broader or deeper than other minima in the radial density distribution for the protein moiety. It seems, therefore, that the RNA must be fitted in a very compact way into the structure of the virus protein. From Fig. 1,[42] it may be seen, further, that the virus particle has a hollow core of radius about 20 Å. The RNA is deeply embedded in the protein, following the protein helix in the form of a *single* molecular chain with the same pitch of 23 Å. and a diameter of 80 Å. (Fig. 2[43]). There are about 50 bases per turn of helix, aligned parallel to the longitudinal axis of the virus particle. The length of the RNA helix, calculated from its diameter and pitch, and from the whole length of the

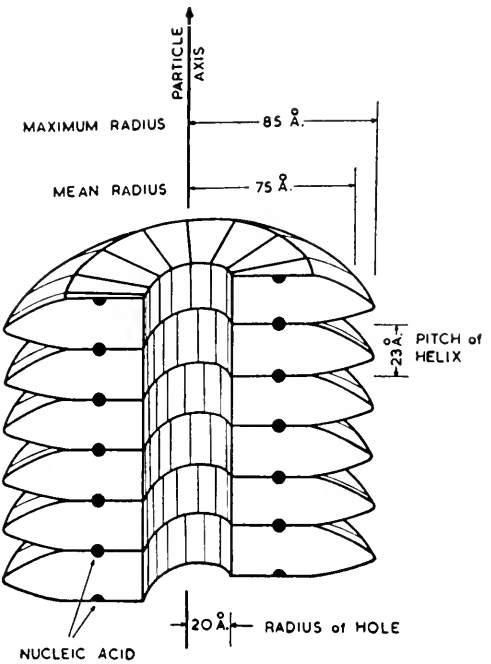

FIG. 1. Schematic representation of a short length of the TMV particle cut in half along a plane through the particle axis, showing the helical arrangement of protein subunits, the helical groove with its accompanying helical ridge, and the hollow axial core. [R. E. Franklin, A. Klug, and K. C. Holmes, *in* "The Nature of Viruses" (G. E. W. Wolstenholme and E. C. P. Miller, eds.), p. 39. Churchill, London, 1957.]

[41] R. E. Franklin, *Nature* **177**, 929 (1956).

[42] R. E. Franklin, A. Klug, and K. C. Holmes, *in* "The Nature of Viruses" (G. E. W. Wolstenholme and E. C. P. Miller, eds.), p. 39. Churchill, London, 1957.

[43] W. Ginoza, *Nature* **181**, 958 (1958).

virus (3000 Å.), is 33,000 Å. A nucleic acid chain of 2×10^6 molecular weight conforms with this length, assuming a normal spacing of about 5 Å. between P atoms along the helix. TMV-RNA occurs, therefore, as a single chain with an "open" arrangement of its bases. This is in contrast to DNA in which every purine base is coupled via H bonds to a pyrimidine base in the complementary helix.[44] It is unlikely that H bonds may link bases from

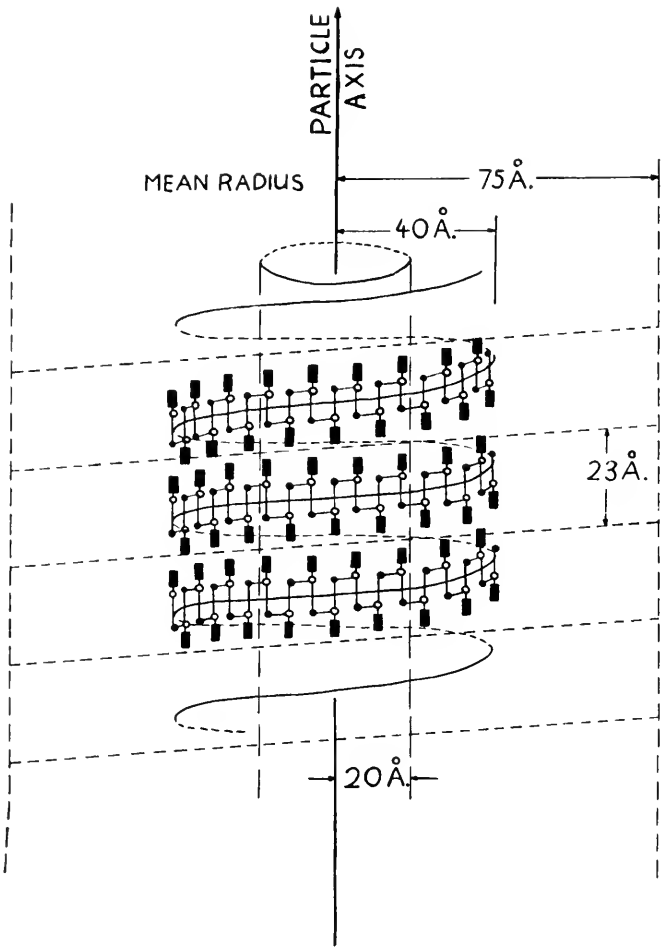

FIG. 2. Schematic representation of a single-strand ribonucleic acid helix of 40 Å. radius in position within the TMV rod. The spacings of the purine and pyrimidine bases, ribose rings, and phosphorus atoms with respect to the helix are made approximately to scale; ● = phosphorus, ○ = sugar, ■ = bases. [W. Ginoza, *Nature* **181**, 958 (1958).]

[44] J. D. Watson and F. H. C. Crick, *Nature* **171**, 737 (1953).

neighboring pitches to each other in the RNA spiral. It is interesting that the radial electron density distribution is similar in four strains of TMV and in cucumber virus 4.[45] The strains differ from one another only in the relative heights of some peaks in protein density, that is, in the weights of protein lying at particular radii.

The results obtained by X-ray analysis have been supported by other studies. Schramm *et al.*[46] were able to show that TMV is gradually degraded at alkaline pH to protein and RNA. In this case, a 2% TMV solution is treated with 0.05 *M* sodium bicarbonate buffer at pH 10. Protein is gradually removed from the intact virus particle so that the naked RNA thread can be observed in the electron microscope.

Similar results were obtained by Hart[47] who treated TMV with the anionic detergent, Duponol C, at an elevated temperature. In this case, the protein seems to unravel from the end of the rod, since shortened rods may be seen in the electron microscope. RNA fibers project from the end of these shortened rods. Further heating of the virus in the presence of Duponol resulted in a shortening and finally in a disappearance of the rods accompanied by the appearance of naked fibers, many of which were 3000 Å. long. After treatment of the detergent-treated virus with ribonuclease, only shortened rods, without any projecting fibers, could be found. This demonstrated that the fibers were most likely the viral RNA.

Schramm and Hart estimated the diameter of the RNA fiber visible in the electron microscope to be 30–40 Å., a value too small compared with that obtained by X-ray crystallography. However, one must remember that the drying of preparations for electron microscopic studies may lead to an unavoidable shrinkage of the RNA fiber, especially since the virus particle has a hollow core. It is possible to observe the core by treating TMV with uranyl acetate[48, 49] which diffuses into the core. The heavy metal thus produces an electron dense core.

The structural studies indicate that the viral RNA possesses a definite "superstructure," namely a single helical chain resulting from its location within the protein coat, which also protects the RNA from enzymic degradation.

In the following chapters, it will be shown how the RNA may be freed from this nucleoprotein complex without loss of biological activity. Furthermore, the molecular structure of such RNA in solution will be discussed.

[45] K. C. Holmes and R. E. Franklin, *Virology* **6**, 328 (1958).
[46] G. Schramm, G. Schumacher, and W. Zillig, *Z. Naturforsch.* **10b**, 481 (1955).
[47] R. G. Hart, *Viroloyg* **1**, 402 (1955); *Proc. Natl. Acad. Sci. U.S.* **41**, 261 (1955).
[48] H. E. Huxley, *Proc. Stockholm Conf. on Electron Microscopy*, **1956** p. 260 (1957).
[49] H. Fernández-Morán and G. Schramm, *Z. Naturforsch.* **13b**, 66 (1958).

b. Preparation of Biologically Active Ribonucleic Acid

Since it has not been possible to test the biological activity of RNA isolated from plant or animal tissues, no definite criteria exist, as yet, as to the relationship of the isolated nucleic acid to that found within the cell. The recent discovery of a well-defined biological activity of at least one type of cellular RNA, the so-called "soluble RNA"[50] and the possibility of isolating this RNA, has led to one approach to the solution of this problem.

The situation is much simpler when one turns to virus RNA since the biological activity of such RNA, the production of certain diseases with well-defined symptoms, is well known. If the biological activity is due solely to the RNA component, one must seek suitable methods to isolate such RNA so that the biological activity remains intact.

Earlier procedures for the isolation of RNA from nucleoproteins, such as treatment with alkali, hot 10% NaCl,[51] guanidine-HCl[52] (see Chapter 11 for a detailed description) do not meet the requirements for isolating a "natural" RNA molecule. Even special techniques formerly employed for isolating TMV-RNA, such as heating the virus in NaCl at neutral pH[53, 54] or splitting the nucleoprotein with $Sr(NO_3)_2$,[55] are not useful owing to the sensitivity of RNA. The splitting of TMV with 67% acetic acid has been employed to obtain native virus protein, but the RNA obtained, thereby, has a very low biological activity.[56] TMV has also been split with amino alcohols,[57] but the applicability of this procedure is not known.

Two methods will be described in some detail since they meet the requirements described above. The phenol method was developed by Zillig *et al.*[58] for the splitting of TMV nucleoprotein. It has proved a most useful method for the preparation of RNA, not only from viruses, but also from plant and animal tissues. The second method, employing detergents, had been used for splitting nucleoproteins for some time. Fraenkel-Conrat[4] employed this technique to isolate an infectious RNA. The phenol method seems to be superior to the detergent method with regard to ease of use and general applicability.

[50] M. B. Hoagland, P. C. Zamecnik and M. L. Stephenson, *Biochim. et Biophys. Acta* **24**, 215 (1957).
[51] J. N. Davidson and C. Waymouth, *Biochem. J.* **38**, 375 (1944).
[52] E. Volkin and C. E. Carter, *J. Am. Chem. Soc.* **72**, 1516 (1951).
[53] S. S. Cohen and W. M. Stanley, *J. Biol. Chem.* **144**, 589 (1942).
[54] C. A. Knight, *J. Biol. Chem.* **197**, 241 (1952).
[55] N. W. Pirie, *Biochem. J.* **56**, 83 (1954).
[56] H. Fraenkel-Conrat, *Virology* **4**, 1 (1957).
[57] P. Newmark and R. W. Myers, *Federation Proc.* **16**, 226 (1957).
[58] H. Schuster, G. Schramm, and W. Zillig, *Z. Naturforsch.* **11b**, 339 (1956).

(1) *Phenol Method.* Westphal *et al.*[59] treated bacteria with a warm homogeneous phenol-water mixture followed by separation of the two phases. Bacterial protein was dissolved in the phenol phase, and nucleic acid and polysaccharide in the aqueous phase. This method proved well-suited, with slight modification, for splitting TMV and isolating an infectious RNA of high molecular weight. The detailed procedure is as follows.

A 3–6% TMV solution in 0.01–0.05 *M* phosphate buffer, pH 6–7, is shaken vigorously for 10–15 minutes in the presence of an equal volume of 80% phenol. After a short centrifugation at low speed, the emulsion is separated into a lower phenol phase containing all of the protein and an upper aqueous phase containing all of the RNA. The aqueous phase is treated with phenol at least twice, in this case by shaking for 5–10 minutes in the presence of a small volume of phenol. The phenol dissolved in the aqueous phase is removed by repeated extraction with peroxide-free ether, and the ether is removed with N_2 gas. The RNA dissolved in the aqueous phase can be used directly for biological tests, or for other chemical and physical studies, or may be precipitated with 2 volumes of alcohol (with the addition of several drops of 1 *M* acetate buffer to facilitate the precipitation), or with 2 *M* NaCl (1 part to 1 part of the RNA solution) prior to use. RNA precipitated by these methods and then redissolved shows no loss in activity.

If no effort is made to have a quantitative yield, the entire procedure can be performed in about an hour. For quantitative extractions, it is sufficient to remove, quantitatively, the aqueous phase immediately after separation of the two phases. This may be accomplished by washing the phenol phase with buffer or water.

TMV-RNA prepared in this manner is biologically active with an infectivity some 0.1–0.5% of that of an equivalent amount of RNA in the intact virus.[3] The advantages of the phenol method are quite obvious. The splitting of nucleoprotein can be performed at low temperatures without employing extreme pH values. The direct contact with phenol leads to an immediate denaturation of any enzymes which may be present, in particular ribonuclease. The denatured enzymes are dissolved in the phenol phase. Thus, the phenol method is generally applicable for the isolation of RNA and especially in those cases where the presence of enzymes, which may degrade RNA, is unavoidable.

If it is necessary to separate nucleic acid from polysaccharides which are simultaneously extracted, as is the case for extraction from complex animal viruses or from tissues, one may precipitate the RNA with NaCl. According to Kirby[60] it is also possible to separate RNA from polysaccharides by extraction with 2-methoxy-ethanol from a solution containing potassium phosphate.

(2) *Detergent Methods.*[4] Four volumes of a 1% solution of TMV containing 10^{-4} *M* Versene is warmed to 50°C., adjusted to pH 8.5 at that temperature, mixed with 1

[59] O. Westphal, O. Lüderitz, and F. Bister, *Z. Naturforsch.* **7b**, 148 (1952).
[60] K. S. Kirby, *Biochem. J.* **64**, 405 (1956).

volume of 5% sodium dodecyl sulfate (Duponol C), and is held at 50 ± 1°C. for 5 minutes. Then the solution is rapidly cooled to 20°C. and brought to 33% saturation by the addition of saturated ammonium sulfate. After a few minutes, the protein precipitate is separated by centrifugation and the supernatant is held in a refrigerator overnight to permit the macromolecular nucleic acid to precipitate. The precipitate is then centrifuged, redissolved in water and reprecipitated with two volumes of cold ethanol with the addition of a few drops of 3 *M* acetate buffer at pH 5 to facilitate flocculation. The alcohol precipitation is repeated and the final aqueous solution is freed from aggregated material and any contaminating virus by ultracentrifugation.

The infectivity of the most active preparation amounted to 0.1–0.3% of the theoretical value, and is, therefore, of the same order of magnitude as in the case of RNA prepared by the phenol method.

(3) *Infectivity Due to the Ribonucleic Acid Component.* The proof that the infectivity obtained after the two extraction methods described is actually due to RNA and not to intact virus particles still present in the preparations, can be obtained in a series of tests. The effect of ribonuclease upon the preparations is the simplest test, since ribonuclease destroys free RNA in a very short time, whereas the activity of intact virus is not influenced. The opposite effect can be obtained with virus antisera which rapidly neutralize intact virus particles without influencing the infectivity of RNA. The marked difference in the sensitivity of virus and virus RNA solutions to heat treatment can also be applied as a test. TMV is relatively heat stable, whereas RNA solutions loose their activity, at least after several days. Finally, the marked difference in the sedimentation constants for virus and virus RNA can be employed as a further control. All these tests, together, have been used to show that the activity of TMV-RNA preparations obtained by either the phenol or the detergent method is due to the nucleic acid alone. On the other hand, well-known analytical tests must be employed to determine the degree of purity of the RNA preparations.

(4) *Protein Content of Infectious Ribonucleic Acid.* It is most important to know the protein content of preparations of infectious RNA in order to exclude the possibility that the infectivity of the preparation is due to the presence of intact or only partially deproteinized virus particles.

(i) *Protein content of phenol RNA.* The protein content of RNA extracted with phenol is less than 2% when measured with the Sakaguchi reaction, and less than 0.4% when measured with a modified biuret reaction.[61] In order to detect smaller quantities of protein, an RNA preparation was hydrolyzed with 1 *M* alkali at 20°C. The nucleotides arising in this hydrolysis were removed by dialysis and the nondialyzable fraction was subjected to acid hydrolysis and tested for the presence of amino acids by paper chromatography. Aspartic acid, glutamic acid, glycine, and serine could be demonstrated in an amount corresponding to approximately 40 μg. of a

[61] R. Markham and J. D. Smith, *Biochem. J.* **46**, 509 (1950).

TMV hydrolyzate which had been treated in an analogous fashion. From the quantity of RNA employed in this experiment, it was possible to calculate a protein content of 0.25%.[58]

The serological test for native TMV protein is significantly more sensitive than the color reactions and chemical methods for protein determination. An impurity amounting to 0.02% protein may be detected using TMV antiserum, and this type of test showed that the content of native TMV protein in the RNA preparations was less than 0.02%.[3]

(ii) *Protein content of detergent-extracted RNA.* Fraenkel-Conrat *et al.*[4] found an upper limit of protein contamination of 0.5% for detergent-extracted RNA using the microbiuret test of Holden and Pirie.[62] After hydrolysis of RNA preparations, a protein content of approximately 0.06% was calculated from the amino acid content. The fact that those amino acids which make up a large percentage of the virus protein were not found in this analysis led to the conclusion that these trace amounts of amino acids are not in the form of viral protein. Protein determination using the phenol reagent[63] gave an estimate of 0.04–0.4%.[64]

These analytical studies show that the infectivity of the RNA preparations must clearly be due to the RNA alone, and the presence of viral protein can be definitively excluded. The presence of small amounts of amino acids or peptides cannot be excluded.

(5) *Other Constituents.* The diphenylamine test shows that there is less than 0.1% DNA in preparations of infectious RNA. It is safe to say that there is no contamination with DNA since a microbiological analysis of thymidine shows that the DNA content is less than 0.001%.[65]

Loring *et al.*[66, 67] studied the metal content of virus and virus RNA preparations. Whereas the content of Cu, Ca, and Mg ions varied, according to the methods of purification, the concentration of iron remained relatively constant, amounting to 8 mg. per 100 gm. of phenol-extracted RNA, and to 1–2 mg. per 100 gm. of detergent-extracted RNA. It was suggested that metal chelates or relatively stable metal complexes appear in the RNA. The significance of metal ions for RNA infectivity was not studied.

[62] M. Holden and N. W. Pirie, *Biochem. J.* **60**, 46 (1955).

[63] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).

[64] L. K. Ramachandran and H. Fraenkel-Conrat, *Arch. Biochem. Biophys.* **74**, 224 (1958).

[65] H. Fraenkel-Conrat and B. Singer, *4th Intern. Congr. Biochem., Vienna, 1958* Symposium No. VII (1958).

[66] H. S. Loring and R. S. Waritz, *Science* **125**, 646 (1957).

[67] H. S. Loring, S. Al-Rawi, and Y. Fujimoto, *J. Biol. Chem.* **233**, 1415 (1958).

c. Size and Structure of the Infectious Ribonucleic Acid

It was shown, in the previous chapters, that TMV has an RNA content of 5% which corresponds to a molecular weight of 2×10^6 for a particle weight of 40×10^6 for the entire virus. An RNA molecule of this size must consist of at least 6,000 mononucleotides of average molecular weight 320. This value is obtained from the average molecular weight of all 4 nucleotides (338) less one mole of water (18).

The RNA obtained by phenol or detergent extraction of TMV is biologically active. It is interesting to consider whether the entire RNA contained in the TMV particle is necessary for the biological activity or whether only part of this is needed. In other words, what is the minimum size of an RNA molecule isolated from TMV which is still capable of initiating an infection? Gierer[11, 68] studied this question by investigating the sedimentation and viscosity, as well as the kinetics of ribonuclease degradation, of infectious RNA. He found that the total amount of RNA contained in a virus particle is necessary for the biological activity. Therefore, RNA molecules of a molecular weight smaller than 2×10^6 are biologically inactive.

(1) *Determination of Molecular Weight from Sedimentation and Viscosity Measurements.* Stanley and Cohen[53] isolated a nucleic acid from TMV by a short heat treatment in NaCl solution, and this RNA had a molecular weight of approximately 300,000. It was, however, unstable and decomposed further to units of molecular weight of approximately 60,000. Light-scattering measurements on RNA, also prepared by heating a TMV solution, gave a molecular weight of 1.7×10^6 under certain conditions of extraction.[69]

The RNA prepared from TMV by the phenol method is not homogeneous. In the analytical ultracentrifuge, it is possible to observe a homogeneous component of high molecular weight (component *A*) as well as an inhomogeneous component of smaller average molecular weight (component *B*).[11, 68] It has not yet been possible to determine whether this smaller component is also present in intact virus, or whether it arises from degraded virus particles, or during the preparation of the RNA by degradation of larger RNA molecules. The inhomogeneity of this component suggests that it arises through degradation. The infectious RNA sediments have the same velocity as component *A*, as could be demonstrated by testing supernatant and sediment for infectivity after varying degrees of centrifugation.

It is possible to determine the molecular weight from the sedimentation constant and viscosity of a particular substance. Employing such data, the

[68] A. Gierer, *Z. Naturforsch.* **13b,** 477 (1958).

[69] G. R. Hopkins and R. L. Sinsheimer, *Biochim. et Biophys. Acta* **17,** 476 (1955).

molecular weight of component A has a value of 1.7×10^6 which is in agreement with the value obtained by light-scattering. However, the molecular weight of the infectious material, component A, must be larger than 1.7×10^6 since the value employed for the viscosity in the calculation of molecular weight is actually too small owing to contributions from the inhomogeneous smaller components (B). In order to determine the molecular weight of component A from the sedimentation constant of this component, it is necessary to investigate the general relationship between sedimentation constant and viscosity of various RNA preparations from TMV-RNA of quite different molecular weights. Figure 3[68] shows this relationship between sedimentation constant and viscosity for different RNA preparations obtained by incubating phenol-extracted RNA with ribonuclease for different lengths of time. It may be seen from Fig. 3 that $[\eta]$ is approximately in-

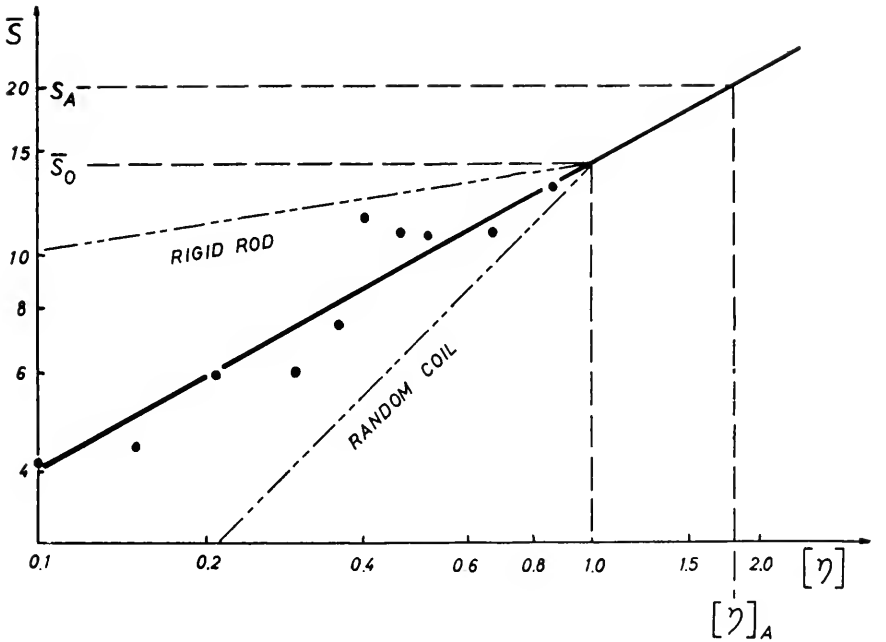

FIG. 3. Relationship between the sedimentation and viscosity constants of TMV-RNA for different molecular weights. Average sedimentation constants $\bar{s}$ are given in Svedberg units and viscosity constants $[\eta]$ are given in relative units by setting $[\eta]_0 = 1$. A 0.2% solution of RNA in 0.02 M phosphate buffer at pH 7.3 was used. From the value $[\eta]_A$, it may be deduced that for the RNA component having a sedimentation constant s_A, the viscosity is a 1.8 multiple of $[\eta]_0$. [A. Gierer, *Z. Naturforsch.* **13b**, 477 (1958).]

versely related to $\bar{s}^2$. The viscosity corresponding to a component of sedimentation constant s_A may be obtained from the equation

$$[\eta]_A/[\eta] = (s_A/\bar{s})^{1.8} \quad (1)$$

It was found, experimentally, that the sedimentation constant of component A (s_A) is 1.4 times as large as the average $\bar{s}$, the sedimentation constant of the entire RNA preparation. Thus, a viscosity 1.8 times as large as the average viscosity of components A and B corresponds to the viscosity of the infectious molecule. From this empirical relationship, it was possible to calculate a molecular weight of 2×10^6 for the infectious RNA.

(2) *Determination of Molecular Weight from Kinetics of Degradation with Ribonuclease. From the correlation between decrease in molecular weight and infectivity due to the action of ribonuclease.* The average molecular weight of an RNA preparation is approximately proportional to its viscosity. The infectivity of the RNA is approximately proportional to the number of local lesions which appear on leaves of *Nicotiana glutinosa* plants upon infection with RNA. A typical example of the measured decrease in infectivity (I), viscosity [η], and mean molecular weight (m) is given in Table V. The decrease in I is much faster than that in m. This indicates that the molecular weight of the infectious component (M) is larger than m. Using the quantitative relationship derived by Gierer[11]

$$1/m_1 - 1/m_2 = \frac{1}{2M} (\ln I_1 - \ln I_2) \quad (2)$$

it is possible to calculate a molecular weight of 2.2×10^6 employing the values given in Table V.

From the correlation of decrease in infectivity with the splitting activity of

TABLE V

THE ALTERATION OF INFECTIOUS TMV-RNA BY TREATMENT WITH RIBONUCLEASE[a]

t (minutes)	[η]	m	I (lesions)
0	300	1.2×10^6	980
5	274	1.1×10^6	786
11	254	1.02×10^6	383
17	232	0.93×10^6	487
22	214	0.86×10^6	272
28	196	0.78×10^6	242
37	168	0.67×10^6	62
65	115	0.46×10^6	1

[a] Decrease of intrinsic viscosity, [η], mean molecular weight (weight average) m, and infectivity, I, with time t. The decrease was induced by 1.5×10^{-3} µg./ml. ribonuclease at 22°C. in a solution of 0.16% ribonucleic acid in 0.02 M phosphate buffer, pH 7. The infectivity, I, is given as the number of lesions per 25 leaves of *Nicotiana glutinosa*, inoculated with RNA. [Taken from A. Gierer, *Nature* **179**, 1297 (1957); *Z. Naturforsch.* **13b**, 485 (1958).]

the enzyme. Since the splitting of RNA, as well as the inactivation, starts without delay upon addition of ribonuclease, both processes can be considered to be single-hit. If the enzymic splitting of the RNA backbone occurs statistically with a probability (α) per bond and per unit of time, and if the biologically active unit consists of N such bonds, the decrease in infectivity (I) with time (t) may be represented by an equation

$$\ln (I/I_0) = N\alpha t \tag{3}$$

(with I_0 = infectivity at time $t = 0$, I = infectivity at time t). When one internucleotide bond has been broken per infective unit by the action of ribonuclease, the infectivity is reduced by a factor e. The molecular weight can be calculated if the activity of the enzyme, expressed as the proportions of bonds split per unit of enzyme concentration and time, is measured under the conditions used for the infectivity test.

The enzymic activity can be determined by the titration of acid phosphate groups released upon splitting, as well as by the increase in UV absorption at 260 mμ which is always observed upon degradation of RNA molecules. Empirically, it was found that the appearance of one acid group per 22 nucleotides (molecular weight = $22 \times 320 = 7000$) corresponds to an increase in UV absorption of 15%. In order to obtain an equivalent increase in absorption in the time in which the infectivity decreases to a value $1/e$, it is necessary to increase the ribonuclease concentration 260 times of that necessary for the measured decrease in infectivity. From these data, Gierer[11] calculated the number of bonds or number of nucleotides as 5700, corresponding to a molecular weight for the infectious RNA molecule of $5700 \times 320 = 1.8 \times 10^6$.

Thus, it was possible, using quite a variety of techniques, to ascribe a molecular weight of approximately 2×10^6 to the infectious RNA within the limited accuracy of the determinations. This value corresponds to the total RNA content of the virus particle. The genetically active substance seems to be associated with approximately 6000 nucleotides which are bound to each other. The experiments presented, so far, do not exclude the possibility that certain sections of this chain may possess no biological function. This question will be discussed later (see Section III, 1, *e*).

Furthermore, it has not yet been proved that all 6000 nucleotides are bonded to each other through covalent bonds (phosphoric acid diester bonds between neighboring nucleosides). Sensitive areas in the polynucleotide chain, for example, labile phosphotriester bonds, are not excluded. However, if subunits of the RNA should exist, these can be biologically active only when they are all bonded together in the specific arrangement of an RNA molecule of molecular weight 2×10^6.

(3) *Chemical Approaches to the Structure of RNA*. It has not yet been

possible to determine the molecular weight or chain length of ribonucleic acids by purely chemical techniques analogous to those successfully employed with proteins and peptides. This is easily understood considering that a nucleic acid of high molecular weight is much larger than protein molecules are and significantly more labile. Furthermore, no homogeneous high molecular RNA has been prepared to date on a preparative scale. Homogeneity or uniformity in molecular size is a prerequisite for structural studies.

If it is assumed that the molecular ratio of base:ribose:phosphoric acid = 1:1:1 is preserved in a high molecular weight polynucleotide and that the nucleotides in the chain are held together by phosphoric acid diester bridges between carbons 3′ and 5′ of adjacent sugar residues, then two types of polynucleotides are possible which are differentiated merely by the position of the singly esterified phosphoric acid on the end of the chain (Fig. 4). According to the findings of Brown and Todd,[70] compound (II), on hydrolysis with alkali, splits as indicated by the dotted lines to yield a nucleoside diphosphate from one end, a nucleoside from the other end, and n nucleoside monophosphates. Compound (I) yields $n + 2$ nucleoside monophosphates and no nucleoside or nucleoside diphosphate (see Chapter 12). After alkaline degradation of TMV-RNA, which had been prepared by heat denaturation or phenol treatment, only nucleotides but no nucleosides or nucleoside-diphosphates could be demonstrated by paper electrophoresis.[71] The limit of sensitivity for the determination of nucleoside di-

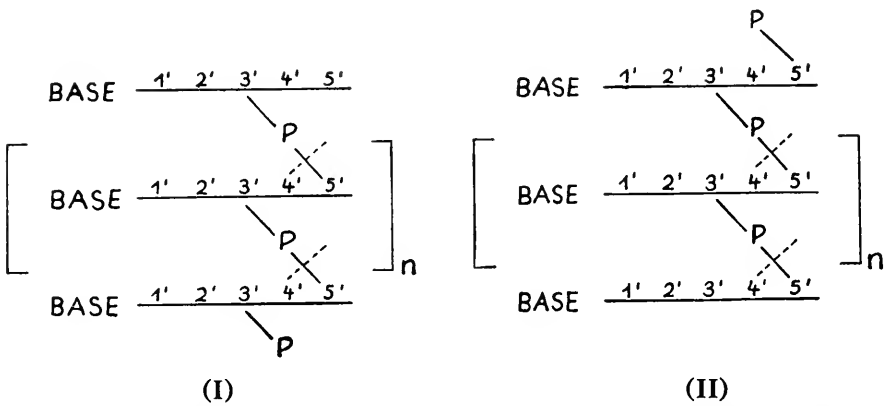

FIG. 4. Schematic representation of RNA structures. A further RNA type may be derived from (I) in which the terminally located phosphoric acid is cyclically linked to both the C-2′ and C-3′ of the terminal ribose.

[70] D. M. Brown and A. R. Todd, *J. Chem. Soc.* p. 52 (1952).
[71] K. K. Reddi and C. A. Knight, *Nature* **180**, 374 (1957).

phosphates by paper electrophoresis is about 1 diphosphate per 500 nucleotides. The nucleosides and nucleoside diphosphates, which had been found in earlier studies after alkali degradation of TMV-RNA,[72] could not be demonstrated after the virus was dialyzed at 37°C. versus 0.1 *M* citrate buffer at pH 7 to remove any adsorbed polynucleotides prior to the extraction of RNA.[73]

In the case of an RNA of mean molecular weight $1–2 \times 10^6$, it is most difficult, if not impossible, to decide whether the RNA is of type (I) or (II). For example, in order to detect one micromole of nucleoside or nucleoside diphosphate, it would be necessary to employ 1–2 gm. of RNA. This indicates how difficult it is to determine the molecular weight of a high molecular weight RNA by chemical methods such as "end-group" analysis.

(4) *Properties of Ribonucleic Acid in Solution. Dependence of RNA structure on ionic strength and pH.* TMV-RNA in solution has the typical properties of a polyelectrolyte. The molecular shape is strongly dependent on the medium, especially on the H ion and salt concentration. Low pH values and high salt concentrations decrease repulsion between charged PO^- groups of the RNA molecule, and hence, increase molecular coiling and association; the association may develop end to end as well as side by side. This effect leads to an increase in sedimentation velocity and a decrease in viscosity. The opposite situation holds for RNA in a solution free of ions or in a solution of very low ionic strength, where the viscosity is extremely high due to the mutual repulsion of PO^- groups, which leads to a stretching of the molecules. The sedimentation constant is accordingly much lower. Although infectious RNA prepared with detergents has a much lower sedimentation constant ($s_{20} = 6–10$ *S*)[4] than that prepared with phenol ($s_{20} > 20$ *S*),[68] it is most likely that the molecular weights are identical when the above mentioned effects are considered since the detergent-extracted RNA was studied in nonionic solution.

Further, as to be expected for a polyelectrolyte, the sedimentation constant of RNA increases with decreasing solute concentration since the mutual interaction of the polymer particles decreases with decreasing concentration.

The viscosity $[\eta]$ is approximately proportional to the average molecular weight and to the 1.8 power of the sedimentation constant in 0.02 *M* phosphate buffer (see Fig. 3). Now in the case of a random coil, viscosity is directly proportional to the sedimentation constant, whereas for a rigid rod a reduction in molecular weight leads to a marked decrease in viscosity, approximately as the square of molecular weight with little change in

[72] R. Markham, R. E. F. Matthews and J. D. Smith, *Nature* **173,** 537 (1954).
[73] R. E. F. Matthews and J. D. Smith, *Nature* **180,** 375 (1957).

TABLE VI

ALTERATION OF THE ULTRAVIOLET ABSORPTION ON DEGRADATION OF TMV-RNA

Substrate	Treatment	Increase in UV absorption at 260 mμ (%)
TMV-RNA	1 *M* NaOH, 8 hours, 37°C.	32
TMV-RNA	Leaf ribonuclease, 8 hours, 37 C.	37
TMV-RNA	Pancreatic ribonuclease 12 hours, 37°C.	15
TMV-RNA "core"	1 *M* NaOH or leaf ribonuclease, 12 hours, 37°C.	21

sedimentation constant. The relationship between $[\eta]$ and s for RNA lies somewhat between these two extremes, suggesting that RNA in solution is a flexible structure, intermediate between a random coil and a rigid rod.

UV absorption. The sum of the absorption at 260 mμ of the component nucleotides of an RNA molecule is higher than the absorption of the undegraded RNA molecule. This so-called hyperchromic effect can be strikingly demonstrated with TMV-RNA.[68, 74] The increase in absorption of TMV-RNA after various treatments is presented in Table VI. The RNA was obtained, in this case, by heat denaturation of TMV. Alkali degradation or treatment with leaf ribonuclease causes a similar increase in UV absorption. This is not unexpected since leaf ribonuclease disrupts all bonds between component nucleotides of RNA, in contrast to pancreatic ribonuclease.[75] Indeed, the increase in UV absorption obtained after incubation of TMV-RNA with pancreatic ribonuclease is less than half that obtained after alkali degradation.

The results with RNA obtained by heat denaturation are quite different from those using RNA extracted with phenol. After alkali degradation of the latter, a 30% increase in UV absorption was observed, as was also true in the former case.[76] But after incubation of phenol-extracted RNA with pancreatic ribonuclease for only ½ hour, the increase in UV absorption was more than 25% (see Fig. 5). Gierer[68] concluded from this observation that the change to higher absorption must occur when the average polynucleotide size has decreased to about 20 nucleotides; therefore, the effect may be connected with the structure of larger RNA molecules in solution.

If the residue resistant to pancreatic ribonuclease ("core") is treated with NaOH or leaf ribonuclease, there is an increase in absorption of 20% which accounts for 60%

[74] K. K. Reddi, *Biochim. et Biophys. Acta* **27**, 1 (1958).
[75] K. K. Reddi, *Biochim. et Biophys. Acta* **28**, 386 (1958).
[76] H. Schuster, unpublished work (1958).

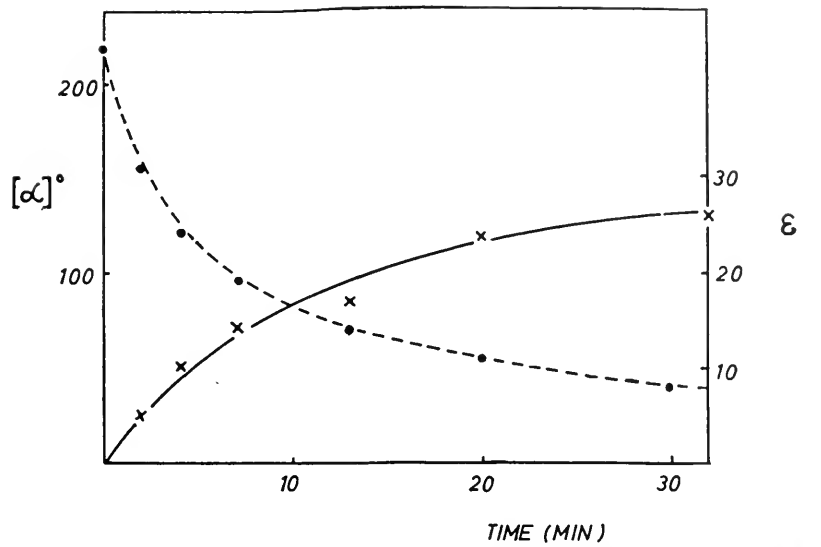

FIG. 5. Alteration of the optical rotation and ultraviolet absorption of TMV-RNA by ribonuclease degradation; ● = rotatory constant $[\alpha]^0$ at 546 mμ, × = UV absorption ϵ at 260 mμ (in per cent of starting material). [A. Gierer, *Z. Naturforsch.* **13b**, 477 (1958).]

of the total hyperchromic effect of the intact molecule. From this observation, Reddi[74] concluded that the molecular size is not critical for the hyperchromic effect since the core has an average chain length of only 6 nucleotides. He suggested that the chemical composition (purine-rich core) may influence the hyperchromic effect. This effect may also be seen in synthetic oligonucleotides[77] from dinucleotides to polynucleotides of an average chain length of 13. It is probably essentially due to the interaction of π-electrons of adjacent rings. Electron orbitals extending over more than one unit are formed, and these are oriented perpendicular to the planes of the ring systems stacked in layers above each other. This represents a new electronic species. Such bond interaction between bases exists even at the dinucleotide level. Since the ionizable groups participate directly in the electron system giving rise to the absorption, this interaction also has an effect on the ionizable groups which is expressed in a displacement of the pK value of mononucleotides, in contrast to oligonucleotides.

Optical rotation. High molecular weight RNA, in comparison to smaller polynucleotides, has a very high optical rotation (Table VII) which is larger than that due to the sum of the nucleotides.[68] This property cannot be due to the individual nucleotides or to the internucleotide bonds, but rather to a structure found in larger RNA molecules in solution.

Ribonuclease degradation causes a decrease in the optical rotation which is scarcely observable during the early drop in viscosity, as is also true for

[77] A. M. Michelson, *Nature* **182**, 1502 (1958).

TABLE VII

Optical Activity of TMV-RNA[a]

Material	Wavelength (mμ)	Specific rotation $[\alpha]^\circ$
TMV-RNA	589	+180°
TMV-RNA	546	+210°
TMV-RNA	436	+400°
Degraded TMV-RNA	546	+20°

[a] Taken from A. Gierer, *Z. Naturforsch.* **13b**, 477 (1958).

the hyperchromic effect. Only after a longer incubation with enzyme, corresponding to that resulting in an increase in UV absorption, is there a decrease in optical rotation. These two effects are shown in Fig. 5 as a function of the time of incubation with enzyme. From a comparison of both curves, one may observe an obvious parallelism in the two effects.

Birefringence. In DNA, according to the Watson-Crick model, the purine and pyrimidine rings are at right angles to the length of the molecule. Since there is a negative birefringence for DNA,[78] and a positive birefringence for TMV-RNA as well as for intact TMV,[79] it is likely that the bases in RNA are parallel to the long axis of the molecule. The magnitude of the birefringence suggests a highly ordered structure.

d. Constitution and Biological Activity

The above sections have discussed the evidence that isolated RNA from TMV is infectious, and that only an RNA molecule having a molecular weight of approximately 2×10^6 is capable of initiating TMV infection, whereas smaller fragments are biologically inactive. We now turn to the problem of macromolecular RNA as a structure capable of transmitting genetic function. It is possible to investigate the problem of alterations in genetic functions induced by controlled alterations in structure and composition and to see how one may alter the molecule without loss of function. It may be possible to distinguish between alterations which lead to no changes in biological activity, those resulting in a complete loss of function, and those resulting in an altered function, leading to mutant forms.

(1) *Action of Physical Agents. The action of X-rays.* RNA isolated from TMV which has been X-irradiated prior to the isolation of the nucleic acid has a viscosity which is lower than that of RNA isolated from unirradiated control TMV.[80] This suggests that the lethal action of X-rays is due to a

[78] M. H. F. Wilkins, R. G. Gosling, and W. E. Seeds, *Nature* **167**, 759 (1951).
[79] R. E. Franklin, *Biochim. et Biophys. Acta* **18**, 313 (1955).
[80] M. A. Lauffer, D. Trkula, and A. Buzzell, *Nature* **177**, 890 (1956).

splitting of the RNA molecule. The inactivation of TMV-RNA in the dry state or in the frozen state follows first-order kinetics.[81] The X-ray dose for 37 % survival ($1/e$ dose) is the same for TMV-RNA and for TMV, amounting to 3.0×10^5 r., and suggests that the X-ray inactivation of intact TMV is due to alterations in the nucleic acid component. The "radiosensitive molecular weight" for RNA, corresponding to a dose of 3.0×10^5 r. is $2.1–4.0 \times 10^6$. A single X-ray induced split is sufficient to inactivate the molecule. Thus, these studies lend further support to Gierer's[11] estimate of the molecular weight of biologically active TMV-RNA based on the kinetics of ribonuclease degradation. The exponential inactivation over a wide range of X-ray doses implies that the infectivity is carried by particles having the same molecular weight and which are not polydispersed with respect to radiation sensitivity. This is in contrast to the findings with respect to the X-ray sensitivity of transforming principle.[82, 83]

Action of UV irradiation on RNA. UV irradiation of virus or RNA results in a loss of biological activity. Whereas X-rays probably inactivate virus RNA by splitting the polynucleotide chain, the mechanism of UV inactivation is not yet understood. After irradiating TMV-RNA in 0.02 *M* phosphate buffer at pH 7.3, McLaren and Takahashi[84] reported no changes in the intrinsic viscosity at 6°C. The only observable change was a slight reduction in optical density at 260 mμ, amounting to less than 1 %. Since the inactivation seems to be a one-hit process, it was suggested that only a local change in the RNA molecule is involved.

Different strains of TMV differ markedly in sensitivity to inactivation with UV light. Two strains (U1, the common strain, and U2) were studied in detail by Siegel *et al.*[85] Upon wet irradiation the two strains differed in sensitivity by a factor of 5.5 (Fig. 6). However, upon irradiation of dried preparations, the same sensitivity was found for both strains and, indeed, the same as the sensitivity of RNA extracted from either strain.[86] The detergent extraction of RNA was employed and irradiation performed at 254 mμ. The sensitivity of dried virus, as well as that of extracted RNA, corresponds to the sensitivity of wet irradiated U2 virus. Thus, it appears as if the protein of U1 virus has a protective action when this strain is irradiated in solution. It is not yet known how this action may occur. It was possible to show that strain U1 is denser than U2 by equilibrium centrifugation of a mixture of the two strains in a density gradient. RNA

[81] W. Ginoza and A. Norman, *Nature* **179**, 520 (1957).
[82] H. Ephrussi-Taylor and R. Latarjet, *Biochim. et Biophys. Acta* **16**, 183 (1955).
[83] J. Marmur and D. J. Fluke, *Arch. Biochem. Biophys.* **57**, 506 (1955).
[84] A. D. McLaren, W. N. Takahashi, *Federation Proc.* **16**, 220 (1957).
[85] A. Siegel, S. G. Wildman, and W. Ginoza, *Nature* **178**, 1117 (1956).
[86] A. Siegel, *Nature* **180**, 1430 (1957).

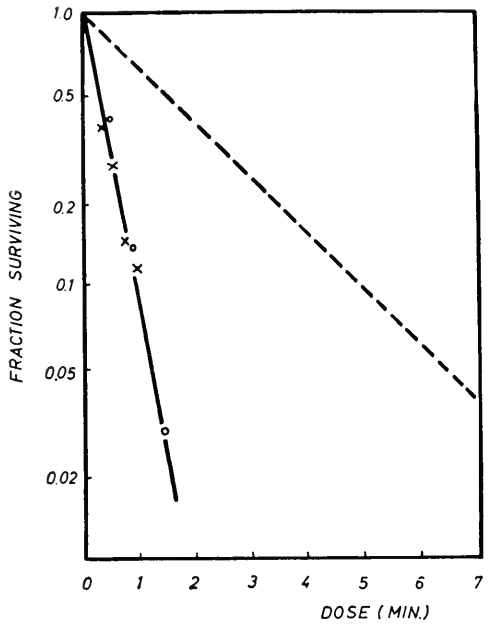

FIG. 6. The ultraviolet sensitivity of TMV strains U1 and U2, and the infectious nucleic acids derived therefrom; × = U1 nucleic acid, ○ = U2 nucleic acid, broken line = U1 intact virus, continuous line = U2 intact virus. [A. Siegel, S. G. Wildman, and W. Ginoza, *Nature* **178**, 1117 (1956).]

is extracted more easily from U2 than from U1 when the detergent method is used. Strain U2 is more sensitive to heat denaturation than U1. These observations led to the hypothesis that the bonding or type of association between protein and RNA is different for the two strains. This might lead to different configurations of the RNA in the two strains, and perhaps the RNA in strain U1 is in such a configuration that it can be better protected from UV radiation.

The two strains, U1 and U2, were also irradiated in solution at different wavelengths of UV light and the resulting action spectrum for inactivation was similar to the absorption spectrum of RNA in the wavelength range 254–280 mμ, indicating that RNA is the principal chromophore responsible for the inactivation of both strains in this range of wavelengths.[87] In the range 226-254 mμ, the action spectrum no longer corresponds to the absorption spectrum of RNA and, further, the sensitivity of both strains is the same at 226 mμ, whereas at 254 and 280 mμ the sensitivities differ by the same factor of 5.5 mentioned above. Thus, at 226 mμ, the inactivation of both strains must be due to a primary absorption of energy in the protein. The quantum yield (number of molecules affected/number of quanta absorbed) of nucleic acid from

[87] A. Siegel and A. Norman, *Virology* **6**, 725 (1958).

either strain was found to 3.4×10^{-4}, assuming a molecular weight of 2.5×10^6. Since many viruses have quite different sensitivites to UV light at 254 mμ, it would be most interesting to see if infectious RNA isolated from different viruses all have the same quantum yield.

The partial reactivation of the activity of UV irradiated bacterial viruses can be accomplished by exposing recently infected host cells to visible light.[88] It has not been possible to induce a similar photoreactivation of UV irradiated TMV after inoculation on tobacco leaves. However, Bawden and Kleczkowski[89] claim that phenol-extracted TMV-RNA, which is inactivated by UV irradiation, can be photoreactivated by exposure to daylight after inoculation on tobacco leaves. In control experiments, intact virus was inactivated with UV light and RNA extracted from such inactive virus. This RNA could not be photoreactivated. Thus, it may be that alterations in the protein component, or more likely in the type of bonding between protein and RNA, are responsible for the UV inactivation which is no longer reversible with visible light.

Heat inactivation. The heat inactivation of TMV-RNA was studied using RNA prepared by the detergent method.[90] Inactivation experiments were performed at neutral pH in the temperature range 37–65°C. The inactivation follows first-order kinetics, as is also the case for intact virus (Fig. 7). From the rate constants for inactivation at different temperatures, the heat of activation ($\Delta H^{\ddagger}$) leading to loss of infectivity was calculated to be 19 kcal./mole. In contrast, the heat of activation for heat denaturation of transforming principle is 93 kcal./mole.[91] This value, which is much higher than that for TMV-RNA, may be interpreted by assuming that some 15 successive pairs of H bonds in the Watson-Crick DNA structure must be broken before an irreversible collapse of the molecule occurs. The lower value of $\Delta H^{\ddagger}$ for TMV-RNA is probably based on a splitting of single phosphate-ester bonds which result in inactivation. It is much too low to be explained in terms of splitting a number of H bonds as in the case of DNA. This is also suggested by the negative value of the entropy of activation (−19.5 entropy units)[90] which may also reflect the formation of a cyclic phosphate triester intermediate in the activated state of the molecule. It indicates that the activated molecule has less degree of freedom because of the added bond between the phosphorus atoms and the 2-OH of the ribose. This cyclic intermediate can be hydrolyzed easily, accompanied by splitting of the polynucleotide chain.

(2) *Action of Enzymes.* Enzymes which can split the polynucleotide chain

[88] R. Dulbecco, *J. Bacteriol.* **59,** 329 (1950).
[89] F. C. Bawden, F. R. S. Kleczkowski, and A. Kleczkowski, *Nature* **183,** 503 (1959).
[90] W. Ginoza, *Nature* **181,** 958 (1958).
[91] S. A. Rice and P. Doty, *J. Am. Chem. Soc.* **79,** 3937 (1957).

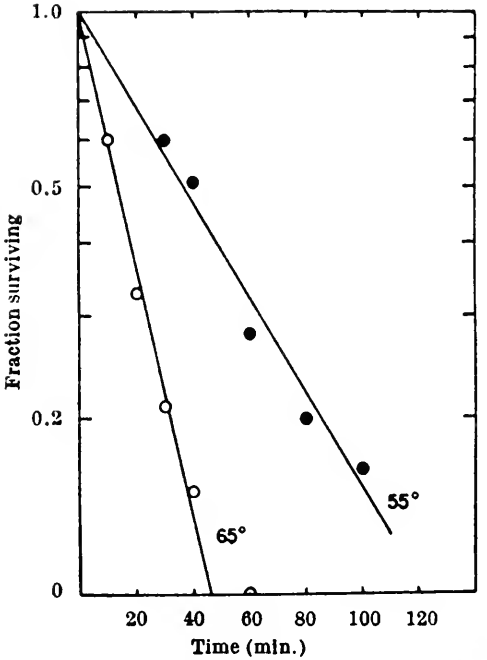

FIG. 7. Survival curves of TMV-RNA heated at pH 7.15 in 0.1 *M* phosphate buffer [W. Ginoza, *Nature* **181**, 958 (1958).]

of RNA hydrolytically, cause a simultaneous inactivation of the nucleic acid molecule, as has been shown with ribonuclease. Ribonuclease splits the ester bond between phosphoric acid and carbon atom 5′ of ribose, but only if the adjacent nucleotide to which phosphoric acid is esterified at carbon atom position 3′ is a pyrimidine nucleotide. In contrast, snake venom phosphodiesterase splits the ester bond between phosphoric acid and carbon atom 3′ of ribose, so that this enzyme must also cause an inactivation of RNA (compare Chapter 15).

Reddi[92] has studied the degradation products obtained by treating TMV-RNA with a purified phosphodiesterase from *Micrococcus pyogenes* var. *aureus*. The 3′-phosphates of all four nucleotides were found along with numerous dinucleotides of the general configuration, nucleoside-3′,5′-nucleoside-3′-phosphate. This enzyme, which also degrades DNA to nucleotides, must, therefore, hydrolytically split phosphoric acid from the carbon atom 5′ of ribose; it does not act on purine or cyclic pyrimidine nucleotides. Chromatographically purified phosphomonoesterase,[93] which is free from diesterase activity, has no effect on the biological activity of TMV-RNA

[92] K. K. Reddi, *Nature* **182**, 1308 (1958).
[93] H. G. Bomann, *Biochim. et Biophys. Acta* **16**, 245 (1955).

under conditions (6 hours, 20°C., pH 5) where all singly esterified phosphoric acid is quantitatively degraded from a commercial preparation of yeast RNA.[76] Phosphate, which must be set free by phosphomonoesterase action, could not be detected in the case of TMV-RNA. If the TMV-RNA molecule with a molecular weight of 2×10^6 is composed of 6000 covalent bonded nucleotides, then the expected amount of phosphates (1 mole phosphate per 6000 moles nucleotides for RNA of either type (I) or type (II), see Fig. 4) must lie well below the limits of sensitivity for phosphate determination.

TMV-RNA was also treated with polynucleotide phosphorylase from *Azotobacter vinelandii*[94] and in this case a slow rate of inactivation was found.[76] This enzyme catalyzes the reversible reaction:

$$n \text{ X-R-PP} \rightleftarrows (\text{X-R-P})_n + n\text{P} \tag{4}$$

where R stands for ribose, PP for pyrophosphate, P for orthophosphate and X for one or more of the following bases: adenine, guanine, uracil, or cytosine. It is, therefore, capable of degrading polynucleotides by phosphorolysis. The products of phosphorolysis, nucleoside-5′-diphosphates of all four bases, were chromatographically determined after a 6-hour incubation of TMV-RNA with the enzyme.[95] While synthetic polynucleotides containing only one kind of nucleotide, such as polyadenylic or polyuridylic acid, are rapidly phosphorolyzed, yeast and bacterial RNA, as well as synthetic polymers containing more than one kind of nucleotide, or the polymer aggregate formed by mixing solutions of polyadenylic and polyuridylic acid are phosphorolyzed slowly. On the contrary, TMV-RNA is phosphorolyzed at an intermediate rate. Ochoa concluded that the slow phosphorolysis of polynucleotides can be related to the fact that they consist largely of multi-stranded rather than single-stranded chains.

By using a homogeneous TMV-RNA, primer free phosphorylase, and employing radioactive phosphorus, it might be possible to determine the terminal nucleotide in the TMV-RNA and, also, to determine how many nucleotides must be split from the terminal position before the biological activity is destroyed.

(3) *Action of Chemical Agents. Reaction of infectious RNA with aldehydes. Formaldehyde.* Treatment of TMV with formaldehyde leads to inactivation of the virus.[96] Since this type of inactivation does not bring about a significant loss or alteration of the serological properties of the virus, it is generally used for the preparation of vaccines, for example, with poliovirus. Since RNA is the infectious component of TMV, the inactivation by formaldehyde must be due to its reaction with RNA, though the aldehyde also reacts with viral protein. Formaldehyde inactivation of TMV obeys first-order kinetics, at least in the early part of the reaction. The reaction

[94] M. Grunberg-Manago, P. J. Ortiz, and S. Ochoa, *Biochim. et Biophys. Acta* **20**, 269 (1956).

[95] S. Ochoa, *Arch. Biochem. Biophys.* **69**, 119 (1957).

[96] W. M. Stanley, *Science* **83**, 626 (1936).

constant is independent of the pH in the range pH 4–8. Groups having pK values lower than 4 or higher than 8, as is the case with the amino groups or the purine and pyrimidine rings of the nucleic acid, must be responsible for the inactivation with formaldehyde.[97]

If TMV-RNA is treated with 1–2% formaldehyde at pH 6.8, the UV absorption maximum shows a gradual increase up to 30% as well as a shift of 3–5 mμ towards higher wavelengths.[98] Similar effects were observed with all the RNA derivatives which contain NH_2 groups. Schiff's base formation ($—N{=}CH_2$) appears to be the most probable reaction in view of the increased absorption and the lability of the product. After treatment with formaldehyde, native DNA did not show this spectral change.

The nature of the formaldehyde reaction with RNA was studied in more detail by Staehelin[99] using C^{14}-labeled formaldehyde. He found that isolated RNA is much more sensitive to formaldehyde than intact virus is. The amount of formaldehyde bound at half inactivation is about 400 molecules per intact virus particle and 20 molecules per RNA. When the reaction with RNA has reached completion, 50–60 molecules of formaldehyde are bound per 100 nucleotides having 71 amino groups. At higher salt concentrations, the formaldehyde binding at RNA is greatly decreased. Since the rate of reaction of adenylic acid with formaldehyde is independent of the salt concentration, the decrease in the reaction rate of formaldehyde with RNA at higher salt concentrations must be due to the aggregation of RNA molecules which results in a decrease of availability of NH_2 groups. Reaction of formaldehyde with RNA proceeds in two steps. The first step leads to a more labile form of binding, whereas most of the formaldehyde becomes firmly bound after a more extensive reaction.

Glyoxal and Kethoxal. Glyoxal and related compounds were found to be antiviral agents.[100] Staehelin[101] observed that glyoxal and Kethoxal (β-ethoxy-α-ketobutyraldehyde) inactivate TMV as well as its infectious RNA. For equivalent molarities of formaldehyde and Kethoxal, TMV is inactivated more rapidly by formaldehyde than by Kethoxal, whereas the situation is reversed for RNA. These differences may be understood if it is considered that formaldehyde is a relatively small molecule so that the nucleic acid in the virus might be more readily accessible to it than to a larger molecule like Kethoxal. Under conditions which lead to approximately 50% inactivation of isolated RNA, only 10–15 molecules of Kethoxal are bound by one molecule of RNA of molecular weight 2×10^6, corresponding to the amount of formaldehyde which is bound per RNA molecule for the same degree of inactivation.

[97] T. E. Cartwright, A. E. Ritchie, and M. Lauffer, *Virology* **2**, 689 (1956).
[98] H. Fraenkel-Conrat, *Biochim. et Biophys. Acta* **15**, 307 (1955).
[99] M. Staehelin, *Biochim. et Biophys. Acta* **29**, 410 (1958).
[100] G. E. Underwood and S. D. Weed, *Proc. Soc. Exptl. Biol. Med.* **93**, 421 (1956).
[101] M. Staehelin, *Biochim. et Biophys. Acta* **31**, 448 (1959).

In contrast to formaldehyde, Kethoxal does not cause a marked increase in the UV absorption maximum of RNA or of mononucleotides. Only the spectrum of guanylic acid is markedly changed after reaction with Kethoxal and glyoxal. The product of the reaction of guanylic acid with the former agent is very stable in contrast to the compounds resulting from the formaldehyde treatment of nucleotides. The spectrum of 1-methylguanine is not altered after treatment with Kethoxal, and thus, the most likely place for a reaction of the dicarbonyl compound is the 2-amino group and imino group in position 1 of the guanine ring. When the reaction has gone to completion, the amount of Kethoxal bound corresponds to less than half the number of amino groups present in the RNA. This also speaks in favor of a synthetic reaction with guanine groups.

The reaction of infectious RNA with other chemical reagents. The reaction of TMV-RNA with nitrous acid has been studied in great detail.[102] It leads to well-defined reaction products. By measuring the rate of inactivation of RNA by nitrous acid, it is possible to calculate the minimum number of amino bases per mole of RNA necessary for the infectivity. Because of its significance for the *in vitro* production of TMV mutants, this method will be discussed in detail in the next chapter. Many other agents can react with infectious RNA resulting in a loss of biological activity. The mode of action of these agents is not yet known and the products of reaction have not yet been identified. Several of these will be discussed, briefly, here.

TMV or TMV-RNA can be rapidly inactivated by treatment with an excess of dimethyl sulfate at neutral pH.[76] The reaction of dimethyl sulfate with virus results in alterations of the protein which make this component insoluble so that it flocculates. RNA is inactivated according to first-order kinetics if the methylation is performed in a highly dilute alcoholic solution of dimethyl sulfate (molar ratio dimethyl sulfate/RNA = 4000:1). Inactivation of RNA is probably due to a substitution of the amino groups and the ring nitrogen atoms of the bases by methyl groups, since the reaction products of the methylation of adenosine with dimethyl sulfate at neutral pH are chiefly N^6-methyladenosine and 1-methyladenosine.[103]

The bromination of RNA in aqueous solution, employing small amounts of bromine, also leads to a rapid inactivation.[76] Sodium periodate, which is a specific oxidizing agent of the *cis*-glycol group (—CHOH—CHOH), also inactivates RNA if the reaction is carried out at a weakly acid pH with an excess of $NaIO_4$ (20 moles per mole RNA nucleotide).[76]

Hydroxylamine inactivates many RNA-containing animal viruses and also destroys the infectivity of TMV-RNA at neutral pH.[104]

(4) *Incorporation of "Unnatural" Bases in RNA.* Certain purine and pyrimidine compounds, which are structural analogs of the bases found in

[102] H. Schuster and G. Schramm, *Z. Naturforsch.* **13b**, 697 (1958).
[103] H. Bredereck, H. Haas, and A. Martini, *Chem. Ber.* **81**, 307 (1948).
[104] R. M. Franklin and H. Schuster, unpublished work (1959).

RNA, have an inhibitory action on virus multiplication due to their antimetabolic activity. Several of these compounds have been investigated in detail with respect to their action on the multiplication of TMV. These are 8-azaguanine, 2-thiouracil, and 5-fluorouracil. 5-Bromouracil, which is incorporated into phage DNA in place of thymine and which greatly reduces the infectivity of phage particles,[105] has no action on TMV. An excellent summary of the studies on purine and pyrimidine analogs has recently been published.[106]

Azaguanine. Azaguanine will inhibit virus multiplication in tobacco leaves infected with TMV.[106] This inhibition can be abolished if plants are treated, at the same time, with several naturally-occurring purines, their ribosides or ribonucleotides. RNA extracted from TMV, grown in leaves in the presence of azaguanine, contains about 3% less guanine than RNA from normal virus. An alkaline hydrolysis of the RNA obtained from "azaguanine-TMV" shows that the compound has been incorporated into the RNA as a nucleotide.[107] The presence of azaguanine in RNA can be demonstrated without great difficulty because azaguanine fluoresces strongly under both acid and alkaline conditions, while guanine compounds fluoresce strongly only under acid conditions. 8-Azaguanylic acid may be demonstrated by paper electrophoresis of the alkaline digest.

TMV preparations, having azaguanine in the RNA, are of lower infectivity than an equivalent amount of normal TMV.[108] Similar observations have been made with turnip yellow mosaic virus. In the latter case, the ratio of azaguanylic acid to guanylic acid could not be determined because the amount of azaguanine incorporated into the nucleic acid was too small.[109] The decrease in specific infectivity is dependent on the length of the time of multiplication of virus in the host cell in the presence of azaguanine. For example, virus particles, from 3 day-old infections, in treated plants show a reduction in infectivity, whereas no difference in infectivity could be detected in material from 14 day-old TMV infections of control and azaguanine treated plants. The reasons for this behavior are not yet clear.

2-Thiouracil. Thiouracil can also be incorporated into TMV if infected tobacco leaves are incubated in a medium containing this compound.[110] Virus multiplication is inhibited in the presence of thiouracil more effectively than by azaguanine.[111] The incorporation of thiouracil into TMV

[105] D. B. Dunn and J. B. Smith, *Nature* **174**, 305 (1954).
[106] R. E. F. Matthews and J. D. Smith, *Advances in Virus Research* **3**, 49 (1955).
[107] R. E. F. Matthews, *Nature* **171**, 1065 (1953).
[108] R. E. F. Matthews, *J. Gen. Microbiol.* **10**, 521 (1954).
[109] R. E. F. Matthews, *Virology* **1**, 165 (1955).
[110] R. Jeener and J. Rosseels, *Biochim. et Biophys. Acta* **11**, 438 (1953).
[111] F. L. Mercer, T. E. Lindhorst, and B. Commoner, *Science* **117**, 558 (1953).

can be reduced or prevented by the presence of uracil in the culture, but not by cytosine or thymine. Thiouracil is incorporated into the RNA of TMV as was shown, using S^{35}-labeled thiouracil.[110, 112, 113] Alkaline hydrolysis or hydrolysis with ribonuclease are best suited for demonstrating the incorporation of thiouracil into RNA. After alkaline hydrolysis of RNA, the mononucleotides were separated by chromatography and it was found that all the radioactivity moved with the R_f expected for pyrimidine mononucleotides. Separation by paper electrophoresis shows that all the activity had about the mobility to be expected for thiouridylic acid. The clearest results were obtained by degrading the RNA with ribonuclease followed by a chromatographic and electrophoretic separation of the products of hydrolysis. Then the radioactivity was found in several components: thiouridine, thiouridylic acid, thiouridine-3′,5′-diphosphate, and thiouracil-containing di- and polynucleotides. Thus, thiouracil seems to occupy certain positions in the nucleic acid which, presumably, are normally occupied by uracil.

The incorporation of thiouracil seems to occur chiefly at the end of the polynucleotide chain since some 25% of the total incorporated S^{35} is located in thiouridine-3′,5′-diphosphate. However, the presence of such a large quantity of thiouridine-3′,5′-diphosphate excludes the possibility that this is found only in a terminal position in a 2×10^6 molecular weight RNA molecule. Approximately 3.5% of the uracil in RNA is replaced by thiouracil, which means that there are about 50 thiouracil molecules per RNA. Therefore, about 12 thiouridine-3′,5′-diphosphate molecules are found per RNA molecule. If this should come only from a terminal position, the polynucleotide can be only 500 nucleotides long, contrary to the conception that the chain is some 6000 nucleotides long.

Virus containing thiouracil in its RNA has approximately the same infectivity per unit weight of virus as normal TMV.[114]

5-Fluorouracil. Fluorouracil also inhibits the multiplication of TMV in excised discs from tobacco leaves. Thereby, the yield of TMV is reduced to some 50% of a control yield.[115] Fluorouracil can replace about $\frac{1}{3}$ of the uracil of TMV-RNA according to base analyses on RNA after hydrolysis with perchloric acid. If RNA is hydrolyzed with snake venom diesterase or by alkaline digestion, it is possible to separate the 5′- or 2′- and 3′-monophosphates of fluorouridine from the corresponding uridine monophosphates by paper electrophoresis. Nucleoside diphosphates could not be demonstrated.

[112] R. E. F. Matthews, *Biochim. et Biophys. Acta* **19**, 559 (1956).
[113] H. G. Mandel, R. Markham, and R. E. F. Matthews, *Biochim. et Biophys. Acta* **24**, 205 (1957).
[114] R. Jeener, *Biochim. et Biophys. Acta* **23**, 351 (1957).
[115] M. P. Gordon and M. Staehelin, *J. Am. Chem. Soc.* **80**, 2340 (1958).

Infectivity was tested again by local lesion count and the infectivity per milligram of virus was not different for virus containing fluorouracil and normal virus.

e. Reaction with Nitrous Acid and Production of Mutants

(1) *The "Sensitive" Size of the Ribonucleic Acid Molecule.* The oxidative deamination of NH_2 groups with HNO_2 is well known in the field of protein chemistry (Van Slyke). The reaction may also be carried out on the amino groups of purines and pyrimidines regardless of the nature of the compounds (free bases, nucleosides, nucleotides, or polynucleotides). Only the reaction rate differs for these different compounds. Under identical conditions, nitrous acid reacts more rapidly with amino acids than with adenine, guanine, or cytosine;[116] the slowest reaction is obtained with nucleotides in a polynucleotide chain.[117, 118]

TMV-RNA is inactivated by HNO_2 according to first-order kinetics in the presence of an excess of HNO_2 at 20°C., in the pH range 4–4.5.[102] In the absence of HNO_2, the RNA is stable over a long period of time in this pH range. The inactivation is not accompanied by a degradation of the polynucleotide chain. The only observable alteration in the bases is deamination, and it is presumably this reaction that leads to the inactivation. The reaction products of adenine, guanine, and cytosine with HNO_2 are hypoxanthine, xanthine, and uracil, respectively. Thus, deamination of the purine bases leads to the presence of "unnatural" bases in RNA and deamination of cytosine produces an alteration of the cytosine/uracil ratio.

The HNO_2 inactivation of RNA occurs in the order of minutes (Fig. 8). The per cent of deaminated nucleotides arising in such short time periods is much too low to allow a direct measurement of them. It is only possible to detect the reaction products after the RNA has been exposed to HNO_2 for hours. This is accomplished by chromatography of a hydrolyzate of RNA. The increase in hypoxanthine, xanthine, and uracil is linear in time under conditions of constant pH and constant nitrite concentration.

If an average of 1 nucleotide out of N nucleotides must be deaminated before inactivation occurs, then the decrease is:

$$I_t = I_0 e^{-N\alpha t} \tag{5}$$

where I_t = infectivity at time t, I_0 = infectivity at time $t = 0$, and α = moles of nucleotides transformed per minute. N may be determined using the experimentally found decrease in infectivity along with the measured

[116] D. D. Van Slyke, *J. Biol. Chem.* **9**, 185 (1911).

[117] H. Bredereck, M. Köthnig, and G. Lehmann, *Ber. deut. Chem. Ges.* **71**, 2613 (1938).

[118] W. E. Fletcher, J. M. Gulland, D. O. Jordan, and H. E. Dibben, *J. Chem. Soc.* p. 30, (1944).

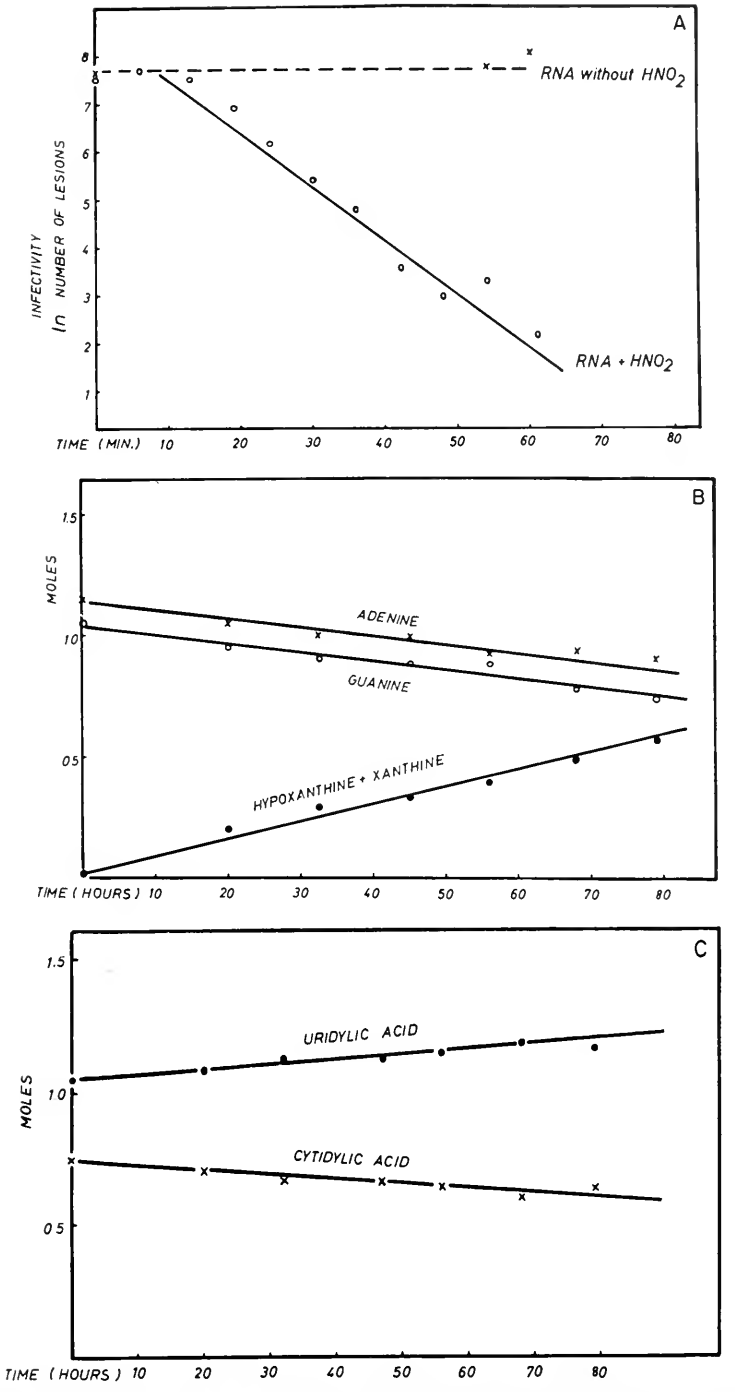

FIG. 8. Inactivation of TMV-RNA with HNO_2 at pH 4.3. (A) Loss in infectivity (tested on *Nicotiana glutinosa*). (B) and (C) Chemical alteration of the RNA nucleotides under the same conditions as in (A). Sum of the purine and pyrimidine bases = 4. [H. Schuster and G. Schramm, *Z. Naturforsch.* **13b**, 697 (1958).]

rate of deamination of bases. In the experiments with TMV-RNA, a value of somewhat over 3000 was found for N. This means that one-hit, that is one deamination, per approximately 3000 nucleotides results in a loss of infectivity of the entire molecule. Since the polynucleotide chain of TMV-RNA is some 6000 nucleotides in length, this would mean that at least half of all the nucleotides are necessary for the transfer of genetic information. One might also say that there is a 50 % probability that one-hit would inactivate an RNA molecule. Since all three amino bases are deaminated at rates which differ very little from each other, it is not possible to decide which alterations lead to inactivation. But from the value of N, it is clear that a certain number of nucleotides can be deaminated without causing a loss in biological activity. It is also conceivable that a deamination of certain bases or of a certain base does not result in inactivation but rather in mutation.

(2) *Production of Mutants.* Gierer and Mundry[119, 120] succeeded in demonstrating that HNO_2 treatment of TMV-RNA or intact TMV actually results in the formation of mutants. TMV-RNA or TMV was incubated with HNO_2 under the conditions described above, and mutants were demonstrated using the Java variety of tobacco. The systemic strain of TMV can only produce chlorotic lesions on the leaves of Java tobacco. Spontaneous mutations, which are often found, give rise to necrotic lesions. Thus, the number of necrotic lesions on Java tobacco plants must be proportional to the concentration of mutated virus particles. It is possible to determine the total concentration of surviving infectious particles by counting the number of necrotic lesions which are produced on leaves of Xanthi or *Nicotiana glutinosa* plants. The number of mutants which are produced by HNO_2 treatment of TMV-RNA are shown in Table VIII. This is expressed as the % necrotic lesions among total chlorotic plus necrotic lesions on Java tobacco plants (see Table VIII, line 6). For any time of treatment with HNO_2, this value lies well above the number of spontaneous mutations which are found with untreated RNA. The inactivation of RNA with HNO_2 is exponential in time [see Eq. (5)]. The concentration of infectious particles n at time t is given by the equation

$$n = n_0 e^{-t/\tau} \tag{6}$$

where n_0 = concentration of infectious particles at time $t = 0$, τ = time for which the number of infectious particles has decreased to $1/e$, (37 % of n_0).

If the alteration of a single base is mutagenic, and if the average number

[119] A. Gierer and K. W. Mundry, *Nature* **182**, 1457 (1958).
[120] K. W. Mundry and A. Gierer, *Z. Vererbungslehre* **89**, 614 (1958).

TABLE VIII

THE PRODUCTION OF MUTANTS OF TMV-RNA BY TREATING THE RNA WITH NITROUS ACID[a]

t(min)	t/τ	Total infections		Mutant infections (Java)	Mutant infections (%) (Java)
		Xanthi	Java		
1	0.05	72.8	183	1.4	0.8
4	0.22	72.4	130	2.5	1.9
8	0.43	89.8	188	4.5	2.4
16	0.86	59.3	97	5.4	5.6
32	1.73	36.1	63	6.6	10.5
64	3.46	10.4	21	2.1	9.8
96	5.2	2.7	3.5	0.5	15.5
Untreated controls (μg./ml. RNA)					
19		155	138	0.3	0.2
1.9		31.2	42	0.1	0.3
0.19		3.1	10	0	—
0.019		0.3	0.8	0	—

[a] TMV-RNA was treated with 1 M $NaNO_2$ solution at pH 4.8 (22°C.). After time t, the RNA is diluted and assayed. Untreated controls of RNA contain an equivalent amount of nitrite (pH 7). Time τ [Eq. (6)] is 18.5 minutes. Total infections are given as numbers of lesions (per leaf) on Xanthi, and as the sum of chlorotic and necrotic lesions on Java; mutant infections as numbers of necrotic lesions per leaf on Java. [Taken from A. Gierer and K. W. Mundry, *Nature* **182**, 1457 (1958).]

of such mutations per RNA molecule in the time τ is p, then the concentration m of such mutants is:

$$m = n_0 p(t/\tau)e^{-t/\tau} = npt/\tau \tag{7}$$

or in terms of the maximum value of m:

$$m/m_{\max.} = e(t/\tau)e^{-t/\tau} \tag{8}$$

Figure 9 shows the production of mutants under different conditions with TMV and with RNA derived from two strains. It should be noted that the mutagenic effect on TMV and on its isolated RNA is equal when the fraction of bases altered in RNA, measured by t/τ, is the same. The alteration of single nucleotides must be mutagenic since the production of mutants also follows a "single-hit" curve.

If one nucleotide out of about 3000 nucleotides is altered,[102] 6% of all the infectious particles have mutated (see Table VIII). That means that the alteration of any out of 180 of the total 6000 nucleotides in the RNA strand would be mutagenic. Nevertheless, it remains undecided which of the three types of chemical changes caused by nitrous acid (alteration of

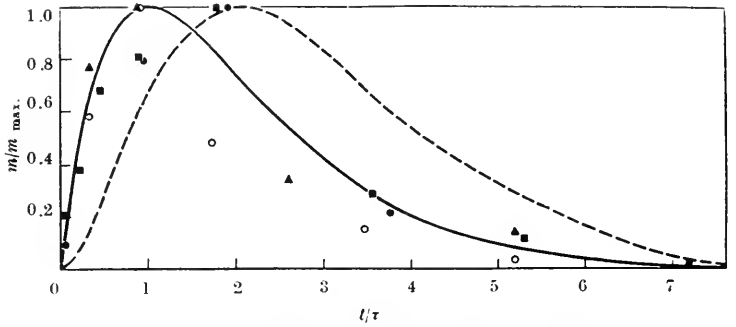

FIG. 9. The production of mutants of TMV by reaction of TMV or its nucleic acid with HNO_2. The dependence of the concentration of mutants m (number of necrotic lesions on Java tobacco relative to maximum value m_x) on the time t of incubation with nitrous acid, related to τ (the average time for one lethal conversion of a base per RNA molecule); continuous line = single-hit curve, broken line = double-hit curve. [A. Gierer and K. W. Mundry, *Nature* **182**, 1457 (1958).]

adenine, guanine, or cytosine) are mutagenic and how the chemically altered RNA is related to its progeny. In the case of a conversion of cytosine to uracil, it is conceivable that the altered RNA undergoes identical reproduction in the host cell.

It is surprising that such a large number of mutants have the same phenotypic expression of necrotic lesions on the given host (Java). This means that a cell of this host is in a position to react with 180 different mutants in such a fashion that the phenotypic effect (local necrotic lesions) is identical in all cases. The 180 mutants differ from one another, however, in that each is produced by deamination of a given nucleotide, and these nucleotides are located at different positions in the nucleotide chain. A variety of other mutants are also produced by nitrous acid. In order to detect them, individual lesions produced by RNA treated with HNO_2 were isolated from Xanthi tobacco and assayed on Java and Samsun plants. Half of the lesions which were reinoculated led to a variety of altered symptoms. The altered symptoms proved genetically stable in a transfer experiment.

The only possible reactions with HNO_2 are adenine → hypoxanthine, guanine → xanthine, and cytosine → uracil, and it may be that any one of these or perhaps only the alteration of the naturally occurring bases, cytosine → uracil may lead to mutation. Now, if a mutant can only arise when the quantitative ratio between amino bases and hydroxy bases changes, it should not be possible to produce RNA mutants by other chemical transformations. Therefore, RNA which had been methylated with dimethyl sulfate was also tested on Xanthi and Java plants. Even after an inactivation of greater than 90% of the RNA, it was not possible to observe necrotic lesions on Java plants.[121] It would be worthwhile to see if any other changes in the nucleotides of RNA lead to mutagenesis. It may be especially interesting to see if the unnatural bases, azaguanine, thiouracil, and fluorouracil, which are incorporated into virus RNA during the multiplication of the virus, produce mutations.

[121] H. Schuster and K. W. Mundry, unpublished work (1958).

f. Infectious Ribonucleic Acid and the Host Cell

TMV produces a general disease in plants of different tobacco races. This disease is expressed in a mosaiclike light and dark green pattern as well as a deformation of the leaves. The virus may be transmitted to plants of different families and, therefore, has a very low host-specificity. For the production of large amounts of TMV, a Samsun strain of *Nicotiana tabacum* is usually employed. *Nicotiana glutinosa* plants are best suited for infectivity assays, since the virus produces local necrosis on leaves of these plants rather than general symptoms. The number of lesions produced by a virus preparation increases with the amount of virus over a wide range of virus concentrations and, therefore, can serve as a measure of the biological activity of a virus preparation. The infection is experimentally initiated by mechanically injuring the cells of the leaves so that the virus can enter the cytoplasm directly.

Cytological studies have shown that the early part of the infection with TMV is characterized by a marked increase in protoplasmic streaming, division, and degeneration of chloroplasts, and the secretion of virus aggregates in the form of crystals.[122] By employing ultraviolet microspectroscopic analysis, it is found that the extinction, $E_{260}/E_{280\,m\mu}$, of the various cell constituents is altered in a characteristic fashion after the entry of virus into the hair cells.[123] This ratio remains unchanged in healthy cells. The relative level of the ratio is determined essentially by the nucleic acid and protein components (viz., the aromatic amino acids). Changes in E_{260}/E_{280} in TMV-infected cells suggest that the ratio of total RNA to protein increases, first in the nucleus and only later in the cytoplasm. Infectious particles may be detected in such infected cells only when the ratio has reached a minimum for the cell nucleus and a maximum for the cytoplasm. However, this method is not suited to determine under which conditions virus specific material is formed.

Some information concerning the course of virus infection resulting from the inoculation of the intact virus or infectious RNA has been obtained by studying the action of UV irradiation on infected leaves. The course of virus synthesis in the plant cells was also studied by assaying infectious material at various times after infection. On the other hand, studies of the effect of ribonuclease on leaves at various times during the infection have produced no clear picture of the infection process.

(1) *Effect of UV Irradiation on Virus Multiplication.* The inactivation of infective centers of *Nicotiana glutinosa* with UV light after inoculation of

[122] H. Zech, *Planta* **40**, 461 (1952).
[123] H. Zech and L. Vogt-Köhne, *Naturwissenschaften* **42**, 337 (1955).

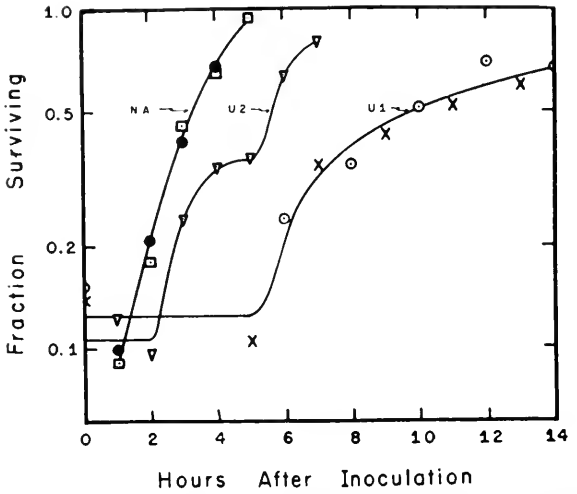

FIG. 10. Ultraviolet light survival of infective centers from TMV strains and nucleic acids derived therefrom. Dose of UV: intact U2 and RNA, 90 seconds; U1, 5 minutes. ● = U1 RNA, ⊡ = U2 RNA, ▽ = intact U2, ⊙ and × = intact U1. NA = RNA. [A. Siegel, W. Ginoza, and S. G. Wildman, *Virology* **3**, 554 (1957).]

TMV and virus RNA from two strains was studied by Siegel *et al.*[124, 125] It was possible to differentiate several phases of the infection. Intact virus from strains U1 or U2 was inoculated on leaves and the infective centers irradiated with a constant dose of UV light at different times after infection. For some time the UV sensitivity of the centers remained unchanged (phase 1). This "lag phase" was 5 hours long for strain U1 and 2.5 hours long for strain U2. This was followed by an increase in the resistance of the infective centers (phase 2), before virus replication begins. On the other hand, if infectious RNA from either strain was inoculated and the resulting centers irradiated, the resistance of the centers increased quite soon after infection (Fig. 10). This difference in sensitivity to UV light when infection is initiated with intact virus or with RNA suggests that the initial events of TMV infection are concerned with the release of RNA from the protein moiety of the virus particle. The locality of this release would be close to the surface of the leaf since the resistance to UV during the lag period is little changed from that of the extracellular virus particle. One might explain the longer lag phase of strain U1 by assuming that RNA is released with greater difficulty from U1 particles. This suggested greater affinity

[124] A. Siegel and S. G. Wildman, *Virology* **2**, 69 (1956).

[125] A. Siegel, W. Ginoza, and S. G. Wildman, *Federation Proc.* **16**, 248 (1957); *Virology* **3**, 554 (1957).

between RNA and protein in strain U1 is revealed, also, by other studies. [See Section III, *d*, (1).]

After direct inoculation of RNA, or after the release of RNA from its protective protein shell, we may imagine that the RNA migrated from the surface to the inside of the cell. Here it may be shielded from the damaging effects of UV light. A further possibility is that RNA combines with some host cell protein which may offer better protection against UV irradiation. It has already been mentioned that the type of RNA-protein bonding may be important for the resistance to UV irradiation.

Before new infectious units appear (phase 4), a plateau of resistance to UV is maintained (phase 3). During phase 4, a second rise in resistance occurs. The timing of all four phases is markedly dependent on temperature.

(2) *Effect of Ribonuclease on Virus Multiplication.* As long as the RNA is enclosed in its protein covering, it is inaccessible to the enzymic action of ribonuclease.[126] Upon release from its protective protein, it is accessible to enzymic degradation. The virus RNA must be separated from the virus protein at some time, in order that the RNA may induce its reduplication. Therefore, it should be possible to obtain some information on the course of infection by adding ribonuclease to infected cells in order, artificially, to increase the ribonuclease concentration within the cell.

Casterman and Jeener[127, 128] infiltrated leaves of *Nicotiana tabacum* with ribonuclease *in vacuo* either before or at various times after inoculation with virus and investigated the resulting action of the enzyme on virus multiplication. If the infiltration of ribonuclease occurred before infection or up to 15 minutes after infection, no virus multiplication occurred. If ribonuclease was infiltrated into the cells 2 hours or more after infection, it was no longer possible to inhibit virus multiplication. Simple immersion of the leaves in the ribonuclease solution, followed by superficial washing with water, had no influence on virus multiplication.

If one postulates that ribonuclease inhibits virus multiplication only by splitting the free virus RNA, these studies can be interpreted by assuming that virus RNA is freed of its protein directly after infection. Two hours after infection, the RNA must be present in a form unaccessible for ribonuclease, possibly by association with some cell structures or by the formation of new intact virus. On the other hand, the inhibitory action of ribonuclease can also be due to the formation of a complex with intact virus. Loring[126] and Kleczkowski[129] have reported that TMV can form nonin-

[126] H. S. Loring, *J. Gen. Physiol.* **25,** 497 (1942).
[127] C. Casterman and R. Jeener, *Biochim. et Biophys. Acta* **16,** 433 (1955).
[128] C. Hamers-Casterman and R. Jeener, *Virology* **3,** 197 (1957).
[129] A. Klezkowski, *Biochem. J.* **40,** 677 (1946).

fectious complexes with ribonuclease under certain conditions. The infiltration of protamine into infected leaves can also inhibit virus multiplication and, therefore, it is doubtful whether the ribonuclease action is specific. These results cannot be compared with the studies employing UV irradiation, since different types of plants were used and the course of infection may be different in different types of cells.

In studies using epidermal hair cells of *Nicotiana langsdorffii*, Benda[130] was able to show that ribonuclease also prevented lesion formation when introduced into an epidermal hair through which the virus must pass to reach the leaf from the inoculated cell. Since the epidermal hair is a column of cells, one cell in diameter, ribonuclease could be introduced into the basal cell while the distal cell is inoculated with virus.

(3) *Mechanism of Plant Virus Infection.* The UV irradiation studies of Siegel *et al.* on the sensitivity of infective centers lead to the hypothesis of a "lag phase" during which the virus protein is stripped from the nucleic acid. One should, therefore, expect that new infectious material will be synthesized sooner if infection is made with RNA than if infection is made with intact virus.

Schramm and Engler[131] infected a parallel series of tobacco plants with TMV and TMV-RNA and then determined the amount of infectious material, at different times after infection, by homogenizing the infected leaves and assaying the homogenate by local lesion test on *Nicotiana glutinosa*. It is possible to detect 10^{-12}–10^{-13} gm. TMV/ml. by this assay. A typical experiment is shown in Fig. 11. After a latent period, virus multiplication is rapid. In both cases, however, it slows down when a concentration of about 10^{-7} gm. virus per gram leaves is reached. If the extrapolation for the two curves is correct, then the latent period for TMV is approximately 30 hours and that for RNA approximately 20 hours. This means that the release of RNA from virus takes several hours, as was also suggested by studies of Siegel *et al.* However, it is not clear why RNA itself has such a long latent period. This difference in the course of infection with TMV or with TMV-RNA is also observed if one studies the times at which local lesions arise on *Nicotiana glutinosa* after infection with TMV or with TMV-RNA.[132]

When infected leaves are homogenized, ribonuclease is set free. Although this can have no effect on intact virus particles, infectious virus RNA, which is not yet enclosed in protein, is destroyed. Therefore, infectious RNA which has been synthesized during the latent period cannot be demon-

[130] G. T. Benda, *Virology* **6**, 718 (1958).

[131] G. Schramm and R. Engler, *Nature* **181**, 916 (1958).

[132] H. Fraenkel-Conrat, B. Singer, and S. Veldee, *Biochim. et Biophys. Acta* **29**, 639 (1958).

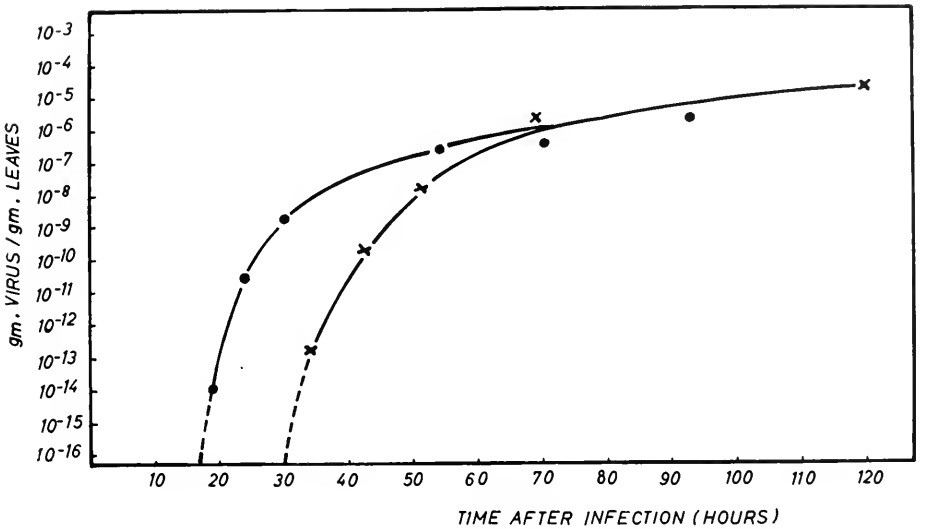

FIG. 11. The synthesis of tobacco mosaic virus. Increase of virus concentration after infection with tobacco mosaic virus (×—×—×) and virus ribonucleic acid (●—●—●) in *Nicotiana tabacum*. [G. Schramm and R. Engler, *Nature* **181**, 916 (1958).]

strated by this procedure. It is only possible to determine the total amount of infectious material synthesized if ribonuclease degradation can be avoided. This can be accomplished if the homogenization is performed in the presence of phenol which immediately destroys all enzyme activity.[133] Engler and Schramm[134] employed this technique to study the multiplication of TMV. The total infectious RNA in infected leaves (*Nicotiana tabacum*) was determined by homogenizing the material in the presence of phenol. The RNA which was already enclosed in protein to form intact virus was determined by homogenizing the leaves in the absence of phenol, followed by incubating the homogenate at 37°C. for 1 hour to destroy all free RNA. Only the RNA within the virus remains intact after this treatment. Then the protein was extracted with phenol and the liberated RNA measured. The infectivity of the total RNA and the virus RNA was assayed by local lesion test on *Nicotiana glutinosa*.

The results of this experiment are shown in Fig. 12. A large amount of free RNA is synthesized before new intact virus is produced. The incorporation of this free RNA into virus protein to form intact virus starts some 10 hours after RNA multiplication has commenced. Synthesis of virus protein starts several hours later than synthesis of virus RNA, but must be much

[133] R. Engler, Doctoral thesis, Tübingen (1957).
[134] R. Engler and G. Schramm, *Nature* **183**, 1277 (1959).

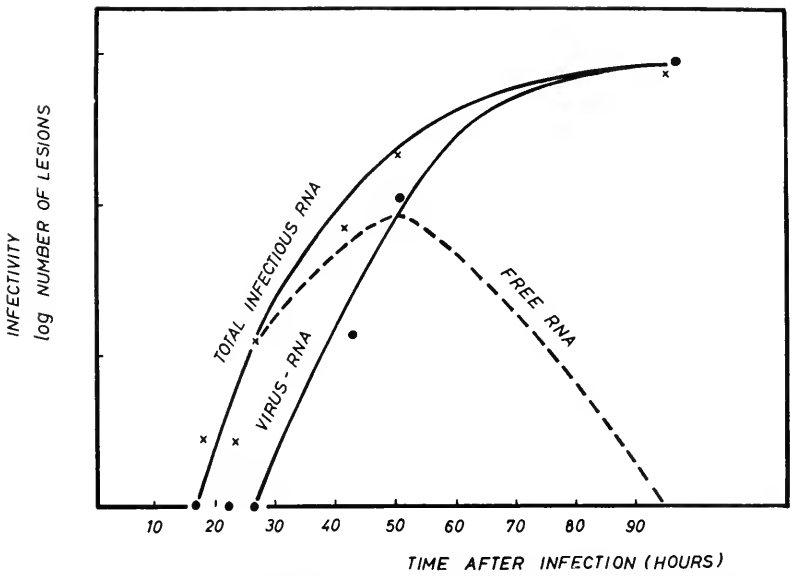

FIG. 12. The synthesis of TMV ribonucleic acid in *Nicotiana tabacum* after infection with TMV (measured by local lesion test in *Nicotiana glutinosa*). [From R. Engler and G. Schramm, *Nature* **183**, 1227 (1959).]

faster since all the RNA is packed into protein after 80–110 hours. Therefore, the amount of free RNA reaches a maximum about 45 hours after infection and decreases afterwards.

By chromatographic studies on extracts from infected plant tissue using cation and anion exchanging cellulose adsorbents, it was possible to identify two infectious components with different chromatographic behavior.[135] One component was not adsorbed on the ion exchange column and lost its infectivity after ribonuclease treatment. This is probably free RNA. The other adsorbed to the column but could be eluated between pH 3.8 and 4.0 near the isoelectric point of TMV, and was not inactivated by ribonuclease. Many typical TMV rods could be seen when this fraction was studied with the electron microscope. Thus, this component is probably intact virus. The relative amounts of the two components are dependent on the length of infection. More RNA was found after shorter infection (9 days) and significantly more virus in extracts of plants infected for 6 months. It is well known that RNA is unstable in the usual tissue extract and, therefore, it is questionable if the quantitative data are significant. Further, it is not known whether the chromatographic treatment has any effect on the infectivity of RNA. This should be tested since it is well known that infectious RNA is sensitive towards certain salts and salt concentrations.

[135] G. W. Cochran and J. L. Chidester, *Virology* **4**, 390 (1957).

g. Reconstitution of Virus from Ribonucleic Acid and Protein

Before the experimental demonstration of the infectivity of TMV-RNA, Fraenkel-Conrat and Williams,[136] and, later, Commoner *et al.*[137, 138] described the formation of infectious particles by mixing inactive protein, which had been prepared by alkaline degradation of TMV at pH 10.5, with nucleic acid which had been prepared by dodecyl sulfate. The two components were inactive when tested separately on *Nicotiana glutinosa*.

After mixing 1% solutions of the two components in the proportion of 10 parts protein to 1 part RNA and incubating at 3°C. for 24 hours (pH 6.0–7.0), nucleoprotein particles carrying virus activity were found. The nucleoprotein particles appeared to be identical in shape and size with TMV except for a greater randomness in length. They contained 5–6% RNA as do TMV particles and were not attacked by ribonuclease. Upon treatment with detergent, there was a partial degradation of virus protein exactly as in the case of native TMV. A central RNA strand was visible after this treatment. The particles proved somewhat more labile to detergent than TMV.

The combination of protein and nucleic acid fractions isolated from different strains of TMV (the so-called mixed reconstitution) was also possible.[139, 140] RNA from 4 different strains of TMV was recombined with the protein component from 3 of these strains. All combinations of recombined nucleoprotein were biologically active. In every case, the reaction products gave the same disease symptoms as did the strain supplying the RNA. The serological characteristics of mixed virus preparations were shown to resemble those of the protein component.

After the preparation of infectious RNA, it became clear that the formation of a biologically active virus could not have resulted from a combination of inactive components. Instead, the comparatively low activity of the RNA component is, simply, significantly increased by addition of the virus protein. This interpretation is also supported by the fact that the activity of TMV particles, which had been partially degraded with detergents, can be increased by about tenfold after addition of native virus protein.[141] The biological activity of the detergent degraded particles could be further reduced by ribonuclease, whereas the particles which had been incubated with protein were resistant to ribonuclease.

[136] H. Fraenkel-Conrat and R. C. Williams, *Proc. Natl. Acad. Sci. U.S.* **41**, 690 (1955).

[137] J. A. Lippincott and B. Commoner, *Biochim. et Biophys. Acta* **19**, 198 (1956).

[138] B. Commoner, J. A Lippincott, G. B. Shearer, E. E. Richman, and J. H. Wu, *Nature* **178**, 767 (1956).

[139] H. Fraenkel-Conrat, *J. Am. Chem. Soc.* **78**, 882 (1956).

[140] H. Fraenkel-Conrat and B. Singer, *Biochim. et Biophys. Acta* **24**, 540 (1957).

[141] R. G. Hart, *Nature* **177**, 130 (1956).

On a molar basis, the intact TMV with a molecular weight of 40×10^6 is about 200 times more active than free RNA. The biological activity of RNA, recombined with protein, is about 60–120 times larger than that of free RNA, or 30–60% of the activity of the original virus. Therefore, the recombination studies suggest that the lower activity of free RNA in solution is probably due to the presence of ribonuclease during inoculation of free RNA and to the sensitivity of free RNA in solution to numerous types of reagents. Thus, the virus protein can be regarded as a stabilizer of the RNA during infection.

The binding of virus protein to RNA in the reconstitution is not highly specific. This is clearly shown by the fact that synthetic ribonucleotide polymers with molecular weights of 10^4–10^5 can recombine with native TMV protein into rods of the same apparent width as TMV, but of various length.[142] These rods contain 4–6% ribonucleotide polymers and incubation with ribonuclease caused no appreciable hydrolysis of the nucleic acid component. As is to be expected, such rods possess no biological activity.

Recombined virus preparations have UV sensitivities which are different from those of normal virus.[85] Many recombined viruses possess the UV sensitivity of their RNA when it is present in a free form, whereas others show sensitivities which lie between that of free RNA and normal virus. The UV sensitivity of recombined virus is never the same as that of normal virus.

In summary, one might say that the higher biological activity of recombined nucleoprotein particles compared with free RNA is most probably due to the stabilizing action of the protein shell which protects the infectious RNA. The viral protein is capable of spontaneous aggregation independent of the presence of other macromolecules. Serum protein, which does not polymerize to form rodlike aggregates, has an inhibitory action on the activity of infectious RNA.[143] Thus, the specificity of the stabilizing action of virus protein probably lies in its ability to form aggregates.

The nature of the bonding between RNA and protein is not well known. It must be of a rather weak type in view of the relative ease of splitting with phenol at neutral pH and in the cold. Reddi[144] has shown that the UV spectrum of TMV is exactly equal to the sum of the spectra of TMV-RNA + TMV protein after correcting for light-scattering. If the purines and pyrimidines of TMV-RNA are involved in the formation of the nucleoprotein complex in a way to suppress some of the chromophores, one would expect that the UV spectrum of TMV minus the contribution of the spectrum of the protein part would have a lower absorption maximum than

[142] R. G Hart and J. D. Smith, *Nature* **178**, 739 (1956).
[143] J. S. Colter, *Progr. in Med. Virol.* **1**, 1 (1958).
[144] K. K. Reddi, *Biochim. et Biophys. Acta* **24**, 238 (1957).

that of protein free TMV-RNA alone. The possibility of a bonding between RNA and protein through triester linkages of RNA phosphoric acid with protein amino acids is ruled out by the experiments of Koshland *et al.*[145] who separated the protein coat of TMV by the action of detergent in the presence of $H_2{}^{18}O$. If such bonds were present, $H_2{}^{18}O$ should be found in RNA phosphate after isolating the RNA by detergent treatment. However such was not the case. The most probable type of binding between RNA and protein may be a type of salt binding between the acid phosphate groups of RNA and the basic groups of amino acids.

2. The Ribonucleic Acids of Other Plant Viruses

Kaper and Steere[146] have isolated an infectious nucleic acid from a spherical plant virus, tobacco ringspot virus (TRSV) by a modification of the heat-denaturation method. One volume of 0.5–1 % virus solution was quickly added to 2 volumes of hot (95–100°C.) *M* NaCl solution, heating was continued for exactly 35 seconds, during which time the mixture was agitated continuously. After cooling the solution, the virus protein was separated from the nucleic acid by centrifugation and the nucleic acid precipitated afterward by ethanol. The yield of RNA varied between 60–70 % of the nucleic acid in the virus. The infectivity of these preparations with a protein content not exceeding 0.7 % ranged from 0.1–1.0 % as compared with the nucleic acid in the virus. Some preparations were also made with the phenol method, but the yield was usually very low and the infectivity not higher than of those prepared by heat denaturation.

The usual tests (incubation with ribonuclease, ultracentrifugation, storage for several days in the cold) conducted with this nucleic acid have given evidence that the associated infectivity cannot be due to contamination with residual whole virus. Also, in contrast to the whole virus, the infectivity of the nucleic acid was influenced by the concentration of phosphate buffer present in the medium used for inoculation; the average lesion count increased gradually up to 0.2 *M* buffer concentration. The molecular weight of the nucleic acid preparation was not determined. Twenty per cent less phosphorus and nitrogen than expected on the basis of the nucleotide composition of the nucleic acid was found, suggesting the presence of a nonnitrogenous impurity.

Up to date, RNA has been isolated from plant viruses other than TMV and TRSV only by methods which do not completely exclude the degradation of the RNA. For example, Dorner and Knight[147] have studied the RNA

[145] D. E. Koshland, Jr., N. S. Simmons, and J. D. Watson, *J. Am. Chem. Soc.* **80**, 105 (1958).

[146] J. M. Kaper and R. L. Steere, *Virology* **7**, 127 (1959).

[147] R. W. Dorner and C. A. Knight, *J. Biol. Chem.* **205**, 959 (1953).

from tomato bushy stunt virus, southern bean mosaic virus, and potato X virus by a method based on treatment of the viruses with detergent, followed by salting out the protein. The virus solutions (~1 %) were heated in detergent (Duponol C) solution (~1 %) for 4 minutes in a boiling water bath. The chilled and dialyzed preparations were heated at 100°C. for 3 minutes in 1 *M* NaCl solution to precipitate the virus protein. The yield in RNA was between 67–87 %. The RNA preparations had sedimentation constants between 2–6 *S* and contained 2–5 % protein.

Turnip yellow mosaic virus (TYMV)-RNA was first isolated by Markham and Smith[148, 149] by treatment of the virus with 30 % (v/v) or more ethanol in neutral solutions at room temperature. Cohen and Schachman[150] determined the molecular weight of TYMV-RNA which had been prepared by alcohol[148] or by heat[53] denaturation of the virus protein. The sedimentation constant lay between $s_{20} = 2$ and 4 *S*. RNA, which had been obtained by heat denaturation of the protein, had a higher sedimentation constant in concentrated solutions than in dilute solutions. This indicates a tendency to aggregate and dissociate reversibly. The RNA solution was polydispersed, which means that only an average molecular weight could be calculated from the sedimentation and viscosity measurements. The weight average molecular weight of the material obtained by heat denaturation was about 10^5 and the corresponding value for RNA liberated by ethanol treatment of the virus was about 5×10^4. According to this, a TYMV particle would contain 20–40 RNA molecules. These values are questionable, however, since an RNA prepared from TMV by the method of heat denaturation also yields a preparation with an average molecular weight of about $2–3 \times 10^5$, whereas TMV-RNA prepared by the phenol method has an average molecular weight of about 10^6.

Ginoza[90] has shown that treating TMV-RNA at 65°C. for 10 minutes results in a loss of biological activity of 40 % of the RNA molecules, probably by hydrolysis of the polynucleotide chain. Therefore, any method of heat denaturation including longer times of heating appears particularly unsuitable for the preparation of high molecular weight RNA. This is also suggested by the fact that RNA isolated from TYMV under milder conditions[151] (10 minutes heating of the virus solution in NaCl phosphate buffer at pH 7.6 and 45°C.) also has a molecular weight less than 10^5. Most of the RNA released is noninfectious. The slight activity which was found may have been due to nondegraded virus particles.

[148] R. Markham and K. M. Smith, *Parasitology* **39**, 330 (1949).
[149] R. Markham and J. D. Smith, *Biochem. J.* **49**, 401 (1951).
[150] S. S. Cohen and H. K. Schachman, *Virology* **3**, 575 (1957).
[151] J. W. Lyttleton and R. E. F. Matthews, *Virology* **6**, 460 (1958).

IV. The Ribonucleic Acids of Animal Viruses

1. Introductory Remarks

As already discussed in Section II, the animal viruses can be divided into two groups depending on whether they contain DNA or RNA. The range of sizes and the composition of animal viruses is much more variable than that of any known plant viruses. The simplest type of animal virus may be represented by poliomyelitis virus, composed of RNA and protein and morphologically rather similar to turnip yellow mosaic virus. The most complex type is represented by vaccinia virus which contains DNA, protein, lipid, riboflavin, biotin, and copper, and may be more closely related to more highly organized biological units. There are many animal viruses which exhibit all degrees of complexity between these two extremes. There is no correlation between the size of a virus particle and the type of nucleic acid found therein. RNA is present in relatively small viruses such as poliovirus as well as in much larger viruses such as Newcastle disease virus. The same is true for DNA-containing viruses. The relatively small papilloma virus as well as the larger vaccinia virus both contain DNA.

It has now been shown that RNA is probably the infectious principle for a large number of animal viruses. This has been accomplished by isolating the virus RNA from tissues infected with the virus under consideration or from a purified virus preparation. In all cases, the extraction was done by the phenol method.[58] The method has not been successful with all viruses which have been studied. For example, no infectious RNA has been isolated from Newcastle disease virus or fowl plague virus, both of which belong to the myxovirus group. It is quite possible that this may be due to methodological difficulties in the extraction, but it is also conceivable that RNA may not be the sole agent responsible for infectivity in the case of all viruses. In some cases, other viral components may be necessary in order to initiate infection.

This discussion of animal virus RNA will be limited to those viruses in which RNA had been demonstrated as the infectious principle and to those cases where at least a chemical analysis has been possible. Of the tumor viruses, the presence of RNA has been well established only in the case of Rous sarcoma virus (about 1% RNA)[152, 153] and the virus of avian myoloblastosis[153a] (about 2% RNA); therefore, those viruses are not included. The studies of Latarjet *et al.*[154, 155] and of Hays *et al.*[156] who have prepared

[152] R. Bather, *Brit. J. Cancer* **11**, 611 (1957).
[153] M. A. Epstein, *Nature* **181**, 1808 (1958).
[153a] J. W. Beard, personal communication (1958).
[154] R. Latarjet and M. de Jaco, *Compt. rend. acad. sci.* **246**, 499 (1958).

nucleic acid which can induce leukemia by detergent extraction of leukemic cells of mice can also be only briefly mentioned since it was not decided whether the active agent was RNA or DNA.

2. Arrangement of the Ribonucleic Acids in Animal Viruses

In most cases in which animal viruses have been morphologically and chemically characterized in some detail, it has been shown that the RNA is in the interior of the infectious particle. The best demonstration of this is the resistance of the virus particles to ribonuclease treatment. For example, the infectivity of poliovirus is not altered when the virus solution is incubated with ribonuclease during the purification procedure.[27] After removal of virus protein by phenol treatment, the infectious virus material in the aqueous phase is highly sensitive to ribonuclease.[157, 158, 159] During the purification of poliovirus, a noninfectious component was isolated which sediments more slowly than the infectious component. The former has little or no RNA and has been observed in the electron microscope as a round flattened particle of somewhat larger diameter than the infective particle. This component seems to be composed mainly of protein and is very similar to the noninfectious nucleic acid-free particle of turnip yellow mosaic virus.[160]

The arrangement of RNA in the more complex viruses has only been studied in some detail for several representatives of the myxovirus group. Influenza and fowl plague viruses, which belong to this group, contain RNA, protein, and carbohydrates. Two subunits of different composition could be distinguished in these viruses; the virus hemagglutinin which contains only protein and carbohydrate, and the so-called g-antigen which contains protein and all the RNA of the virus. The hemagglutinin must be part of the surface of the whole particle since it possesses the biological surface characteristics of the infective particle. The ribonucleoprotein component, which is antigenic, is only detectable in appreciable amounts after the particles are carefully disrupted.

By electron microscopic studies of the virus after degradation with enzymes, it may be concluded that the virus particle has an external shell of

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[155] R. Latarjet, N. Rebeyrotte, and E. Moustacchi, *Compt. rend. acad. sci.* **246**, 853 (1958).

[156] E. F. Hays, N. S. Simmons, and W. S. Beck, *Nature* **180**, 1419 (1957).

[157] G. Koch, H. E. Alexander, I. M. Mountain, K. Sprunt, and O. Van Damme, *Federation Proc.* **17**, 256 (1958).

[158] H. E. Alexander, G. Koch, I. M. Mountain, K. Sprunt, and O. Van Damme, *Virology* **5**, 172 (1958).

[159] H. E. Alexander, G. Koch, I. M. Mountain, and O. Van Damme, *J. Exptl. med.* **108**, 493 (1958).

[160] R. Markham, *Discussions Faraday Soc.* **11**, 221 (1951).

protein and an internal nucleoprotein ring having RNA on its external surface.[161, 162] In the case of Newcastle disease virus (NDV), which is also a myxovirus and which is considerably larger than influenza and fowl plague viruses, the RNA is probably arranged on a ringlike structure that is surrounded by a trypsin-sensitive shell.[162] The phospholipid of the virus seems to be superficially situated.

3. Preparation of Biologically Active Ribonucleic Acids

An infectious RNA component has now been isolated from 8 different animal viruses. These are: Mengo and West Nile encephalitis viruses, equine encephalomyelitis virus (type east and west), poliomyelitis virus, mouse encephalomyelitis virus, murine encephalomyocarditis virus, foot and mouth disease virus, Semliki forest virus.

In all cases, the RNA component has been isolated from tissues or cells infected with the virus in question and in the case of poliovirus, mouse encephalomyelitis virus, and western equine encephalomyelitis virus, nucleic acid has also been isolated from highly purified virus preparations.

a. RNA form Mengo and West Nile Encephalitis Viruses[163, 164]

(1) *Preparation.* Mengo and West Nile viruses were grown in cells of the Ehrlich ascites carcinoma. A saline suspension of samples of mouse brain, which had been infected with the virus in question, was injected intraperitoneally into mice in which the carcinoma had grown for several days. After some days, the infected cells were collected, washed, and frozen. The frozen tissues were ground to a fine powder, mixed with buffer at pH 7, and centrifuged. The nucleic acid was extracted from the supernatant by the phenol method.

The infectivity of the aqueous phase, which contains the extracted nucleic acid of virus and cells, was determined for both viruses by intracerebral injection into mice. The viruses produced were recovered from the infected mouse brains and identification was accomplished by means of specific immune sera.

The infectivity of nucleic acid preparations was of the order of 0.1% of the virus suspensions from which they were prepared. This is similar to the infectivity of TMV-RNA. Virus RNA may occur in infected cells in a free form synthesized by the host cells in response to virus infection, in intact virus particles, or in both forms in the same cell. In order to distinguish between these possibilities, a homogenate of tumor cells, infected with Mengoencephalitis, was incubated with ribonuclease before phenol treatment. This results in a degradation of any virus RNA or precursor of this which is present in free form, as well as cellular RNA. However, this treatment had little if any effect on the infectivity of the active component which was isolated with phenol after the ribonuclease treatment. Thus, the infectious RNA probably originates from intact virus particles.

(2) *Proof that Infectivity Was Due to RNA.* The nitrogen/phosphorus ratio of

[161] R. C. Valentine and A. Isaacs, *J. Gen. Microbiol.* **16,** 195 (1957).
[162] R. C. Valentine and A. Isaacs, *J. Gen. Microbiol.* **16,** 680 (1957).
[163] J. S. Colter, H. H. Bird, and R. A. Brown, *Nature* **179,** 859 (1957).
[164] J. S. Colter, H. H. Bird, A. W. Moyer, and R. A. Brown, *Virology* **4,** 522 (1957).

the infectious material was 1.6–1.7, in good agreement with rather pure nucleic acid preparations. The material contained no protein which could be detected either by the biuret reaction or by acid hydrolysis followed by paper chromatography of amino acids.

A short incubation of the RNA preparations with ribonuclease or a longer incubation of 6 hours at 37°C. without enzymes destroyed the activity. Neither treatment had any significant effect on the infectivities of the corresponding viruses. The infectious material could be precipitated from 1 *M* NaCl solutions, whereas the virus could not. When samples of virus and RNA preparations were centrifuged at 30,000 r.p.m. for 60 minutes—conditions under which virus but not viral RNA sedimented—the infectivity was markedly reduced in the supernatants of virus preparations, whereas that of RNA was but slightly affected.

The infectivity of RNA from Mengo encephalitis virus was destroyed by treatment with either Mengo antiserum (monkey) or normal monkey serum. On the other hand, the infectivity of the virus was reduced only by specific antiserum. The loss of infectivity, in this case, is probably due to the fact that the serum contains ribonuclease activity.[159]

b. RNA from Equine Encephalomyelitis Virus[165, 166]

An infectious RNA component was isolated from the brains of mice infected with equine encephalomyelitis virus of both eastern and western type (EEE virus, WEE virus) after treatment with phenol. The infectivity could be tested in mice by intracerebral injection of 0.03 ml. RNA or in embryonated eggs by injection of 0.2 ml. RNA in the allantois sac. Infectious material could be obtained, only, by adding phenol to the tissues before homogenization. If the tissues were homogenized prior to phenol extraction, the yield of RNA was significantly lower and the preparation was not infectious. This loss in infectivity and the low yield is probably due to ribonuclease activity. Since ribonuclease has little or no effect on the virus infectivity, it is quite possible that the infectious component isolated by phenol treatment is essentially free virus RNA or a precursor of this.

This was supported by the fact that no infectious RNA could be isolated from a purified concentrate of WEE virus by phenol treatment in the cold. After treatment of this virus with hot phenol for 5 minutes at 40 or 50°C., the RNA could be extracted in essentially quantitative yields, but the biological activity of this RNA was only some 10^{-5}% of the original virus preparation.[167]

If bacteria are extracted with hot phenol, nucleic acid and lipopolysaccharides are found in the aqueous phase[59] and, therefore, it may be that the lipid coat of EEE virus, which has some 50% lipid, is only dissolved by treating with hot phenol. All the preparations of infectious RNA were sensitive to ribonuclease and heat treatment (4.5 hours, 37°C.) The activity could be precipitated with alcohol with no loss in infectivity, whereas virus was inactivated by similar treatment.

c. RNA from Poliomyelitis Virus

Infectious RNA was extracted from the brains and spinal cords of hamsters which had been infected 18–24 hours previously by intracerebral injection with a suspension of type II MEF_1 strain of poliomyelitis virus.[164] The isolation and characterization

[165] E. Wecker and W. Schäfer, *Z. Naturforsch.* **12b**, 415 (1957).
[166] E. Wecker, *Z. Naturforsch.* **14b**, 370 (1959).
[167] E. Wecker, *Virology* **7**, 241 (1959).

of virus RNA was essentially the same as that for Mengo and West Nile encephalitis viruses.

Alexander *et al.*[157, 158, 159] isolated RNA from purified type I and type II polio virus which was grown in either monkey kidney or human amnion cells in monolayer. Osmotic shock and dodecyl sulfate treatment of the virus did not yield detectable amounts of infectious RNA, but treatment of the virus with phenol, in the cold, yielded infectious RNA. The infectivity of the RNA solution was tested on HeLa and human amnion cell monolayers on which the virus forms plaques. The RNA preparations produced confluent cytopathogenic effects on these monolayers if an undiluted preparation was tested directly after the phenol treatment. The cytopathogenic effect occurs even when the period of contact is only 30 seconds. When diluted with physiological saline solutions, the infectivity is greatly decreased, but after dilution in hypertonic salt solutions (1 *M* NaCl with 0.04 *M* phosphate buffer, pH 7.2) a high degree of infectivity is maintained. In appropriate environments, the degree of infectivity (plaque-forming units) of the RNA is proportional to the dilution factor. The infectivity is almost 1% of the infectivity of the virus and remains stable for 6–8 weeks when stored at −70°C.

The RNA preparations showed a UV spectrum characteristic for nucleic acid. The protein content was not determined. Proteolytic enzymes (chymotrypsin and papain) had no effect on the infectivity. Ribonuclease destroyed the activity and so did normal monkey serum which contains ribonuclease. Deoxyribonuclease, normal globulin, and polio-immune gamma globulin from rabbit serum, all of which showed no ribonuclease activity, had no effect upon the infectivity.

d. Ribonucleic Acid from Other Viruses

Franklin *et al.*[168] prepared an infectious nucleic acid fraction from brains of mice infected with mouse encephalomyelitis virus (ME virus) by cold and hot phenol treatment. In contrast to the greatly reduced infectivity of EEE virus-RNA prepared with hot phenol, the infectivity of ME virus-RNA obtained with hot phenol treatment was the same as that of RNA isolated from ME virus-infected tissues by cold phenol extraction. The procedure used for Mengo encephalitis virus was also used here. This consists of ribonuclease treatment of homogenates of infected tissues prior to phenol extraction. The results of this experiment indicate that the infectious RNA is present in the cell in a form which is resistant to ribonuclease, probably as intact virus. This is in contrast to the situation with EEE virus. Infectious RNA was also isolated from virus which had been partially purified using the fluorocarbon Arcton 63. This agent does not affect the infectivity of ME virus, whereas an extensive treatment with Arcton 63 does destroy the infectious principle in EEE virus-infected tissues which are the source of infectious EEE virus-RNA.[169] It was also possible to isolate infectious RNA from a highly purified preparation of ME virus. In all cases, the infectious ME virus-RNA was assayed by intracerebral injection in mice. Discrete plaques could be produced on monolayers of Earle's L-cells but were not suitable for quantitative tests. Huppert and Sanders[170] employed the phenol method to prepare an infectious RNA component from cells of the Krebs 2 mouse ascites carcinoma which had been infected with murine encephalomyocarditis virus. The authors indicate that intact virus cannot be the sole source of infectious RNA

[168] R. M. Franklin, E. Wecker, and C. Henry, *Virolgy* **7**, 220 (1959).
[169] R. M. Franklin and E. Wecker, unpublished data (1959).
[170] J. Huppert and F. K. Sanders, *Nature* **182**, 515 (1958).

in this case. Infected tissue culture fluid of tumor cells was centrifuged until most of the virus was sedimented. The infectious RNA obtained by phenol extraction of the supernate was of much higher titer than was to be expected if RNA were extracted solely from virus. On the other hand, treatment of infected cell homogenates with ribonuclease removed UV absorbing material without affecting the infectivity which could afterwards be extracted by phenol. This indicates that the potentially infective material must normally be present intracellularly in a form not identical with the virus but resistant to the action of ribonuclease. The origin and cellular location of this material and the part it plays in virus synthesis are, at present, completely unclear.

It has also been possible to prepare infectious RNA by phenol extraction of pig kidney tissue cultures and from the muscle and heart of suckling mice which had been infected with foot and mouth disease virus.[171, 172] Infectivity of the RNA was tested in pig kidney cell monolayer cultures as well as by intramuscular or intracerebral injection in mice. The various tests designed to differentiate infectious RNA from infectious virus were also employed here. There was a further differential test in that infectious RNA had little or no infectivity when tested by the intraperitoneal route, whereas virus is highly infectious when inoculated by this route.

The phenol extract of mice brains infected with Semliki forest virus, a virus which can also multiply in mosquitoes, yielded an RNA fraction which was also studied in comparison with intact virus by the known differentiating procedures.[173] Virus and RNA also reacted differently after treatment with sodium deoxycholate. This reagent, which has been shown to inactivate many of the arthropod-borne viruses,[174] also inactivates the Semliki forest virus but does not affect the infectivity of RNA.

4. Size of Infectious Ribonucleic Acids

The determination of the molecular weight of infectious RNA from animal viruses presents certain technical difficulties. The main difficulty lies in the preparation of sufficient virus RNA in pure form. This difficulty is present in the extraction of RNA from purified virus preparations, as well as from infected cells. In the latter case, the entire cell RNA will be extracted and this will interfere with any characterization of the virus RNA. On the other hand, it is relatively easy to separate the total RNA from cellular DNA by employing certain salts during the phenol extraction which prevent the release of DNA from cell structures into the aqueous phase,[175] or by making use of the different solubilities of the two types of nucleic acid in 1 *M* NaCl. RNA, itself, can also be fractionated with 1 *M* NaCl. Only the high molecular weight RNA can be precipitated in 1 *M* NaCl,[176, 177]

[171] F. Brown, R. F. Sellers, and D. L. Stewart, *Nature* **182**, 535 (1958).
[172] M. Mussgay and K. Strohmaier, *Zentr. Bakteriol., Parasitenk. Infektionskrankh. u. Hyg.* **173**, 163 (1958).
[173] P. Y. Cheng, *Nature* **181**, 1800 (1958).
[174] M. Theiler, *Proc. Soc. Exptl. Biol. Med.* **96**, 380 (1957).
[175] K. S. Kirby, *Biochem. J.* **66**, 495 (1957).
[176] F. F. Davis and F. W. Allen, *J. Biol. Chem.*, **227**, 907 (1957).
[177] J. S. Colter and R. A. Brown, *Science* **124**, 1077 (1956).

whereas low molecular weight RNA remains in solution and can be precipitated only with alcohol.

Colter *et al.*[143, 177, 178] prepared RNA from Ehrlich ascites carcinoma cells by phenol extraction. This was not homogeneous in that $^2/_3$ of the material exhibited a sedimentation constant of less than 3 *S*, and the remaining RNA sedimented into two components with sedimentation constants of 32 and 15 *S*. A comparison was made between RNA extracted from noninfected Ehrlich carcinoma cells with that from cells infected with Mengo and West Nile viruses. A similar comparison was made between RNA extracted from noninfected hamster brains and brains from hamsters infected with poliovirus. In all cases, there was no qualitative difference in the ultracentrifuge patterns of RNA from infected and noninfected material. The RNA components having sedimentation constants of 15 and 32 *S* could be quantitatively precipitated with 1 *M* NaCl and the infectivity was always recovered quantitatively in the precipitate. The infective component probably sedimented with the 32 *S* component.

Studies on nucleic acid isolated from chicken embryos infected with EEE virus led to similar results.[166] Three components could be identified in the ultracentrifuge. Two high molecular weight components sedimented with s_{20} approximately 31 and 19 *S* and the cellular DNA sedimented with $s_{20} \sim 12$ *S* when this was present in the preparation. Also, in this case, it is most likely that the infectivity sediments with the 31 *S* component.

Strohmaier and Mussgay[179] centrifuged infectious RNA from foot and mouth disease virus in a density gradient (D_2O-H_2O), and found a value of 37 *S* for the infectious component.

In general, it is possible to determine the molecular weight of infectious RNA by measurements of sedimentation and viscosity [see Section III, *c*, (1)]. However, virus RNA is only an insignificant fraction of the total RNA extracted from virus-infected cells and, therefore, it is not possible to determine the molecular weight of virus RNA from measurements of sedimentation and viscosity on such extracts since the viscosity of virus RNA cannot be determined in the presence of an overwhelming higher concentration of cellular RNA. However, the molecular weight may be estimated from the sedimentation constant alone by employing the empirical relationship between sedimentation constant s and molecular weight m determined for TMV-RNA.[68]

$$m = 1100\, s^{2.2} \tag{9}$$

This formula can be applied, generally, only under two conditions: (1)

[178] R. A. Brown, M. C. Davies, J. S. Colter, J. B. Logan, and D. Kritchevsky, *Proc. Natl. Acad. Sci. U.S.* **43**, 857 (1957).

[179] K. Strohmaier and M. Mussgay, *Z. Naturforsch.* **14b**, 171 (1959).

RNA from different viruses should have approximately the same density. (2) The different nucleic acids must have a basic structure similar to that of TMV-RNA. Similarities in molecular size and structure between TMV-RNA and an RNA component from plant and animal tissues have recently been demonstrated.[180] On the other hand, there have not yet been any comparative studies between nucleic acids from different viruses. If the above mentioned equation is used to calculate the molecular weight of the virus RNA specimens which have been studied, the molecular weight of RNA from West Nile, Mengo, and poliomyelitis virus is about 2×10^6, and that from foot and mouth disease is about 3×10^6. Hence, it seems likely that, also, in case of RNA from animal viruses, only a high molecular weight component possesses the ability to induce infection.

5. Reaction of Infectious Ribonucleic Acids with Chemical Agents

The reaction of infectious RNA of animal viruses with chemical agents has only been studied in detail for hydroxylamine and nitrous acid. Chemical methods have not yet been employed to characterize this type of RNA in more detail.

a. Reaction with Hydroxylamine

Certain bacterial viruses can be inactivated with hydroxylamine and in this case the inactivation seems to be due to alteration of virus protein, probably by splitting thiolester bonds.[181] A series of animal RNA viruses have been inactivated with hydroxylamine, and among these are mouse encephalomyelitis virus, WEE virus, fowl plaque virus, and swine influenza.[182] Other RNA-containing viruses, such as NDV and mumps virus, are resistant to this compound. Infectious RNA from ME virus is inactivated by hydroxylamine at a rate comparable to that of the virus itself. Thus, it appears likely that hydroxylamine inactivates the ME virus by reacting with the RNA component rather than with the protein. This is supported by the fact that certain properties of virus protein are not influenced by hydroxylamine inactivation. Among such properties which have been tested are serological properties of ME virus, serological properties of fowl plague virus, as well as enzymic activity and hemagglutinating activity of fowl plague virus.

The nature of the reaction of hydroxylamine with RNA is not yet known. Model experiments were made using high molecular weight RNA from various sources and these showed that hydroxylamine apparently does not split the phosphoester linkages of the RNA backbone. Further, the products

[180] A. Gierer, *Z. Naturforsch.* **13b,** 788 (1958).
[181] L. M. Kozloff, M. Lute, and K. Henderson, *J. Biol. Chem.* **228,** 511 (1957).
[182] R. M. Franklin and E. Wecker, *Nature.* **184,** 343 (1959).

of alkaline and acid hydrolysis of treated nucleic acid showed no differences from those of untreated nucleic acid. The fact that hydroxylamine can inactivate free RNA from animal viruses as well as from TMV,[101] but does not attack all RNA-containing viruses, suggests that this agent attacks a special configuration in the nucleic acid component of certain viruses since it appears that all nucleic acids have certain chemical properties, such as types of bases and phosphoester backbone, in common. Franklin and Wecker[182] concluded that this configuration could be an amino acyl ester or some other acyl group in an ester linkage, located at a terminal position on the high molecular weight RNA chain as is the case with the low molecular weight soluble RNA from liver cells. They based their assumption on the similarity between the stability of infectious RNA and the amino acyl ester bond of the soluble RNA at different pH values, as well as on similarities in behavior with hydroxylamine and hydrazine. Neither hydrazine nor semicarbazide caused an inactivation of infectious RNA from ME virus. A more direct approach to this problem is difficult because the direct determination of probably one amino acyl group in one molecule of RNA seems to be impossible.

b. Reaction with Nitrous Acid

The ribonucleic acid of mouse encephalomyelitis virus can be inactivated by nitrous acid as was the case for TMV and its nucleic acid.[183] Since it is still quite difficult to obtain large amounts of infectious RNA from animal viruses, and since the biological test for infectious RNA from animal viruses is not yet completely satisfactory, kinetics of inactivation with HNO_2 were studied in more detail with intact viruses. All viruses which have been tested up to date were inactivated with nitrous acid. Among these viruses are poliomyelitis virus, mouse encephalomyelitis virus, Newcastle disease virus, and influenza virus. The reaction kinetics showed that all viruses studied are inactivated more rapidly than the free RNA from TMV.[102] In the case of poliovirus and influenza virus, the RNA content per virus particle is the same as that per TMV particle. Furthermore, it is unlikely that the amino bases in RNA from different sources react differently with nitrous acid. Thus, the more rapid inactivation of animal viruses may be due to an alteration of other viral components by HNO_2.

ACKNOWLEDGMENT

The author is much indebted to Dr. R. M. Franklin for the translation of the manuscript.

[183] W. Schäfer, H. Schuster, T. Zimmermann, and R. Rott, *Z. Naturforsch.* **14b**, 632 (1959).

CHAPTER 35

Biosynthesis of Purine Nucleotides*

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* The following are the systematic names corresponding to the trivial names used in this chapter: 2-amino-*N*-ribosylacetamide-5′-phosphate, glycinamide ribonucleotide; 2-formamido-*N*-ribosylacetamide-5′-phosphate, formylglycinamide ribonucleotide; 2-formamido-*N*-ribosylacetamidine-5′-phosphate, formylglycinamidine ribonucleotide; 5-amino-1-ribosylimidazole-5′-phosphate, 5-aminoimidazole ribonucleotide; 5-amino-1-ribosyl-4-imidazolecarboxylic acid 5′-phosphate, 5-amino-4-imidazolecarboxylic acid ribonucleotide; *N*-(5-amino-1-ribosyl-4-imidazolecarbonyl)-L-aspartic acid 5′-phosphate, 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide; 5-amino-1-ribosyl-4-imidazolecarboxamide-5′-phosphate, 5-amino-4-imidazolecarboxamide ribonucleotide; 5-formamido-1-ribosyl-4-imidazolecarboxamide-5′-phosphate, 5-formamido-4-imidazolecarboxamide ribonucleotide; [6-(succinylamino)-9-(ribofuranosyl 5′-phosphate) purine], adenylosuccinic acid.

The following abbreviations have been used: adenosine triphosphate, ATP; adenosine diphosphate, ADP; adenylic acid, AMP; guanosine triphosphate, GTP; guanosine diphosphate, GDP; 5,6,7,8-tetrahydrofolic acid, FH_4; orthophosphate, P_i.

The number of the atom of the purine ring may be designated as follows: nitrogen atom 1, N-1, carbon atom 2, C-2, etc.

Since primary emphasis will be placed on the enzymic synthesis of the purine nucleotides, the reader is referred to the chapter on the Biosynthesis of Purines and Pyrimidines by Reichard[1] in Volume II of this book for earlier work on this subject. Also reference may be made to two recent reviews on the synthesis of purine nucleotides which treat the subject either in more detailed form or from another point of view.[2, 3]

[1] P. Reichard, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. II, p. 277. Academic Press, New York, 1955.

[2] S. C. Hartman and J. M. Buchanan, *Ergeb. Physiol. u. exptl. Pharmakol.* **50**, 71 (1959).

[3] J. M. Buchanan and S. C. Hartman, *Advances in Enzymol.* **21**, 200 (1959).

I. Introduction

In this chapter it is proposed to review the major lines of evidence upon which the current formulation of the reactions of purine synthesis *de novo* is based. This evidence has come from isotopic experiments with the intact animal and with extracts and homogenates of tissues and finally from experiments with isolated enzyme systems. The use of microbial mutants and of systems blocked with metabolic poisons has been of great value. From a technical point of view the modern armamentarium of methods for the analysis, isolation, and purification of new compounds and enzymes has provided the means of solving complex problems of biosynthesis which would not have readily been amenable to study a decade or more ago. Possibly the greatest contributing factor to the solution of the problem of purine nucleotide synthesis has come from the fact that avian liver represents a source from which all the enzymes of purine synthesis can be obtained in soluble form and in relatively high activity.

II. Precursors of the Purines

The study of the synthesis of the purine nucleotides in isolated enzyme systems was preceded by an investigation of the metabolic precursors of the purines (Volume II, Chapter 23). Schoenheimer and his colleagues had shown that excretory purines and purines of nucleic acids and nucleotides are readily formed from the nitrogen of administered ammonium salts.[4, 5] In contrast, purine or pyrimidine bases, with the exception of adenine,[6] were not major sources of tissue purines in the rat. Likewise, urea,[3] histidine,[7] and arginine[8] are not precursors as such of the purine ring. These experiments served to indicate that purines as well as pyrimidines are formed from simple metabolic units rather than from preformed dietary materials of more complex structure.

The precursors of the carbon atoms of uric acid were determined by feeding certain compounds labeled with C^{13} followed by measuring the C^{13} concentration of the individual carbon atoms of the excreted uric acid.[9-12] The individual carbon atoms of the purine ring have their origin in the precur-

[4] F. W. Barnes, Jr., and R. Schoenheimer, *J. Biol. Chem.* **151,** 123 (1943).
[5] A. A. Plentl and R. Schoenheimer, *J. Biol. Chem.* **153,** 203 (1944).
[6] G. B. Brown, P. M. Roll, A. A. Plentl, and L. F. Cavalieri, *J. Biol. Chem.* **172,** 469 (1948).
[7] C. Tesar and D. Rittenberg, *J. Biol. Chem.* **170,** 35 (1947).
[8] K. Bloch, *J. Biol. Chem.* **165,** 477 (1947).
[9] J. C. Sonne, J. M. Buchanan, and A. M. Delluva, *J. Biol. Chem.* **166,** 395 (1946).
[10] J. M. Buchanan and J. C. Sonne, *J. Biol. Chem.* **166,** 781 (1946).
[11] J. C. Sonne, J. M. Buchanan, and A. M. Delluva, *J. Biol. Chem.* **173,** 69 (1948).
[12] J. M. Buchanan, J. C. Sonne, and A. M. Delluva, *J. Biol. Chem.* **173,** 81 (1948).

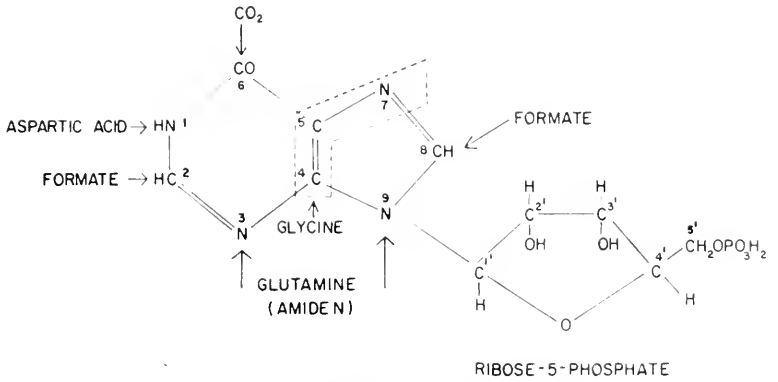


FIG. 1. Precursors of inosinic acid.

sors indicated in Fig. 1. Glycine[13, 14] plays the central role in the synthesis, the carboxyl and α carbon atoms and the nitrogen atom contributing to carbon atoms 4 and 5 and nitrogen atom 7 of the purine ring. CO_2 is the precursor of carbon atom 6, and formate of carbon atoms 2 and 8. It was more difficult, however, to determine the precursors of the three remaining nitrogen atoms by *in vivo* experiments since the rapid metabolism of some of the common nitrogenous compounds made it difficult to distinguish between their reactions and those of ammonium salts. With the discovery by Greenberg[15] that synthesis of the purine compound, hypoxanthine, could be accomplished from radioactive precursors in homogenates of pigeon liver it was possible to continue studies on the remaining nitrogenous precursors. When soluble extracts[16, 17] were used as the source of enzyme, the side reactions of the amino acids were substantially reduced and ammonium salts were not a major source of purine nitrogen.

In pigeon liver extracts[18] the labeled substrates glycine, formate, and CO_2 were utilized for purine synthesis in the ratio of 1:2:1. In double-labeling experiments two moles of amide N^{15} of glutamine and one mole of N^{15}-labeled nitrogen of aspartic (or glutamic) acid were utilized for every mole of C^{14}-labeled glycine.[19] Upon degrading the labeled purine formed it was found that N-3 and N-9 were derived from the amide nitrogen of glutamine and N-1 was derived from aspartic acid.[20] Although glutamic acid

[13] J. L. Karlsson and H. A. Barker, *J. Biol. Chem.* **177**, 597 (1949).
[14] D. Shemin and D. Rittenberg, *J. Biol. Chem.* **167**, 875 (1947).
[15] G. R. Greenberg, *Arch. Biochem.* **19**, 337 (1948).
[16] G. R. Greenberg, *Federation Proc.* **10**, 192 (1951).
[17] M. P. Schulman and J. M. Buchanan, *Federation Proc.* **10**, 244 (1951).
[18] M. P. Schulman, J. C. Sonne, and J. M. Buchanan, *J. Biol. Chem.* **196**, 499 (1952).
[19] J. C. Sonne, I. Lin, and J. M. Buchanan, *J. Biol. Chem.* **220**, 369 (1956).
[20] B. Levenberg, S. C. Hartman, and J. M. Buchanan, *J. Biol. Chem.* **220**, 379 (1956).

may donate its nitrogen atom to N-1 of hypoxanthine, it does so only indirectly through transamination to form the more direct precursor, aspartic acid.

To the list of purine precursors must be added ribose phosphate. In his studies on the synthesis of purine compounds in pigeon liver homogenates, Greenberg[21] demonstrated that two radioactive purine compounds, hypoxanthine and inosinic acid, are derived from radioactive formate. By comparison of the change in specific activity of the two products he concluded that the nucleotide was formed first and then degraded to the base. The synthesis of inosinic acid could be stimulated by the addition of ribose-phosphate to the incubation medium. Ribose-5-phosphate or nucleosides which yielded ribose-1-phosphate on phosphorolysis served equally well as stimulatory agents of purine synthesis.

These experiments of Greenberg were based in part on earlier findings of Krebs and his associates[22, 23] who found that hypoxanthine is formed *de novo* in pigeon liver slices from unknown precursors. The synthesis was stimulated by the addition of glutamine and oxalacetic acid to the slices, but at the time of these experiments in 1936 it was not possible to determine the role of these substances in the synthesis. In light of the N^{15} experiments described above it is evident that glutamine served as the source of two of the nitrogen atoms directly and that oxalacetate participated in the synthesis of the other nitrogen donor, aspartic acid.

The finding by Edson, Model, and Krebs[22] that hypoxanthine accumulated in pigeon liver slices emphasized the relative importance of the reduced purine compounds and contributed to the later discovery that formate rather than CO_2 was the precursor of the carbon atoms 2 and 8 of the purine ring. While hypoxanthine is oxidized to uric acid in the livers of most animals, this compound accumulates in pigeon liver preparations because of the absence of the enzyme, xanthine oxidase, in this tissue (Volume II, Chapter 23).

III. Enzymic Reactions of Inosinic Acid Synthesis *de Novo*

In general terms the procedure for the detection and isolation of the enzymes and intermediates concerned with the synthesis of the purine nucleotides has been to incubate radioactive precursors with crude extracts of pigeon or chicken liver and then to follow the accumulation of radioactive nonpurine intermediates. Separation of these new compounds was usually accomplished by use of ion-exchange or paper chromatographic procedures. A number of the intermediates are heterocyclic amine deriva-

[21] G. R. Greenberg, *J. Biol. Chem.* **190,** 611 (1951).
[22] N. L. Edson, H. A. Krebs, and A. Model, *Biochem. J.* **30,** 1380 (1936).
[23] Å. Orström, M Orström, and H. A. Krebs, *Biochem. J.* **33,** 990 (1939).

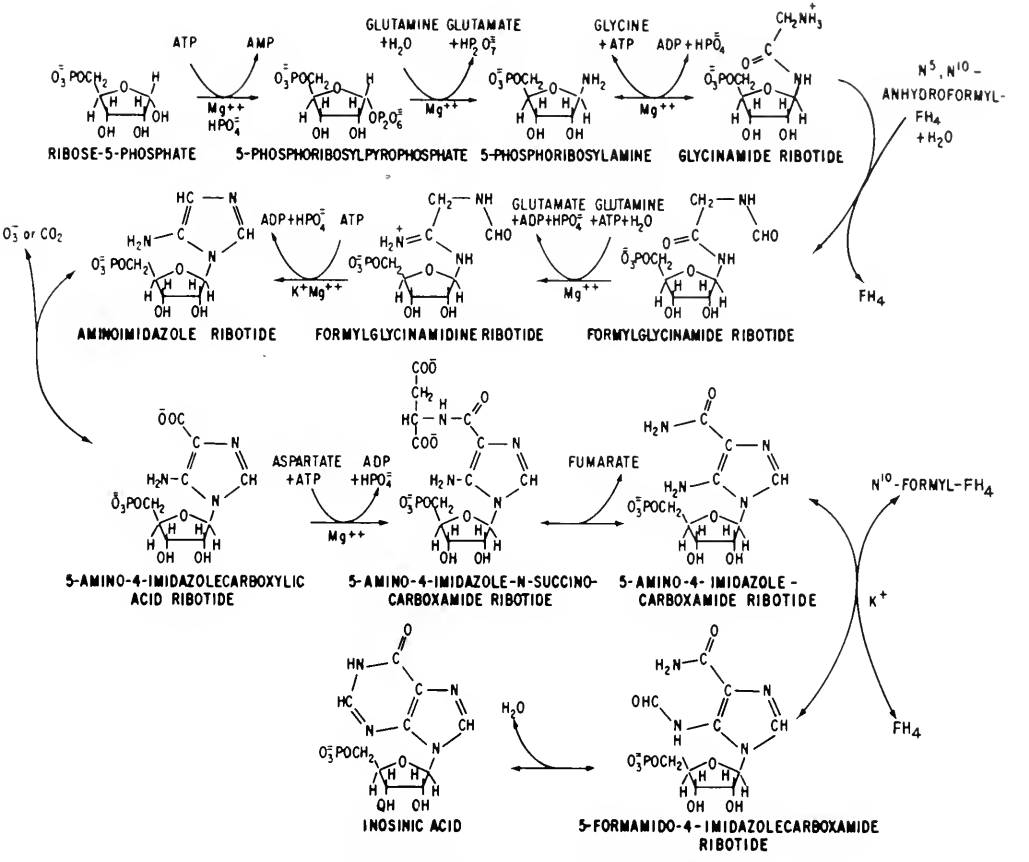


FIG. 2. Enzymic synthesis of inosinic acid *de novo*.

tives which are able to undergo diazotization and coupling with N-1-naphthylethylenediamine according to the procedure of Bratton and Marshall[24] to yield colored products with characteristic absorption spectra. These circumstances have permitted the identification and differentation of these cyclic intermediates. These heterocyclic intermediates may also be distinguished as a group from such arylamines as *p*-aminobenzoic acid by virtue of the fact that they are not readily acetylated by acetic anhydride whereas the carbocyclic amines are.[25] The compounds and reactions concerned with the synthesis of inosinic acid from its precursors are shown in Fig. 2, and the reactions for the synthesis of guanylic and adenylic acids from inosinic acid are shown in Fig. 3.

[24] A. C. Bratton and E. K. Marshall, Jr., *J. Biol. Chem.* **128**, 537 (1939).
[25] J. M. Ravel, R. E. Eakin, and W. Shive, *J. Biol. Chem.* **172**, 67 (1948).

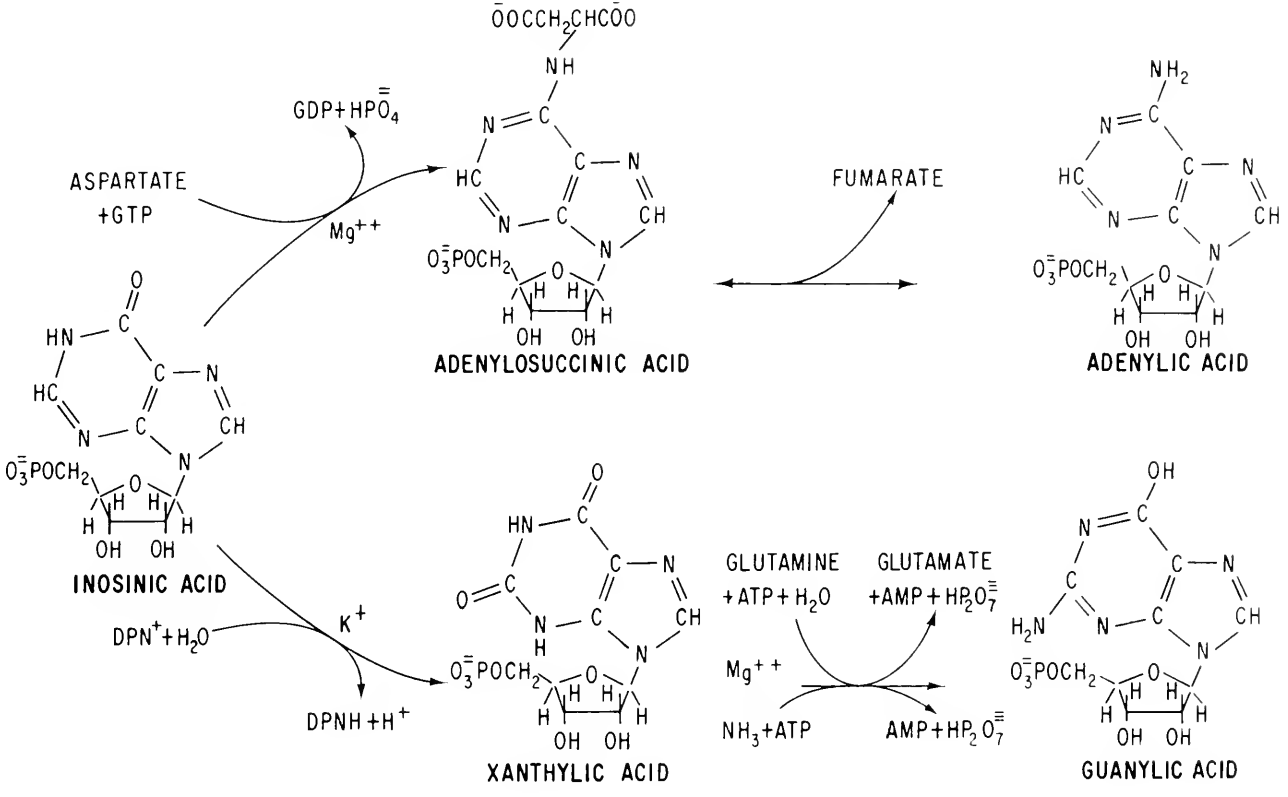


FIG. 3. Enzymic synthesis of adenylic and guanylic acids from inosinic acid.

Because of the central location in the purine ring of the carbon and nitrogen atoms derived from glycine, it was considered likely that this precursor would participate in one of the early reactions of purine biosynthesis. This indeed proved to be the case. Goldthwait *et al.*[26] found that two phosphoribosyl derivatives of glycinamide accumulated when glycine, glutamine, formate, ribose-5-phosphate, and ATP were incubated with crude extracts of pigeon liver. The two compounds[26-29] have been isolated, purified, and demonstrated to have the structures shown in Fig. 4. These compounds have been given the trivial names, glycinamide ribonucleotide and formylglycinamide ribonucleotide. In the absence of formate or of tetrahydrofolic acid or its formyl derivative, formylglycinamide ribonucleotide is not formed:

$$\text{Ribose-5-phosphate} + \text{ATP} \rightarrow \text{5-phosphoribosyl-1-pyrophosphate} + \text{AMP} \quad (1)$$

The discovery that phosphoribosyl derivatives were involved in purine biosynthesis had initiated a series of investigations to determine the nature of the reaction and the intermediates involved in the synthesis of these compounds. Toward this end the synthesis of inosinic acid from hypoxanthine, ribose-5-phosphate, and ATP was studied by Williams and Buchanan,[30] and by Korn *et al.*[31, 32] From this model system a new mechanism for the synthesis of nucleotides from bases was discovered which involved the reaction of ribose-5-phosphate and ATP [Eq. (1)] to yield a new ribosyl intermediate first identified by Kornberg and his associates[33] as 5-phosphoribosyl-1-pyrophosphate. It is now known that the two terminal phosphate groups of ATP are transferred as a unit to ribose-5-phosphate in the presence of the enzyme 5-phosphoribose pyrophosphokinase.[34] 5-Phosphoribosylpyrophosphate may undergo condensation with a number of bases with the liberation of inorganic pyrophosphate in the presence of a

[26] D. A. Goldthwait, R. A. Peabody, and G. R. Greenberg, *J. Am. Chem. Soc.* **76,** 5258 (1954).

[27] S. C. Hartman, B. Levenberg, and J. M. Buchanan, *J. Am. Chem. Soc.* **77,** 501 (1955).

[28] S. C. Hartman, B. Levenberg, and J. M. Buchanan, *J. Biol. Chem.* **221,** 1057 (1956).

[29] R. A. Peabody, D. A. Goldthwait, and G. R. Greenberg, *J. Biol. Chem.* **221,** 1071 (1956).

[30] W. J. Williams and J. M. Buchanan, *J. Biol. Chem.* **203,** 583 (1953).

[31] E. D. Korn and J. M. Buchanan, *Federation Proc.* **12,** 233 (1953).

[32] E. D. Korn, C. N. Remy, H. C. Wasilejko, and J. M. Buchanan, *J. Biol. Chem.* **217,** 875 (1955).

[33] A. Kornberg, I. Lieberman, and E. S. Simms, *J. Biol. Chem.* **215,** 389 (1955).

[34] H. G. Khorana, J. F. Fernandes, and A. Kornberg, *J. Biol. Chem.* **230,** 941 (1958).

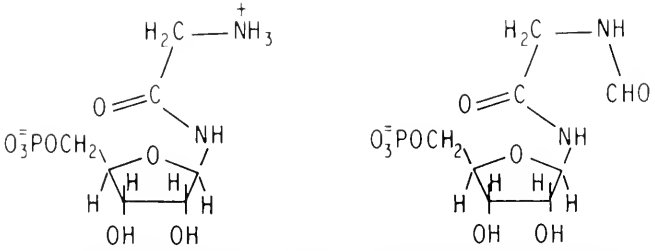


FIG. 4. Structures of glycinamide ribonucleotide and formylglycinamide ribonucleotide.

class of enzymes called nucleotide pyrophosphorylases.[35-37] A nucleotide phosphorylase has been isolated which utilizes the bases hypoxanthine, guanine, and 6-mercaptopurine[38] and is different from another enzyme of the same class which reacts adenine and 5-amino-4-imidazolecarboxamide.[39] Still other nucleotide pyrophosphorylases are involved in the synthesis of the pyrimidine nucleotides.[36, 40]

5-Phosphoribosylpyrophosphate is a reagent of great versatility in the enzymic reactions concerned with the synthesis of phosphoribosyl compounds. Of considerable interest is the fact that it is also involved in the synthesis of glycinamide ribonucleotide from glycine.[27] An enzyme[41, 42] 5-phosphoribosylpyrophosphate amidotransferase has been isolated from avian liver which catalyzes the reaction of 5-phosphoribosylpyrophosphate with glutamine to yield 5-phosphoribosylamine, glutamic acid, and inorganic pyrophosphate according to Eq. (2). Although 5-phosphoribosyl-

$$\text{5-Phosphoribosylpyrophosphate} + \text{glutamine} + H_2O \rightarrow \text{5-phosphoribosylamine} + \text{glutamic acid} + \text{pyrophosphate} \quad (2)$$

amine is presumed to be the product of Eq. (2), it has not been isolated so far because of its chemical instability. Incubation of the purified enzyme with P^{32}-labeled pyrophosphate or with C^{14}-labeled glutamic acid and other participants of the reaction does not lead to the formation of radioactive 5-phosphoribosylpyrophosphate or glutamine. These experiments are taken as evidence of the essential irreversibility of the reaction. There is evidence that an inversion of the configuration at position 1 of the ribose takes place

[35] C. N. Remy, W. T. Remy, and J. M. Buchanan, *J. Biol. Chem.* **217,** 885 (1955).
[36] I. Lieberman, A. Kornberg, and E. S. Simms, *J. Biol. Chem.* **215,** 403 (1955).
[37] A. Kornberg, I. Lieberman, and E. S. Simms, *J. Biol. Chem.* **215,** 417 (1955).
[38] L. N. Lukens and K. A. Herrington, *Biochim. et Biophys. Acta* **24,** 432 (1957).
[39] J. G. Flaks, M. J. Erwin, and J. M. Buchanan, *J. Biol. Chem.* **228,** 201 (1958).
[40] I. Crawford, A. Kornberg, and E. S. Simms, *J. Biol. Chem.* **226,** 1093 (1957).
[41] D. A. Goldthwait, *J. Biol. Chem.* **222,** 1051 (1956).
[42] S. C. Hartman and J. M. Buchanan, *J. Biol. Chem.* **233,** 451 (1958).

in this reaction by a nucleophilic attack of the nitrogen atom of glutamine upon the carbon atom bearing the pyrophosphate substituent. 5-Phosphoribosylpyrophosphate has been demonstrated to be of the α configuration. 5-Phosphoribosylamine is presumed to be a β-ribofuranosyl derivative since during the further reactions of purine synthesis the β-nucleotides are formed without any other apparent opportunity for change in configuration on the part of the purine nucleotide intermediates. It has been suggested that the several operations of the reaction, i.e., the release of pyrophosphate, the transfer of the amide group, and the introduction of the elements of water are catalyzed by one enzyme without the formation of a discrete covalent intermediate such as *N*-(5-phosphoribosyl)glutamine.

The second step in purine biosynthesis[41, 43] involves the reaction of 5-phosphoribosylamine, ATP, and glycine to yield glycinamide ribonucleo-

$$\text{5-Phosphoribosylamine} + \text{glycine} + \text{ATP} \rightleftarrows \text{glycinamide ribonucleotide} + \text{ADP} + \text{orthophosphate} \quad (3)$$

tide according to Eq. (3). This reaction is freely reversible. The amide bond is not broken in the splitting of glycinamide ribonucleotide unless both ADP and orthophosphate are present. Arsenate may replace orthophosphate but ADP is still an essential component of the system. In addition the oxygen of orthophosphate labeled with O^{18} is transferred to the carboxyl carbon of glycine during its formation from glycinamide ribonucleotide. These observations have led to the postulation that the synthesis and breakdown of glycinamide ribonucleotide occurs when all three substrates (either 5-phosphoribosylamine, glycine, and ATP or glycinamide ribonucleotide, ADP, and orthophosphate depending upon the direction of reaction) are present and can interact simultaneously. The enzyme system responsible for this synthesis has been called glycinamide ribonucleotide kinosynthase.

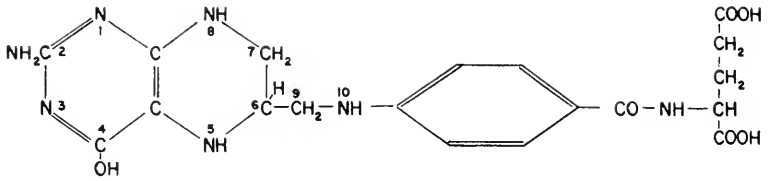
The formylation of glycinamide ribonucleotide to formylglycinamide ri-

$$N^5,N^{10}\text{-Anhydroformyl-FH}_4 + \text{glycinamide ribonucleotide} + \text{H}_2\text{O} \rightarrow \text{formylglycinamide ribonucleotide} + \text{FH}_4 + \text{H}^+ \quad (4)$$

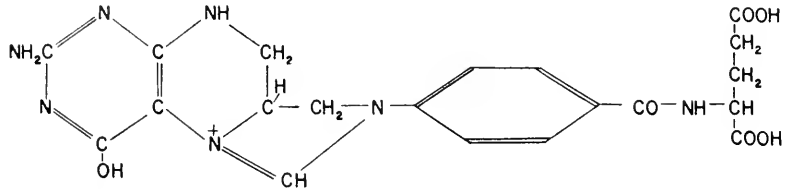
bonucleotide[26, 44] takes place according to Eq. (4). This reaction is very easily measured by determining the amount of tetrahydrofolic acid (FH_4) formed. Under the acid conditions of the Bratton and Marshall procedure tetrahydrofolic acid breaks down to *p*-aminobenzoyl glutamate, which may be diazotized and coupled with *N*-1-naphthylethylenediamine to form a

[43] S. C. Hartman and J. M. Buchanan, *J. Biol. Chem.* **233**, 456 (1958).

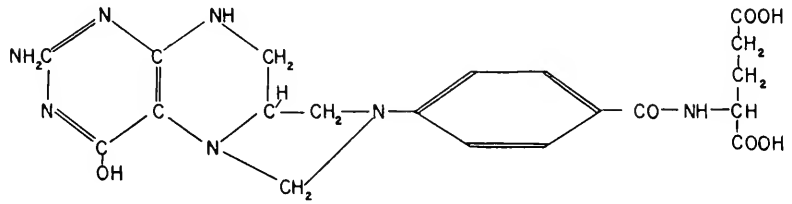
[44] D. A. Goldthwait, R. A. Peabody, and G. R. Greenberg, *J. Biol. Chem.* **221**, 569 (1956).



5,6,7,8-TETRAHYDROFOLIC ACID (FH_4)



N^5,N^{10}-ANHYDROFORMYL TETRAHYDROFOLIC ACID *



N^5,N^{10}-METHYLENE TETRAHYDROFOLIC ACID

FIG. 5. Structure of formylated derivatives of tetrahydrofolic acid. (*N^5,N^{10}-anhydroformyltetrahydrofolic acid has also been called N^5,N^{10}-methenyltetrahydrofolic acid.)

purple-colored product. The enzyme, glycinamide ribonucleotide transformylase, has been purified approximately sixtyfold from chicken liver and has been separated completely from the transformylase responsible for the incorporation of formate carbon into position 2 of the purine ring.[45] The enzyme is sensitive to metal impurities and is inhibited about 50% by a concentration of cupric ion of 10^{-4} M. This inhibition may be relieved by the addition of cyanide.

With crude preparations of enzyme, both N^5,N^{10}-anhydroformyltetrahydrofolic acid (Fig. 5) and N^{10}-formyltetrahydrofolic acid may donate formyl groups to glycinamide ribonucleotide. The fact that both forms are utilized is due to the presence of cyclohydrolase, an enzyme which carries out the hydration of the N^5,N^{10}-anhydroformyltetrahydrofolic acid to the

[45] L. Warren and J. M. Buchanan, *J. Biol. Chem.* **229**, 613 (1957).

N^{10}-formyl derivative [Eq. (5)]. The reaction is reversible. Upon purifica-

$$N^5,N^{10}\text{-Anhydro-FH}_4 + H_2O \rightleftarrows N^{10}\text{-formyl-FH}_4 + H^+ \quad (5)$$

tion of glycinamide ribonucleotide transformylase the cyclohydrolase is lost. It is then possible to demonstrate[46] that the actual donor of the formyl group to glycinamide ribonucleotide is the N^5,N^{10}-anhydroformyl-FH_4 as shown in Eq. (4).

The next step in purine nucleotide biosynthesis[47, 48] involves the reaction of formylglycinamide ribonucleotide with glutamine and ATP to yield

$$\begin{aligned}\text{Formylglycinamide ribonucleotide} + \text{glutamine} + \text{ATP} + H_2O \rightarrow \\ \text{formylglycinamidine ribonucleotide} + \text{ADP} + \text{orthophosphate} \\ + \text{glutamic acid}\end{aligned} \quad (6)$$

formylglycinamidine ribonucleotide according to Eq. (6). Studies with highly purified preparations of the enzyme which catalyze Eq. (6) suggest that this reaction proceeds without the formation of free intermediates. Incubation of P^{32}-labeled orthophosphate or C^{14}-labeled glutamic acid with the enzymic system does not lead to the synthesis of radioactive ATP or glutamine. It is therefore believed that, like the previous glutamine reaction of this series, Eq. (6) is also in effect irreversible.

This enzyme is readily inhibited by low concentrations of the antitumor agents, azaserine and 6-diazo-5-oxonorleucine (Fig. 6)[49] (see also Chapter 39). They compete with the natural substrate of the reaction, glutamine, for the enzyme site. The degree of inhibition is thus a function of the ratio of glutamine to inhibitor. With the chicken liver enzyme there is half inhibition of the reaction when the ratio of concentration of glutamine to azaserine is 18 or when the ratio of concentration of glutamine to 6-diazo-5-oxonorleucine is 727. Mole-for-mole, 6-diazo-5-oxonorleucine is therefore

$NH_2—CO—CH_2—CH_2—CHNH_2—COOH$
Glutamine

$\overset{-\ +}{N{=}N{=}CH—CO—O—CH_2—CHNH_2—COOH}$
Azaserine

$\overset{-\ +}{N{=}N{=}CH—CO—CH_2—CH_2—CHNH_2—COOH}$
6-Diazo-5-oxonorleucine

FIG. 6. Structures of glutamine, azaserine, and 6-diazo-5-oxonorleucine.

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[46] S. C. Hartman and J. M. Buchanan, *4th Intern. Congr. Biochem., Vienna,* **13,** 97 (1958).

[47] B. Levenberg and J. M. Buchanan, *J. Biol. Chem.* **224,** 1019 (1957).

[48] I. Melnick and J. M. Buchanan, *J. Biol. Chem.* **225,** 157 (1957).

[49] B. Levenberg, I. Melnick, and J. M. Buchanan, *J. Biol. Chem.* **225,** 163 (1957).

a better inhibitor than azaserine by a factor of 40. When either inhibitor is incubated with the enzyme in the absence of glutamine there is rapid formation of an inhibitor-enzyme complex which does not dissociate even upon addition of glutamine. The enzyme is thus irreversibly inactivated. Recently radioactive azaserine has been prepared and has been found to react with the purified enzyme in approximately stoichiometric amounts. Because of the combination of azaserine with the enzyme even in the presence of glutamine the inhibition is partly competitive and partly noncompetitive in nature.

Formylglycinamidine ribonucleotide is converted enzymically into the first imidazole intermediate by reaction with ATP according to Eq. (7).[47, 50] The enzyme responsible for this reaction has been isolated from chicken

Formylglycinamidine ribonucleotide + ATP →
5-aminoimidazole ribonucleotide + ADP + orthophosphate (7)

liver and purified with some difficulty due to its relative instability. The activity of the enzyme during incubation shows a pronounced requirement for potassium ions. Likewise, the enzyme is able to withstand dialysis and prolonged storage only in solutions of potassium salts. The enzyme catalyzes an irreversible reaction in which the energy for the closure of the imidazole ring is obtained through the hydrolysis of an anhydride bond of ATP with ADP and orthophosphate as products. 5-Aminoimidazole ribonucleotide may be isolated by a procedure similar to that used for the isolation of the other intermediates of these reactions. The ribonucleotide is isolated by chromatography on an anion exchange column and is then precipitated as the barium salt at neutral pH. 5-Aminoimidazole ribonucleotide reacts with the Bratton and Marshall reagents to yield an orange-colored product with an absorption maximum at 500 mμ. The ribonucleotide itself has only a nonspecific end absorption in the ultraviolet region. The ribonucleotide is destroyed by heating at 100° in acid solution for a short time. The relative instability of the intermediate under even moderate conditions has made it difficult to isolate in pure form.

5-Aminoimidazole reacts with CO_2 (or HCO_3^-) in the next step[51] of this reaction sequence to yield 5-amino-4-imidazolecarboxylic acid ribonucleotide [Eq. (8)]. This compound exhibits a specific absorption maximum at 249 mμ, a property which has provided an excellent means for its determination.

5-Aminoimidazole ribonucleotide + CO_2 ⇌
5-amino-4-imidazolecarboxylic acid ribonucleotide (8)

[50] B. Levenberg and J. M. Buchanan, *J. Biol. Chem.* **224**, 1005 (1957).
[51] L. N. Lukens and J. M. Buchanan, *J. Am. Chem. Soc.* **79**, 1511 (1957).

When reacted with the Bratton and Marshall reagents, 5-amino-4-imidazolecarboxylic acid ribonucleotide yields a red-purple-colored product with an absorption maximum at 519 mμ. Like its immediate precursor, 5-amino-4-imidazolecarboxylic acid ribonucleotide is difficult to isolate and purify. It is relatively stable in alkaline solution but in acid solution it is decarboxylated. Likewise, at 100° in acid solution 5-amino-4-imidazolecarboxylic acid ribonucleotide is completely destroyed as measured by the Bratton and Marshall test.

The enzyme responsible for the carboxylation reaction, 5-aminoimidazole ribonucleotide carboxylase, has been purified over fortyfold. The equilibrium constant for Eq. (8) is in the neighborhood of 3.3 liters per mole. The carboxylation reaction proceeds appreciably only in the presence of high concentrations of bicarbonate or under conditions in which the product of the carboxylation is removed by further reaction.

Under physiological conditions 5-amino-4-imidazolecarboxylic acid ribonucleotide is removed by reaction with aspartic acid and ATP to yield 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide [Eq. (9)].[51] Despite the fact that fairly acidic conditions must be used during the chromatographic isolation of this highly negatively-charged molecule, it is relatively stable under these conditions. It has been obtained as the barium salt in purified form. The succinocarboxamide ribonucleotide has an absorption maximum at 268 mμ. When reacted with the Bratton and Marshall reagents at room temperature the succino compound does not yield a colored

5-Amino-4-imidazolecarboxylic acid ribonucleotide + aspartic acid + ATP ⇌

5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide (9)

+ ADP + orthophosphate

product as would be expected for a heterocyclic amine of this type. It is now known that the failure of the succinocarboxamide ribonucleotide to yield a product is due to the fact that the diazotized intermediate is unstable at room temperature and breaks down before coupling with the *N*-1-naphthylethylenediamine takes place. When the reaction is carried out at 0°, however, a purple-colored product is formed with an absorption maximum at 560 mμ. The fact that the succinocarboxamide ribonucleotide is the only one of the unsubstituted heterocyclic amines which does not yield a colored product at room temperature has proved to be a useful tool for its determination.

Equation (9) is readily reversible. In the presence of orthophosphate and ADP, 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide breaks down to 5-aminoimidazolecarboxylic acid ribonucleotide, ATP, and aspartic acid. Arsenate may replace phosphate in the reaction but ADP is still required in catalytic quantities to achieve maximal rate of reaction. The

adenine nucleotides cannot be replaced in the reaction by the other purine and pyrimidine nucleotides.

During its further conversion to inosinic acid, 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide is cleaved to fumaric acid and 5-amino-4-imidazolecarboxamide ribonucleotide [Eq. (10)].[52] This reaction has an equilibrium constant of 2.3×10^{-3}. The enzyme responsible for Eq. (10)

5-Amino-4-imidazole-*N*-succinocarboxamide ribonucleotide $\rightleftarrows$ 5-amino-4-imidazolecarboxamide ribonucleotide + fumaric acid (10)

is believed to be identical with adenylosuccinase for reasons which will be presented in a later section.

5-Amino-4-imidazolecarboxamide ribonucleotide has an absorption maximum at 267 mμ and reacts with the Bratton and Marshall reagents to yield a purple-colored product which absorbs maximally at 540 mμ. In contrast to some of the heterocyclic amines mentioned previously, treatment of the carboxamide ribonucleotide briefly at 100° in acid solution does not affect the color yield obtained in the Bratton and Marshall procedure.

5-Amino-4-imidazolecarboxamide which accumulates with its ribosyl and phosphoribosyl derivatives in cultures of *Escherichia coli* poisoned with sulfonamides, was first isolated as the free base by Stetten and Fox[53] and soon thereafter identified by Shive and his co-workers.[54] By more refined methods it was possible to isolate the ribonucleoside and in small quantities the ribonucleotide from these cultures.[55] The ribonucleotide may also be formed enzymically by phosphorylation of the ribonucleoside[56] or by reaction of the base with 5-phosphoribosylpyrophosphate.[39]

The final steps in the synthesis of inosinic acid *de novo*[57-60] involve the formylation of 5-amino-4-imidazolecarboxamide ribonucleotide by N^{10}-for-

N^{10}-Formyl-FH_4 + 5-amino-4-imidazolecarboxamide ribonucleotide $\rightarrow$ 5-formamido-4-imidazolecarboxamide ribonucleotide + FH_4 (11)

myltetrahydrofolic acid according to Eq. (11). The formamide compound does not accumulate since the transformylase responsible for Eq. (11) also

[52] R. W. Miller, L. N. Lukens, and J. M. Buchanan, *J. Am. Chem. Soc.* **79**, 1513 (1957).
[53] M. R. Stetten and C. L. Fox, Jr., *J. Biol. Chem.* **161**, 333 (1945).
[54] W. Shive, W. W. Ackermann, M. Gordon, M. E. Getzendaner, and R. E. Eakin, *J. Am. Chem. Soc.* **69**, 725 (1947).
[55] G. R. Greenberg and E. L. Spilman, *J. Biol. Chem.* **219**, 411 (1956).
[56] G. R. Greenberg, *J. Biol. Chem.* **219**, 423 (1956).
[57] G. R. Greenberg, *Federation Proc.* **13**, 745 (1954).
[58] G. R. Greenberg, L. Jaenicke, and M. Silverman, *Biochim. et Biophys. Acta* **17**, 589 (1955).
[59] J. G. Flaks, M. J. Erwin, and J. M. Buchanan, *J. Biol. Chem.* **229**, 603 (1957).
[60] L. Warren, J. G. Flaks, and J. M. Buchanan, *J. Biol. Chem.* **229**, 627 (1957).

contains an enzymic component, inosinicase, which is capable of cyclizing the formamido compound to inosinic acid [Eq. (12)].[59] The transformylase requires potassium ions for activity while inosinicase does not.

5-Formamido-4-imidazolecarboxamide ribonucleotide $\rightleftarrows$ inosinic acid + H_2O (12)

5-Amino-4-imidazolecarboxamide ribonucleotide transformylase[59] may be determined by measuring the disappearance of nonacetylatable diazotizable amine catalyzed by the enzyme in the presence of N^{10}-formyltetrahydrofolic acid. Since tetrahydrofolic acid, which is produced in the reaction, is decomposed to an acetylatable arylamine during the course of the Bratton and Marshall procedure an acetylation step must be introduced to prevent its reaction in the test.

Although 5-formamido-4-imidazolecarboxamide ribonucleotide does not accumulate in the enzymic reactions it may be synthesized chemically by formylating the free amine in the presence of a mixture of concentrated formic acid and acetic anhydride. The formamido compound has the same spectral characteristics as the free amine with an absorption maximum at 267 mμ. Since this material is a substituted amine it does not react in the Bratton and Marshall procedure but may readily be converted quantitatively to the free amine by a brief hydrolysis in 0.1 *N* HCl at 100°.

The formamido compound is readily converted enzymically to inosinic acid. The enzyme is specific for the phosphoribosyl compound and does not catalyze the cyclization of the corresponding ribonucleoside or the free base. It has been possible to demonstrate the reversibility of Eq. (12) even though the equilibrium of the reaction greatly favors the formation of inosinic acid. At equilibrium the ratio of inosinic acid to 5-formamido-4-imidazolecarboxamide ribonucleotide is 16,000 to 1.[60]

At the present time it has not been possible to separate the carboxamide ribonucleotide transformylase from inosinicase. During purification of the enzyme from chicken liver the ratio of the two activities remains constant. In certain mutants of *Neurospora crassa* loss of one activity will be accompanied by loss of the other. There are a few instances, however, where inosinicase activity may be retained while the transformylase is lost. It thus is difficult to decide whether both activities reside in one protein or not. In any event carboxamide ribonucleotide transformylase and inosinicase are closely associated with each other structurally, functionally, and genetically.

In contrast to the formylation of glycinamide ribonucleotide, the formylation of 5-amino-4-imidazolecarboxamide ribonucleotide requires specifically the N^{10}-formyltetrahydrofolic acid as the formyl donor.[46] Preparations of the carboxamide ribonucleotide transformylase which contain cyclohydrolase may utilize either the N^{10}-formyl or the N^5,N^{10}-anhydro-

formyltetrahydrofolic acid. When cyclohydrolase is eliminated, however, the reaction becomes specific for the N^{10}-formyl derivative.

The two transformylase reactions of purine biosynthesis may be coupled with each other in the direction indicated in Eq. (13).[60] This composite

$$\text{Inosinic acid} + \text{glycinamide ribonucleotide} + H_2O \rightarrow \text{5-amino-4-imidazolecarboxamide ribonucleotide} + \text{formylglycinamide ribonucleotide} \quad (13)$$

reaction requires the participation of inosinicase, the carboxamide ribonucleotide transformylase, cyclohydrolase, and glycinamide ribonucleotide transformylase in the order named. Equation (13) is essentially irreversible because of the irreversibility of the last reaction of the series, the formylation of glycinamide ribonucleotide.

IV. Enzymic Synthesis of Formyltetrahydrofolic Acid Compounds

The enzymic formation of the formylated derivatives of tetrahydrofolic acid has now been studied extensively. N^{10}-Formyltetrahydrofolic may be formed directly by the coupling of formic acid and tetrahydrofolic acid in the presence of ATP according to Eq. (14).[58, 61] N^5,N^{10}-Anhydroformyl-

$$\text{Formic acid} + FH_4 + \text{ATP} \rightleftarrows N^{10}\text{-formyl-}FH_4 + \text{ADP} + \text{orthophosphate} \quad (14)$$

tetrahydrofolic acid, which is also referred to as N^5,N^{10}-methenyltetrahydrofolic acid, may be derived from serine[62-67] by reactions shown in Fig. 7 (see also footnote 11 in Chapter 39).[68-70] In certain bacteria which catabolize purines as a source of energy, N^5,N^{10}-anhydroformyltetrahydrofolic acid may be formed from formiminoglycine by reactions included in Fig. 7. Formiminotetrahydrofolic acid may also be formed from formiminoglutamic acid which is produced in the catabolism of histidine in mammalian liver.[71, 72] A third form of formyltetrahydrofolic acid, the N^5 derivative, has been

[61] J. C. Rabinowitz and W. E. Pricer, Jr., *Federation Proc.* **17**, 293 (1958).
[62] N. Alexander and D. M. Greenberg, *J. Biol. Chem.* **214**, 821 (1955).
[63] N. Alexander and D. M. Greenberg, *J. Biol. Chem.* **220**, 775 (1956).
[64] R. L. Kisliuk, *Federation Proc.* **15**, 289 (1956).
[65] L. Jaenicke, *Federation Proc.* **15**, 281 (1956).
[66] G. R. Greenberg and L. Jaenicke, *in* "The Chemistry and Biology of the Purines" (G. E. W. Wolstenholme and C. M. O'Connor, eds.), p. 204. Churchill, London, 1957.
[67] M. J. Osborn and F. M. Huennekens, *Biochim. et Biophys. Acta* **26**, 646 (1957).
[68] J. C. Rabinowitz and W. E. Pricer, Jr., *J. Am. Chem. Soc.* **78**, 5702 (1956).
[69] J. C. Rabinowitz and W. E. Pricer, Jr., *J. Am. Chem. Soc.* **78**, 1513 (1956).
[70] J. C. Rabinowitz and W. E. Pricer, Jr., *J. Am. Chem. Soc.* **78**, 4176 (1956).
[71] A. Miller and H. Waelsch, *Arch. Biochem. Biophys.* **63**, 263 (1956).
[72] H. Tabor and J. C. Rabinowitz, *J. Am. Chem. Soc.* **78**, 5705 (1956).

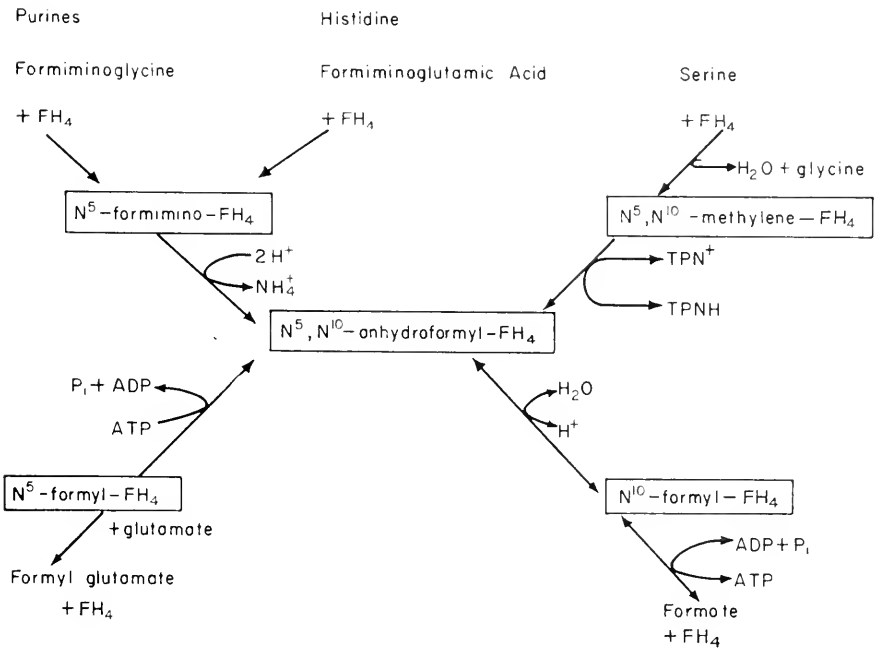

FIG. 7. The enzymic formation of the formylated derivatives of tetrahydrofolic acid.

isolated from natural sources as the citrovorum factor.[73] The N^5 derivative may be converted enzymically in the presence of ATP into the N^5,N^{10}-anhydroformyl derivative[57, 74] or a compound closely related to the cyclic derivative so that N^5-formyltetrahydrofolic acid may serve indirectly as a source of formyl groups in purine biosynthesis. However, N^5-formyltetrahydrofolic acid may donate formyl groups directly to glutamic acid to yield formylglutamic acid.[75] It is thus seen that the nature of the formyl donor in any given instance depends upon the enzymic system involved.

V. Synthesis of Adenylic Acid from Inosinic Acid

The precursors of the extra-ring nitrogens of adenylic and guanylic acids have been shown to be aspartic acid and the amide nitrogen of glutamine, respectively.[76] The enzymic synthesis of adenylic acid (Fig. 3) has been studied in bone marrow, yeast, and *Escherichia coli*. The synthesis of ad-

[73] H. E. Sauberlich, *J. Biol. Chem.* **195**, 337 (1952).
[74] J. M. Peters and D. M. Greenberg, *J. Am. Chem. Soc.* **80**, 2719 (1958).
[75] M. Silverman, J. C. Keresztesy, G. J. Koval, and R. C. Gardiner, *J. Biol. Chem.* **226**, 83 (1957).
[76] R. Abrams and M. Bentley, *J. Am. Chem. Soc.* **77**, 4179 (1955).

enylic acid from inosinic acid is catalyzed by two enzymes and proceeds via the intermediate adenylosuccinic acid.[77] The reaction sequence is in several respects analogous to other reactions in which the α-amino nitrogen of aspartic acid is transferred to form an amide [Eqs. (15 and 16)]. Both Eqs.

$$\text{Inosinic acid} + \text{aspartic acid} + \text{GTP} \rightleftarrows \text{adenylosuccinic acid} + \text{GDP} + \text{orthophosphate} \tag{15}$$

$$\text{Adenylosuccinic acid} \rightleftarrows \text{adenylic acid} + \text{fumaric acid} \tag{16}$$

(15) and (16) are reversible. The involvement of guanosine compounds in Eq. (15) has been definitely proven in the enzyme system isolated from *E. coli*.[78] A similar function for guanosine compounds has also been demonstrated for the system in bone marrow but here there is a cooperating function of adenosine triphosphate which is not at present completely understood.[76] When inosinic acid is labeled in position 6 with O^{18}, O^{18} is found in the orthophosphate formed. There are many similarities between Eq. (9), the synthesis of 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide, and Eq. (15). The major difference is the preference of the nucleoside triphosphate, which by the hydrolysis of a phosphoanhydride bond provides the energy for the synthesis of the carbon to nitrogen bond.

The splitting of adenylosuccinic acid to adenylic acid and fumaric acid is in close analogy to the splitting of 5-amino-4-imidazole-*N*-succinocarboxamide ribonucleotide to 5-amino-4-imidazolecarboxamide ribonucleotide and fumaric acid. In fact, present evidence indicates that adenylosuccinase is responsible for both functions.[52] Upon purifying the enzyme over 200-fold, activites toward both substrates are maintained in a constant ratio during the various steps of purification. Moreover, in a certain class of mutant of *Neurospora crassa* loss of activity for one substrate is always accompanied by loss of activity for the other. Similar observations have been made with mutants of *E. coli* and *Salmonella typhimurium*.[79] This is thus an unusual circumstance in which one genetic event results in the loss of enzymic activities for two nonsequential steps in a biosynthetic pathway.

VI. Synthesis of Guanylic Acid from Inosinic Acid

The first step in the formation of guanylic acid from inosinic acid (Fig. 3) involves the oxidation of inosinic acid to xanthylic acid by DPN [Eq. (17)].

$$\text{Inosinic acid} + \text{DPN} + \text{H}_2\text{O} \rightleftarrows \text{xanthylic acid} + \text{DPNH} + \text{H}^+ \tag{17}$$

The enzyme responsible for this reaction has been called inosine-5′-phosphate dehydrogenase. The further amination of xanthylic acid to guanylic

[77] C. E. Carter and L. H. Cohen, *J. Biol. Chem.* **222**, 17 (1956).
[78] I. Lieberman, *J. Am. Chem. Soc.* **78**, 251 (1956).
[79] J. S. Gots and E. G. Gollub, *Proc. Natl. Acad. Sci. U. S.* **43**, 826 (1957).

acid has been studied by three groups of investigators.[80-84] Enzymes have been isolated from pigeon liver, rabbit bone marrow, and calf thymus which are capable of effecting the amination of xanthylic acid to guanylic acid

$$\text{Xanthylic acid} + \text{glutamine} + \text{ATP} + \text{H}_2\text{O} \rightleftarrows \text{guanylic acid} + \text{glutamic acid} + \text{AMP} + \text{pyrophosphate} \tag{18}$$

according to Eq. (18). An enzyme obtained from *Aerobacter aerogenes*, xanthosine-5′-phosphate aminase utilizes ammonia as the nitrogen donor rather than glutamine in a similar amination reaction. The enzyme isolated from animal sources may also utilize ammonium ions as a nitrogen donor but much less effectively than the preferred substrate, glutamine.

Isotopic experiments indicate that like other nitrogen transfer reactions involving glutamine Eq. (18) is essentially irreversible. It differs from the reaction of formylglycinamidine ribonucleotide synthesis in that the nucleoside phosphate products of the reaction are AMP and pyrophosphate rather than ADP and orthophosphate. O^{18}-transfer experiments show that the nucleophilic displacement on ATP must occur at the innermost phosphorus atom rather than at the terminal one. Azaserine will inhibit the synthesis of guanylic acid from xanthylic acid. The inhibition constant for this reaction is much higher than it is for the amination of formylglycinamide ribonucleotide.

VII. Summary

The synthesis of inosinic acid from elementary precursors can be considered as taking place by coupling of Eqs. (19) and (20). In these reactions

$$2\text{NH}_4^+ + 2\text{HCOO}^- + \text{HCO}_3^- + \text{glycine} + \text{aspartate} + \text{ribose-5-phosphate} \rightarrow \text{inosinic acid} + \text{fumarate} + 9\text{H}_2\text{O} \tag{19}$$

$$9\text{ATP} + 9\text{H}_2\text{O} \rightarrow 8\text{ADP} + 8\text{HPO}_4^{=} + \text{AMP} + \text{HP}_2\text{O}_7^{=} + 9\text{H}^+ \tag{20}$$

the assumption is made that the two ammonium ions are being incorporated first into the actual nitrogen donor, glutamine, by reaction with glutamic acid and ATP. At least in theory aspartate could be derived from fumarate and NH_4^+ according to Eq. (21)[85] so that Eq. (19) may be reduced

$$\text{Fumarate} + \text{NH}_4^+ \rightarrow \text{aspartate} \tag{21}$$

$$\text{Glycine} + \text{ribose-5-phosphate} + 3\text{NH}_4^+ + \text{HCO}_3^- + 2\text{HCOO}^- \rightarrow \text{inosinic acid} + 9\text{H}_2\text{O} \tag{22}$$

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[80] R. Abrams and M. Bentley, *Arch. Biochem. Biophys.* **79,** 91 (1959).
[81] U. Lagerkvist, *J. Biol. Chem.* **233,** 138 (1958).
[82] U. Lagerkvist, *J. Biol. Chem.* **233,** 143 (1958).
[83] B. Magasanik, H. S. Moyed, and L. B. Gehring, *J. Biol. Chem.* **226,** 339 (1957).
[84] H. S. Moyed and B. Magasanik, *J. Biol. Chem.* **226,** 351 (1957).
[85] V. R. Williams and R. T. McIntyre, *J. Biol. Chem.* **217,** 467 (1955).

to Eq. (22). It can be seen that the driving force for inosinic acid biosynthesis according to Eq. (22) is derived, in effect, from the hydrolysis of 9 equivalents of ATP (including 2 equivalents required for glutamine formation and 2 equivalents for formate activation). Suggestions have been made in preliminary form to explain the formation of carbon to nitrogen bonds by a coordinated attack on a carbon atom (such as a carboxyl carbon) by the nucleophilic nitrogen donor, and by the electrophilic nucleoside triphosphates. Further studies along these lines should crystallize our present tentative concepts of how phosphate compounds are integrally involved in the synthesis of a variety of carbon to nitrogen bonds not only in purine reactions but in the synthesis of other nitrogen-containing compounds.

CHAPTER 36

Biosynthesis of Pyrimidine Nucleotides

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I. Introduction

Information regarding the mechanism of pyrimidine nucleotide biosynthesis has been considerably consolidated since the reviews of Reichard[1] and Schlenk[2] were written. The emphasis of the experimental approaches to the investigation of the synthetic mechanisms has shifted from isotope incorporation studies to the study of the relevant enzymes in cell-free systems. It is the purpose of this chapter to review briefly the mechanisms involved in pyrimidine nucleotide biosynthesis with particular reference to recent investigations of thymidylic acid synthesis.

II. Biosynthesis of Uridine Nucleotides

1. Biosynthesis of Uridine-5′-phosphate

a. Biosynthesis from Small Molecules

Early investigations of pyrimidine nucleotide biosynthesis involved either a study of the incorporation of labeled small molecule precursors into the polynucleotide pyrimidines of microorganisms, whole animals, tissue slices,

[1] P. Reichard, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. II, p. 277. Academic Press, New York, 1955.

[2] F. Schlenk, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. II, p. 309. Academic Press, New York, 1955.

or homogenates, or a study of growth factor requirements of microbial mutants.

The work of Barnes and Schoenheimer[3] using N^{15}-ammonium citrate clearly showed that mammalian and avian polynucleotides could be derived from small molecule precursors. N-1 has been shown[4] to be derived from ammonia, C-2 from CO_2,[5] while the remainder of the pyrimidine ring (N-3, C-4, C-5, and C-6) is derived from aspartic acid.[6, 7]

The implication of the *Lactobacillus bulgaricus* 09 growth factors, carbamylaspartic acid (ureidosuccinic acid), and orotic acid (uracil-4-carboxylic acid) in pyrimidine synthesis was indicated by their incorporation into the polynucleotides of that organism.[8] Confirmation of the role of orotic acid as a pyrimidine precursor has been provided by the observation of its incorporation into the polynucleotides of *Escherichia coli* B,[9] yeast,[10] mammalian liver slices,[11, 12] and tumors[13] and by its incorporation into the uridine-5′-phosphate of the acid-soluble fraction of rat liver.[14-16]

The synthesis of orotic acid in liver slices has been studied by Reichard and Lagerkvist.[7] In an extensive investigation they have studied the incorporation of N^{15}-labeled ammonium chloride, bicarbonate-C^{13}, L-aspartate-N^{15}, L-aspartate-1, 4-C^{13}, L-aspartate-2, 3-C^{14} and L-carbamylaspartate-N^{15} into an added pool of orotic acid. The results obtained confirm and amplify those of previous investigations and together with other isotopic and microbiological evidence clearly implicate aspartic acid, carbamylaspartic acid, and orotic acid (or close derivatives thereof) as biosynthetic precursors of uridine-5′-phosphate (Fig. 1). The implication of dihydroorotic acid in this pathway is indicated by its conversion to orotic acid by rat liver homogenates[17] and by its ability to support the growth of *L. bulgaricus* 09.[18]

[3] F. W. Barnes and R. Schoenheimer, *J. Biol. Chem.* **151,** 123 (1943).
[4] U. Lagerkvist, *Arkiv Kemi* **5,** 569 (1953).
[5] M. R. Heinrich and D. W. Wilson, *J. Biol. Chem.* **186,** 447 (1950).
[6] U. Lagerkvist, P. Reichard, and G. Ehrensvard, *Acta Chem. Scand.* **5,** 1212 (1951).
[7] P. Reichard and U. Lagerkvist, *Acta Chem. Scand.* **7,** 1207 (1953).
[8] L. D. Wright, C. S. Miller, H. R. Skeggs, J. W. Huff, L. L. Weed, and D. W. Wilson, *J. Am. Chem. Soc.* **73,** 1898 (1951).
[9] L. L. Weed and S. S. Cohen, *J. Biol. Chem.* **192,** 693 (1951).
[10] M. Edmonds, A. M. Delluva, and D. W. Wilson, *J. Biol. Chem.* **197,** 251 (1952).
[11] L. L. Weed and D. W. Wilson, *J. Biol. Chem.* **189,** 435 (1951).
[12] P. Reichard and S. Bergström, *Acta Chem. Scand.* **5,** 190 (1951).
[13] L. L. Weed, *Cancer Research* **11,** 470 (1951).
[14] R. B. Hurlbert and V. R. Potter, *J. Biol. Chem.* **195,** 257 (1952).
[15] R. B. Hurlbert, *Federation Proc.* **11,** 234 (1952); **12,** 222 (1953).
[16] E. Herbert, V. R. Potter, and L. I. Hecht, *J. Biol. Chem.* **225,** 659 (1957).
[17] C. Cooper, R. Wu, and D. W. Wilson, *J. Biol. Chem.* **216,** 37 (1955).
[18] C. S. Miller, J. T. Gordon, and E. L. Engelhardt, *J. Am. Chem. Soc.* **75,** 6086 (1953).

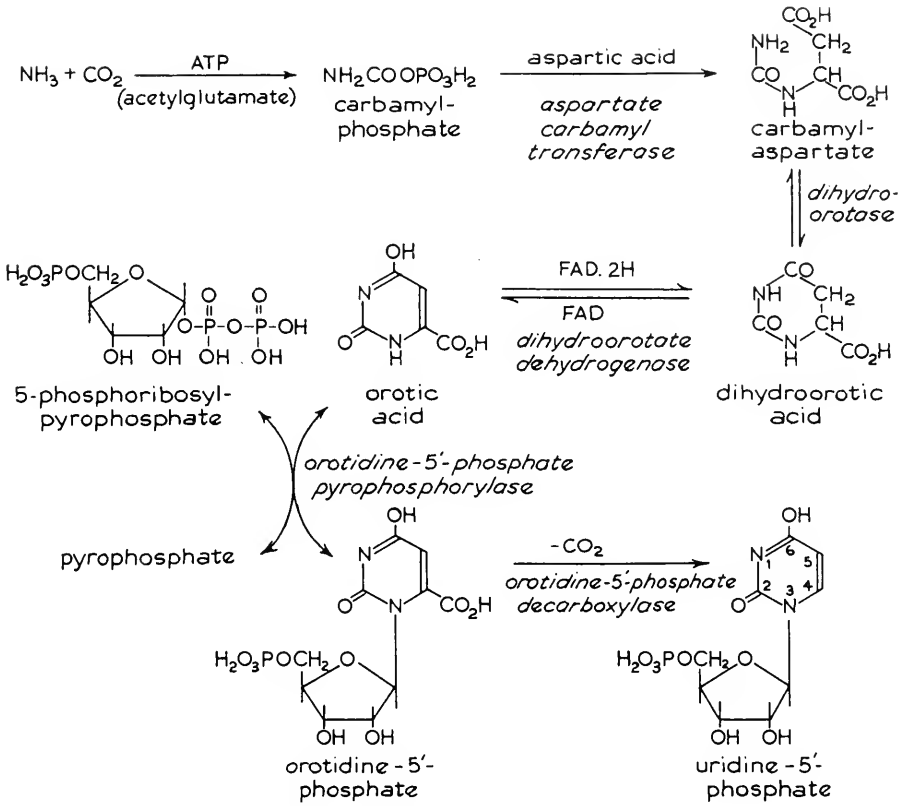

FIG. 1. The pathway of *de novo* synthesis of uridine-5′-phosphate.

b. Biosynthesis in Cell-Free Systems

In organisms capable of *de novo* pyrimidine synthesis the operation of the sequence of reactions shown in Fig. 1 has been generally confirmed in those cases investigated.

The first reaction, the formation of carbamylaspartic acid from aspartic acid is formally analogous to citrulline formation from ornithine. Grisolia and Cohen[19] have demonstrated a requirement for adenosine-5′-triphosphate (ATP), Mg^{++}, and acetylglutamate in the formation of the activated derivative of NH_3 and CO_2 (compound X) involved in citrulline synthesis. The involvement of compound X in carbamylaspartic acid synthesis in an isolated mammalian enzyme system has also been demonstrated.[19a] Jones *et al.*[20] have made the important discovery that carbamyl phosphate is

[19] S. Grisolia and P. P. Cohen, *J. Biol. Chem.* **198**, 561 (1952).
[19a] P. Reichard and G. Hanshoff, *Acta Chem. Scand.* **8**, 1102 (1954); **9**, 519 (1955).
[20] M. E. Jones, L. Spector, and F. Lipmann, *J. Am. Chem. Soc.* **77**, 819 (1955).

formed in extracts of *Streptococcus faecalis* and that carbamyl phosphate is a substrate of ornithine carbamyl transferase. Using an extract of rat liver and a purified *E. coli* enzyme Reichard and Hanshoff[21] have confirmed the role of carbamyl phosphate (compound X) in aspartate carbamyl transferase action and have established the nonidentity of the aspartate and ornithine carbamyl transferases. The coliform aspartate carbamyl transferase has been considerably purified and good stoichiometry for the reaction: carbamyl phosphate + aspartate → carbamylaspartate + inorganic phosphate, has been obtained. Evidence for reversibility was obtained only by the use of inorganic phosphate-P^{32}. The mechanism of the reaction apparently does not involve a carbamyl-enzyme complex as indicated by negative isotope exchange experiments of the type (*a*) carbamyl phosphate + inorganic phosphate-P^{32} + enzyme and (*b*) aspartate-C^{14} + carbamylaspartate + enzyme. The mechanism probably involves a nucleophilic attack of the N-atom of aspartic acid on the carbonyl group of carbamyl phosphate subsequent to the binding of the substrates on the enzyme.

Lieberman and Kornberg[22] have described a ureidosuccinase enzyme obtained from cells of *Zymobacterium oroticum* grown anaerobically on orotic acid as the sole energy source. The enzyme effects the hydrolysis of carbamylaspartic acid to NH_3, CO_2, and aspartic acid. A requirement for a sulfhydryl compound and Mn^{++} but not for adenosine-5′-diphosphate (ADP) has been reported (cf. the ATP-yielding cleavage of citrulline[23]). The highly exergonic character of the reaction together with its effective irreversibility led to the postulation of the synthesis of carbamylaspartic acid from aspartic acid via arginosuccinate, a compound previously implicated in pyrimidine biosynthesis on the basis of the observed utilization of citrulline-ureido-C^{14} for polynucleotide pyrimidine synthesis in *Neurospora*[24] and pigeon liver homogenates[25] and for orotic acid synthesis in rat liver slices.[26] These observations on citrulline utilization have been integrated, however, with the pathway described in Fig. 1 by the observation by Smith and Reichard[27] of carbamyl phosphate formation from citrulline by a rat liver mitochondrial extract in the presence of ATP, Mg^{++}, and acetylglutamate. The carbamyl group of citrulline may also be transferred to aspartic acid to yield carbamylaspartate through the coupling of the ornithine and aspartate carbamyl transferases. The equilibrium of the coupled system is in favor of carbamylaspartate formation.[27a]

[21] P. Reichard and G. Hanshoff, *Acta Chem. Scand.* **10,** 548 (1956).
[22] I. Lieberman and A. Kornberg, *J. Biol. Chem.* **212,** 909 (1955).
[23] V. A. Knivett, *Biochem. J.* **56,** 602 (1954).
[24] M. R. Heinrich, V. C. Dewey, and G. W. Kidder, *J. Am. Chem. Soc.* **76,** 3102 (1954).
[25] M. P. Schulman and S. J. Badger, *Federation Proc.* **13,** 292 (1954).
[26] L. H. Smith, Jr., and D. Stetten, *J. Am. Chem. Soc.* **76,** 3864 (1954).
[27] L. H. Smith and P. Reichard, *Acta Chem. Scand.* **10,** 1024 (1956).
[27a] P. Reichard, *Acta Chem. Scand.* **11,** 523 (1957).

The interconversion of carbamylaspartic acid and orotic acid in cell-free extracts of *Z. oroticum* has been reported by Lieberman and Kornberg.[28] The extracts contain dihydroorotase and dihydroorotic acid dehydrogenase (a flavin nucleotide-linked enzyme[29]) catalyzing, respectively, the interconversion of carbamylaspartic acid and dihydroorotic acid and of dihydroorotic acid and orotic acid. The adaptive nature of these *Zymobacterium* enzymes has been demonstrated[30] and the possibility that they may not be concerned with normal pyrimidine biosynthetic pathways may be considered. Yates and Pardee[30] have, however, demonstrated the presence of corresponding enzymes in extracts of *E. coli* B grown on minimal medium, the activities of the isolated enzymes being sufficient to account for their participation in polynucleotide pyrimidine synthesis in the parent cells.

The pathway of conversion of orotic acid to uridine-5′-phosphate has been clearly defined by the recent work of Lieberman *et al.*[31] These authors have described a purified yeast enzyme, orotidine-5′-phosphate pyrophosphorylase, which effects a condensation of orotic acid with 5-phosphoribosylpyrophosphate[32] to yield orotidine-5′-phosphate which in turn is converted to uridine-5′-phosphate irreversibly by orotidine-5′-phosphate decarboxylase. The occurrence of these enzymes in mammalian liver[33] satisfactorily explains the precursor-product relationship between orotic acid and acid-soluble uridine-5′-nucleotides previously noted.[16, 33]

The structure of orotidine-5′-phosphate as the 5′-phosphate ester of 3-D-ribofuranosyluracil-4-carboxylic acid is indicated[31] by its apparent identity with the product obtained enzymically by phosphate transfer to orotidine.[34] The structure of orotidine as 3-D-ribofuranosyluracil-4-carboxylic acid has been substantiated recently on the basis of a spectrophotometric study of N-alkylorotic acid derivatives.[34a]

The yeast orotidine-5′-phosphate pyrophosphorylase is specific to orotic acid, no reaction with DL-carbamylaspartic acid, L-dihydroorotic acid, uracil, or cytosine having been observed. No evidence has been adduced for the participation of an open-chain glycoside in the reactions leading to orotidine-5′-phosphate formation.

It may be noted, parenthetically, that Handschumacher[35] has presented evidence indicating that the growth inhibitory action of *as*-triazine-3,5-

[28] I. Lieberman and A. Kornberg, *Biochim. et Biophys. Acta* **12,** 223 (1953).
[29] H. C. Friedman and B. Vennesland, *J. Biol. Chem.* **233,** 1398 (1958).
[30] R. A. Yates and A. B. Pardee, *J. Biol. Chem.* **221,** 743 (1956).
[31] I. Lieberman, A. Kornberg, and E. S. Simms, *J. Biol. Chem.* **215,** 403 (1955).
[32] I. Lieberman, A. Kornberg, and E. S. Simms, *J. Biol. Chem.* **215,** 389 (1955).
[33] R. B. Hurlbert and P. Reichard, *Acta Chem. Scand.* **9,** 251 (1955).
[34] A. M. Michelson, W. Drell, and H. K. Mitchell, *Proc. Natl. Acad. Sci. U. S.* **37,** 396 (1951).
[34a] J. J. Fox, N. Yung, and I. Wempen, *Biochim. et Biophys. Acta* **23,** 295 (1957).
[35] R. E. Handschumacher, *Federation Proc.* **17,** 236 (1958).

dione (4-azauracil) with respect to a number of species of microorganisms is due to an inhibition of orotidine-5′-phosphate decarboxylase. The orotic acid analogs, uracil-4-sulfonic acid, uracil-4-sulfonamide, and uracil-4-methylsulfone, inhibit the growth of certain orotic acid-requiring strains of *Lactobacillus bulgaricus*. These analogs have been shown recently to inhibit competitively the conversion of orotic acid to orotidine-5′-phosphate by a partially purified orotidine-5′-phosphate pyrophosphorylase of yeast.[35a] No action on orotidine-5′-phosphate decarboxylase could be detected (see Chapter 39).

Yates and Pardee[35b] have demonstrated a control of pyrimidine nucleotide synthesis in *E. coli* by feed-back inhibition. They have shown that cytidine and especially cytidine-5′-phosphate are competitive inhibitors with aspartic acid and carbamyl phosphate for carbamylaspartic acid formation. Uracil, uridine, uridine-5′-phosphate, and cytosine do not inhibit appreciably.

c. Biosynthesis from Preformed Pyrimidines

The ability of tissues to incorporate uracil, uridine, and uridine-2′-, 3′-, and 5′-phosphates into polynucleotides has been the subject of some investigation.[36-38] Plentl and Schoenheimer[39] and Rutman *et al.*[40] have clearly indicated the inability of rat liver to utilize uracil for ribonucleic acid synthesis. Cytosine is similarly not utilized.[41] On the other hand, pyrimidine nucleosides and nucleotides do function as precursors of tissue polynucleotide pyrimidines.[38, 42-44] It must be noted, however, that no evidence for the utilization of intact pyrimidine ribonucleotides has been presented. Liebman and Heidelberger[45] have shown that the extent of incorporation of P^{32}-labeled pyrimidine-2′-, 3′-, and 5′-ribonucleotides into the polynucleotides of Ehrlich ascites carcinoma cells, rat liver slices, and the Flexner-

[35a] W. L. Holmes, *J. Biol. Chem.* **223,** 677 (1956).
[35b] R. A. Yates and A. B. Pardee, *J. Biol. Chem.* **221,** 757 (1956).
[36] E. Hammarsten and P. Reichard, *J. Biol. Chem.* **183,** 105 (1950).
[36a] U. Lagerkvist, P. Reichard, B. Carlsson, and G. B. Grabosz, *Cancer Research* **15,** 164 (1955).
[37] F. J. Di Carlo, A. S. Schultz, P. M. Roll, and G. B. Brown, *J. Biol. Chem.* **180,** 329 (1949).
[38] P. M. Roll, G. B. Brown, F. J. Di Carlo, and A. S. Schultz, *J. Biol. Chem.* **180,** 333 (1949).
[39] A. A. Plentl and R. Schoenheimer, *J. Biol. Chem.* **153,** 203 (1944).
[40] R. J. Rutman, A. Cantarow, and K. E. Paschkis, *Cancer Research* **14,** 119 (1954).
[41] A. Bendich, H. Getler, and G. B. Brown, *J. Biol. Chem.* **177,** 565 (1949).
[42] E. Hammarsten, P. Reichard, and E. Saluste, *Acta Chem. Scand.* **3,** 433 (1949).
[43] W. H. Prusoff, *J. Biol. Chem.* **231,** 873 (1958).
[44] L. I. Hecht and V. R. Potter, *Cancer Research* **16,** 999 (1956).
[45] K. C. Liebman and C. Heidelberger, *J. Biol. Chem.* **216,** 823 (1955).

Jobling carcinoma is consistent with a prior dephosphorylation and equilibration with inorganic phosphate. Roll *et al.*[46] have confirmed this result for a number of rat tissues using pyrimidine-3′(2′-)-ribonucleotides uniformly labeled with C^{14} and with P^{32}. The nucleoside units of the nucleotides were, however, incorporated extensively into tissue ribonucleic acid (RNA) without rupture of the glycoside linkage. Extracellular nucleotides are utilized for *E. coli* nucleic acids only after dephosphorylation, the nucleotide-P being equilibrated with the inorganic phosphate of the medium.[46]

In contrast to the negligible utilization of free pyrimidines for rat liver polynucleotide formation, Rutman *et al.*[40] have shown that uracil-2-C^{14} is incorporated into the RNA of hepatomas induced by 2-acetylaminofluorene. Heidelberger *et al.*[47] have demonstrated a similar precursor role of uracil-2-C^{14} in rat intestinal mucosa and in the Flexner-Jobling carcinoma.

Canellakis[48] has recently reinvestigated the incorporation of uracil by rat tissues. At low extracellular concentrations, uracil, uridine, and uridine-5′-phosphate are extensively degraded to CO_2 by rat liver slices. In contrast to this and to previous results uracil at high extracellular concentration is utilized as effectively as uridine or uridine-5′-phosphate for polynucleotide synthesis. In agreement with these observations Canellakis[49] has demonstrated the occurrence in a high-speed supernatant fraction of rat liver of a uridine phosphorylase (cf. Cardini *et al.*,[50] Paege and Schlenk[51]) and a uridine kinase which together define a pathway for uracil utilization (Fig. 2). Cytosine is not a substrate of the phosphorylase. The same supernatant fraction also contains the catabolic enzymes degrading uracil to CO_2 via 4,5-dihydrouracil, β-ureidopropionic acid, and β-alanine[52] (cf. Grisolia and Wallach,[53] Fink *et al.*,[54] Fritzson,[55] and Fritzson and Pihl[56]). It would appear that there is an inverse relationship between the RNA turnover of a tissue and its content of the uracil catabolic enzymes. It is suggested that the balance between the anabolic and catabolic pathways may constitute part of a homeostatic mechanism governing the rate of RNA synthesis.

[46] P. M. Roll, H. Weinfeld, and E. Carroll, *J. Biol. Chem.* **220,** 455 (1956).
[47] C. Heidelberger, K. C. Liebman, E. Harbers, and P. M. Bhargava, *Cancer Research* **17,** 399 (1957).
[48] E. S. Canellakis, *J. Biol. Chem.* **227,** 701 (1957).
[49] E. S. Canellakis, *J. Biol. Chem.* **227,** 329 (1957).
[50] C. E. Cardini, A. C. Paladini, R. Caputo, and L. F. Leloir, *Acta Physiol. Latinoamer.* **1,** 57 (1950).
[51] L. M. Paege and F. Schlenk, *Arch. Biochem. Biophys.* **52,** 488 (1954).
[52] E. S. Canellakis, *J. Biol. Chem.* **221,** 315 (1956).
[53] S. Grisolia and D. P. Wallach, *Biochim. et Biophys. Acta* **18,** 449 (1955).
[54] R. M. Fink, C. McGaughey, R. E. Cline, and K. F. Fink, *J. Biol. Chem.* **218,** 1 (1956).
[55] P. Fritzson, *J. Biol. Chem.* **226,** 223 (1957).
[56] P. Fritzson and A. Pihl, *J. Biol. Chem.* **226,** 229 (1957).

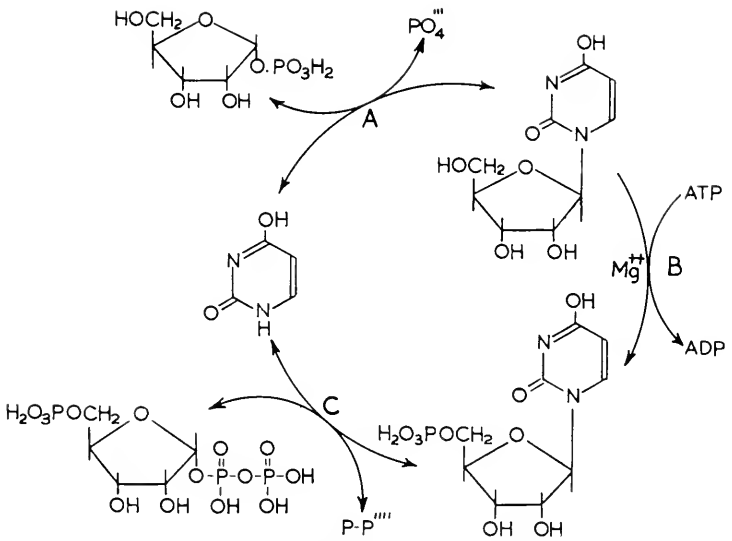

FIG. 2. The uridine phosphorylase (A), uridine kinase (B), and uridine-5′-phosphate pyrophosphorylase (C) reactions.

This concept is further supported by the occurrence of the enzymes associated with RNA synthesis in the same high speed cellular supernatant fraction.

Reichard and Skold[57] have also reported the occurrence of uridine phosphorylase and uridine kinase in acetone powder extracts of Ehrlich ascites tumor. The extracts possessed in addition a feeble uridine-5′-phosphate pyrophosphorylase capable of condensing uracil and 5-phosphoribosylpyrophosphate (Fig. 2). Uridine-5′-phosphate pyrophosphorylase is apparently absent in rat liver.

Crawford *et al.*[58] have investigated uridine-5′-phosphate synthesis in extracts of alumina-ground cells of a number of species of *Lactobacilli*. A clear correlation between pyrimidine growth requirements and the presence of a specific nucleotide pyrophosphorylase has been obtained. Thus *Lactobacillus bifidus* which requires either uracil or orotic acid for growth yields an extract containing nucleotide pyrophosphorylase activities towards orotic acid and uracil. The purified uracil enzyme is not active with orotic acid. *Lactobacillus arabinosus* which in certain growth phases required uracil specifically for growth had during this period an enzyme for uracil but not orotic acid. *L. bulgaricus* 09, which utilizes orotic acid but not uracil for growth, yields an enzyme active toward orotic acid but not uracil. Canella-

[57] P. Reichard and O. Sköld, *Acta Chem. Scand.* **11**, 17 (1957).
[58] I. Crawford, A. Kornberg, and E. S. Simms, *J. Biol. Chem.* **226**, 1093 (1957).

kis[49] has reported that sonic extracts of a uracil-requiring *L. bulgaricus* contain uridine-5′-phosphate pyrophosphorylase. *E. coli* B extracts have been shown[58] to contain no uridine-5′-phosphate pyrophosphorylase. On the other hand, extracts of *E. coli* W and of its cytosineless, uracilless mutant *E. coli* Wc⁻ show a small but significant conversion of uracil to the 5′-nucleotide in the presence of 5-phosphoribosylpyrophosphate.

2. Biosynthesis of Uridine-5′-di- and triphosphates

The isolation by ion-exchange procedures of purine and pyrimidine nucleoside-5′-mono-, di-, and triphosphates from several sources has been reported.[59-62] Potter *et al.*[63] have shown that inorganic phosphate-P^{32} rapidly equilibrates with the di- and triphosphates of rat liver *in vivo*. Herbert *et al.*[64] have subsequently investigated nucleotide phosphorylation in various cellular subfractions obtained by differential centrifugation of rat liver homogenates. They have reported that the cytoplasmic fraction can phosphorylate uridine-5′-phosphate (UMP) to uridine-5′-diphosphate (UDP) and uridine-5′-triphosphate (UTP). Isolated mitochondria effect a phosphorylation of UDP to UTP but have no action on UMP, while the supernatant fraction from the mitochondria is able to phosphorylate UMP to UDP and some UTP. The phosphorylation capacity of the cytoplasmic fraction toward uridine nucleotides is not unexpectedly dependent upon oxidative phosphorylation reactions or when these are prevented upon addition of ATP. Other groups[65-67] have reported enzymic interactions of ATP and UMP analogous to adenylate kinase (myokinase). Lieberman *et al.*[67] have described a partially purified yeast enzyme which effects the following interconversions:

$$\text{ATP} + \text{UMP} \rightleftharpoons \text{ADP} + \text{UDP} \tag{1}$$

$$\text{UTP} + \text{AMP} \rightleftharpoons \text{UDP} + \text{ADP} \tag{2}$$

$$\text{UTP} + \text{UMP} \rightleftharpoons 2\ \text{UDP} \tag{3}$$

The reactions may be followed using the coupled pyruvate phosphokinase-lactic dehydrogenase reactions as a spectrophotometric method for

[59] H. Schmitz, R. B. Hurlbert, and V. R. Potter, *J. Biol. Chem.* **209,** 41 (1954).
[60] L. I. Hecht, V. R. Potter, and E. Herbert, *Biochim. et Biophys. Acta* **15,** 134 (1954).
[61] H. Schmitz, *Biochim. et Biophys. Acta* **14,** 160 (1954).
[62] R. Bergkvist and A. Deutsch, *Acta Chem. Scand.* **7,** 1307 (1953); **8,** 1880, 1889 (1954).
[63] V. R. Potter, E. Herbert, Y. Takagi, P. Siekevitz, and A. F. Brumm, *Federation Proc.* **13,** 276 (1954).
[64] E. Herbert, V. R. Potter, and Y. Takagi, *J. Biol. Chem.* **213,** 923 (1955).
[65] J. L. Strominger, L. A. Heppel, and E. S. Maxwell, *Arch. Biochem. Biophys.* **52,** 488 (1954).
[66] A. Munch-Petersen, *Acta Chem. Scand.* **8,** 1102 (1954).
[67] I. Lieberman, A. Kornberg, and E. S. Simms, *J. Biol. Chem.* **215,** 429 (1955).

ADP or UDP assay. The uridylate kinase [reaction (3)] can be distinguished from adenylate kinase on the basis of differential heat lability at pH 7. The possibility of the catalytic involvement of an adenine nucleotide in reaction (3) could not however be dismissed.[67] Strominger *et al.*[65] have reported similar transphosphorylation reactions in calf liver preparations but no evidence for uridylate kinase activity [reaction (3)] was obtained. Berg and Joklik[68, 69] and Lieberman *et al.*[67] have described a nucleoside-5′-diphosphate kinase of widespread occurrence which effects the following transphosphorylation:

$$\text{ATP} + \text{UDP} \rightleftharpoons \text{ADP} + \text{UTP}$$

UTP synthesis from UDP by the action of pyruvate phosphokinase has also been reported.[70] In contrast to these enzymes of an anabolic character, a Mg^{++}-requiring nucleoside-5′-diphosphatase has been described by Gibson *et al.*[71]:

$$\text{UDP} \xrightarrow[H_2O]{Mg^{++}} \text{UMP} + PO_4'''$$

ADP is not a substrate of the enzyme. It is apparent that there are several mechanisms available for the synthesis and rearrangements of uridine-5′-di- and triphosphate. To these must be added polynucleotide phosphorylase first described in *Azotobacter vinelandii* by Grunberg-Manago and coworkers.[72, 73] The enzyme is capable (*1*) of exchange of inorganic phosphate-P^{32} with UDP and (*2*) of synthesis of a 3′,5′-linked polynucleotide from UDP or other nucleoside-5′-diphosphates.

The biosynthesis of uridine nucleotide coenzymes has been reviewed recently[74-77] and consequently will not be discussed here.

III. Biosynthesis of Cytidine and Deoxycytidine Nucleotides

1. Biosynthesis of Cytidine-5′-mono-, di-, and triphosphates

Herbert *et al.*[78] have extended their investigations of uracil nucleotide phosphorylations by rat liver fractions to a study of cytidine-5′-phosphate

[68] P. Berg and W. K. Joklik, *Nature* **172,** 1008 (1953).
[69] P. Berg and W. K. Joklik, *J. Biol. Chem.* **210,** 657 (1954).
[70] A. Kornberg, *in* "Phosphorus Metabolism" (W. D. McElroy and B. Glass, eds.), Vol. I, p. 392. Johns Hopkins Press, Baltimore, 1951.
[71] D. M. Gibson, P. Ayangar, and D. R. Sanadi, *Biochim. et Biophys. Acta* **16,** 536 (1955).
[72] M. Grunberg-Manago and S. Ochoa, *J. Am. Chem. Soc.* **77,** 3165 (1955).
[73] M. Grunberg-Manago, P. J. Ortiz, and S. Ochoa, *Science* **122,** 907 (1955).
[74] H. M. Kalckar and H. Klenow, *Ann. Rev. Biochem.* **23,** 527 (1954).
[75] C. E. Carter, *Ann. Rev. Biochem.* **25,** 123 (1956).
[76] J. Baddiley and J. G. Buchanan, *Quart. Revs.* **12,** 152 (1958).
[77] M. F. Utter, *Ann. Rev. Biochem.* **27,** 245 (1958).
[78] E. Herbert and V. R. Potter, *J. Biol. Chem.* **222,** 453 (1956).

(CMP) phosphorylation. As with the uracil analog CMP is phosphorylated by a cytoplasmic fraction during oxidative phosphorylation to cytidine-5′-di- and triphosphates (CDP and CTP, respectively). The adenylate kinase of mitochondria has apparently no action on CMP. Evidence obtained through the use of inorganic phosphate-P^{32} is consistent with the role of ATP as an intermediate phosphate acceptor during the incorporation of inorganic phosphate into CDP and CTP.

Strominger *et al.*[65] have described a liver enzyme preparation which effects the following transphosphorylation:

$$\text{ATP} + \text{CMP} \rightleftharpoons \text{ADP} + \text{CDP}$$

Maley[79] has similarly reported the preparation of a purified cytidine monophosphokinase from *A. vinelandii*. The preparation also contains an ATP-linked nucleoside diphosphokinase converting CDP to CTP. Strominger[80] has also reported the phosphorylation of CDP by pyruvate phosphokinase, the system apparently not involving adenine nucleotides as intermediate phosphate donors.

Brawerman and Chargaff[81-83] have described a nucleoside phosphotransferase in crude extracts of a variety of cell types. CMP is formed exclusively from cytidine and an organic phosphate by plant and bacterial enzymes whereas CMP and some cytidine-3′-phosphate are formed by the corresponding mammalian system. The variation in transferase activity with the growth phase of the parent cell points to a role of the phosphotransferase in nucleotide biosynthesis. As no *de novo* pathway of cytidine (or uridine) synthesis other than via the corresponding 5′-nucleotides has been described, the actual role of the nucleoside phosphotransferase enzyme may be merely that of a salvage mechanism.

A connection between cytosine and uracil derivatives is achieved through amination and deamination reactions. Lieberman[84, 85] has reported the conversion of UTP to CTP by an *E. coli* extract in the presence of NH_3 and ATP. Aspartic acid, asparagine, glutamic acid, and glutamine failed to replace NH_3. Uracil, uridine, and UMP were similarly not utilized. UDP was utilized for CTP formation but the evidence available indicated its prior conversion to UTP. That the amination mechanism in mammalian cells may differ from that in *E. coli* is indicated by the work of Eidinoff

[79] F. Maley, *Federation Proc.* **17**, 267 (1958).
[80] J. L. Strominger, *Biochim. et Biophys. Acta* **16**, 616 (1955).
[81] G. Brawerman and E. Chargaff, *Biochim. et Biophys. Acta* **15**, 549 (1954).
[82] G. Brawerman and E. Chargaff, *Biochim. et Biophys. Acta* **16**, 524 (1955).
[83] M. Tunis and E. Chargaff, *Biochim. et Biophys. Acta* **21**, 205 (1956).
[84] I. Lieberman, *J. Am. Chem. Soc.* **77**, 2661 (1955).
[85] I. Lieberman, *J. Biol. Chem.* **222**, 765 (1956).

et al.,[86] who have reported that the incorporation of carbamylaspartic acid and orotic acid into the polynucleotide cytosine of some rat tissues was significantly depressed relative to the effect on uracil and thymine by the glutamine analog, 6-diazo-5-oxo-L-norleucine (DON) (see Chapter 39). DON had little effect on the utilization of cytidine-3′(2′-)-phosphate-G-C^{14} by tumor slices for polynucleotide pyrimidine synthesis in marked contrast to its effect on the incorporation of uridine-3′(2′-)-phosphate-G-C^{14} into polynucleotide cytosine. Salzman *et al.*[87] have also reported that the 6-amino group of polynucleotide cytosine is derived from the glutamine amide group rather than ammonia. Kammen and Hurlbert[88] have recently extended these observations and have described a synthesis of cytidine nucleotides from orotic acid-6-C^{14} and uridine-5′-phosphate-6-C^{14} by a soluble enzyme system from the Novikoff hepatoma. For full activity, the Dowex-1-(formate)-treated enzyme required glutamine, ATP, guanosine-5′-phosphate (GMP), and an ATP-generative system. GMP could be replaced by the corresponding triphosphate. Low levels of DON were inhibitory (cf. the antagonistic effect of DON on other glutamine amide transfer reactions[89]). The complexity of kinase and pyrophosphatase activities present in the system obscured the nature of the substrate of the amination reaction but the involvement of UTP is considered likely on the basis of the evidence available.

The uracil and cytosine ring systems are also interrelated through the activities of the cytosine and cytidine deaminases described in a variety of mammalian and microbial cells. The systems are, however, generally considered to be catabolic with respect to the cytosine ring rather than anabolic with respect to uracil derivatives. Thus cytidine-3′-phosphate is catabolized by a high speed supernatant fraction of rat liver homogenates, the products being uracil, ammonia, and inorganic phosphate.[90] The available evidence implicated cytidine and uridine but not uridine-3′-phosphate or cytosine as intermediates.

Both CDP and CTP have been shown to participate in polynucleotide synthesis. CDP is a substrate of polynucleotide phosphorylase[72] and CTP has been implicated[91] as a proximal precursor during the incorporation of CMP into end groups of the RNA of the supernatant fraction of Ehrlich ascites carcinoma cells.

[86] M. L. Eidinoff, J. E. Knoll, B. Marano, and L. Cheong, *Cancer Research* **18**, 105 (1958).
[87] N. P. Salzman, H. Eagle, and E. D. Sebring, *J. Biol. Chem.* **230**, 1001 (1958).
[88] H. O. Kammen and R. B. Hurlbert, *Biochim. et Biophys. Acta* **30**, 195 (1958).
[89] B. Levenberg, I. Melnick, and J. M. Buchanan, *J. Biol. Chem.* **225**, 163 (1957).
[90] M. J. Simcock, G. Sneyd, and R. D. Blatt, *Arch. Biochem. Biophys.* **71**, 62 (1957).
[91] L. I. Hecht, P. C. Zamecnik, M. L. Stephenson, and J. F. Scott, *J. Biol. Chem.* **233**, 954 (1958).

The biosynthesis of cytidine nucleotide coenzymes has been reviewed recently by Baddiley and Buchanan[76] and will not be discussed here.

2. Biosynthesis of Deoxycytidine-5′-mono-, di-, and triphosphates

Deoxycytidine nucleotides have been isolated from the acid-soluble fraction of thymus.[92, 93] Deoxycytidine-5′-phosphate (dCMP) can be phosphorylated by ATP in the presence of the cytidine monophosphate kinase described by Maley[79] and the product further phosphorylated to the corresponding triphosphate (dCTP) by a nucleoside diphosphate kinase. Lehman *et al.*[94] have purified similar kinases from *E. coli* B. The 5′-di- and triphosphates of deoxycytidine (and thymidine) have been isolated following the ATP-linked phosphorylation of the corresponding deoxynucleoside-5′-phosphate by rat liver homogenates and by a high-speed supernatant fraction of regenerating rat liver.[95]

The *de novo* synthesis of deoxycytidine nucleotides probably involves a pathway of the following type:

$$\rightarrow \text{UMP} \rightarrow \text{UDP} \rightarrow \text{UTP} \rightarrow \text{CTP} \rightarrow ? \rightarrow \text{dCMP}$$

The available evidence indicates that dCTP is not formed by amination of deoxyuridine-5′-triphosphate by analogy with the UTP/CTP conversion. Thus C^{14}-deoxyuridine is an effective precursor of DNA-thymine but not of DNA-cytosine or RNA-pyrimidines of regenerating liver,[96] intestinal mucosa,[96] minced chicken embryo,[97] and suspensions of rabbit and chicken bone marrow cells.[97] Further, Friedkin and Kornberg[98] have shown in a study of the 5′-deoxynucleotide kinase activity of *E. coli* extracts that no activity with respect to deoxyuridine-5′-phosphate (dUMP) could be detected.

Implicit in the proposed pathway of *de novo* deoxycytidine nucleotide formation is the conversion of a ribose residue to a 2-deoxyribose residue. The problem of deoxyribose synthesis will be discussed later but it may be noted that a considerable body of evidence exists pointing to a ribose to deoxyribose conversion at the level of a nucleoside or nucleotide without the

[92] R. L. Potter and S. Schlesinger, *J. Am. Chem. Soc.* **77,** 6714 (1955).
[93] R. L. Potter, S. Schlesinger, V. Buettner-Janusch, and L. Thompson, *J. Biol. Chem.* **226,** 381 (1957).
[94] I. R. Lehman, M. J. Bessman, E. S. Simms, and A. Kornberg, *J. Biol. Chem.* **233,** 163 (1958).
[95] E. S. Canellakis and R. Mantsavinos, *Biochim. et Biophys. Acta* **27,** 643 (1958).
[96] P. Reichard, *Acta Chem. Scand.* **9,** 1275 (1955).
[97] M. Friedkin and D. Roberts, *J. Biol. Chem.* **220,** 653 (1956).
[98] M. Friedkin and A. Kornberg, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 609. Johns Hopkins Press, Baltimore, 1957.

fission of the *N*-glycoside linkage. Thus cytidine-G-C^{14} has been shown by Rose and Schweigart[99] to be incorporated intact into the deoxycytidine residues of DNA of the rat. This result has been confirmed for a variety of avian[100] and mammalian tissues[101], [102] and for a cytidineless mutant of *Neurospora*.[103] The level at which the sugar interconversion occurs is not clear but Reichard[100] has presented evidence in favor of a reaction at the nucleoside-5′-monophosphate level.

Together with other deoxynucleotide-5′-triphosphates, dCTP has been shown[94, 104] to be a substrate of an *E. coli* polymerase enzyme which effects net synthesis of DNA in the presence of primer DNA, Mg^{++}, and the other three deoxynucleotide-5′-triphosphates (see Chapter 31).

A new type of deaminase, deoxycytidine-5′-phosphate deaminase, has been reported recently by Scarano[105] in homogenates and acetone powder extracts of *Paracentrotus lividus*. The function of this enzyme is not yet clear.

3. Biosynthesis of 5-Hydroxymethyldeoxycytidine-5′-phosphate

5-Hydroxymethylcytosine has been identified[106, 107] as a constituent of T-even *E. coli* bacteriophage DNA in which it occurs more or less fully glucosylated on the 5-hydroxymethyl group.[108, 109] Bacterial DNA-cytosine is known to serve as a precursor of both viral 5-hydroxymethylcytosine and thymine whereas bacterial thymine cannot be transformed to 5-hydroxymethylcytosine.[110] The β-carbon of serine is incorporated into the hydroxymethyl and methyl groups of 5-hydroxymethylcytosine and thymine, respectively.[111]

Flaks and Cohen[112] have recently described a synthesis of 5-hydroxymethyldeoxycytidine-5′-phosphate from formaldehyde-C^{14} and dCMP in the presence of a phosphate buffer extract of alumina-ground $T6r^{+}$- infected

[99] I. A. Rose and B. S. Schweigert, *J. Biol. Chem.* **202,** 635 (1953).
[100] P. Reichard, *Biochim. et Biophys. Acta* **27,** 434 (1958).
[101] P. M. Roll, H. Weinfeld, and E. Carroll, *J. Biol. Chem.* **220,** 455 (1956).
[102] P. Reichard, *Acta Chem. Scand.* **11,** 11 (1957).
[103] W. S. McNutt, *J. Biol. Chem.* **233,** 189 (1958).
[104] M. J. Bessman, I. R. Lehman, E. S. Simms, and A. Kornberg, *J. Biol. Chem.* **233,** 171 (1958).
[105] E. Scarano, *Biochim. et Biophys. Acta* **29,** 459 (1958).
[106] S. S. Cohen, *Cold Spring Harbor Symposia Quant. Biol.* **18,** 221 (1953).
[107] G. R. Wyatt and S. S. Cohen, *Biochem. J.* **55,** 774 (1953).
[108] E. Volkin, *J. Am. Chem. Soc.* **76,** 971 (1954).
[109] R. L. Sinsheimer, *Proc. Natl. Acad. Sci. U. S.* **42,** 502 (1956).
[110] L. L. Weed and S. S. Cohen, *J. Biol. Chem.* **192,** 693 (1951).
[111] S. S. Cohen and L. L. Weed, *J. Biol. Chem.* **209,** 789 (1954).
[112] J. G. Flaks and S. S. Cohen, *Biochim. et Biophys. Acta* **25,** 667 (1957).

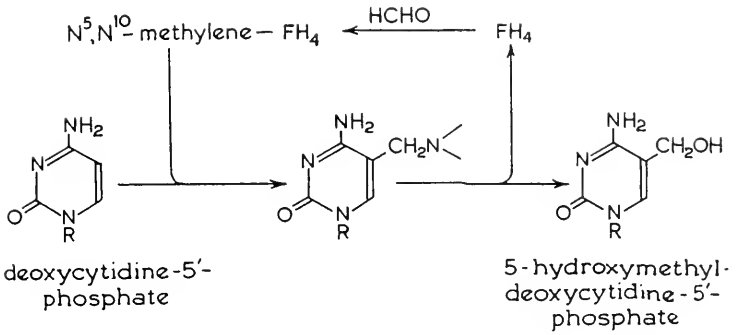

FIG. 3. The 5-hydroxymethyldeoxycytidine-5′-phosphate synthetase reaction (R = 5′-phosphodeoxyribosyl).

cells of *E. coli*. A requirement for tetrahydrofolic acid (FH_4) was demonstrated using Dowex-1-(chloride)-treated enzyme. The reaction may be envisaged as a Mannich-type base formation followed by hydrolysis (Fig. 3) or as a Knoevenagel-type reaction.

The possible biosynthetic relationship between 5-hydroxymethyl and 5-methyl pyrimidine derivatives will be considered later. Little is known of the mechanism of biosynthesis of the 5-methylcytosine ring. Normally considered as a constituent of plant and mammalian DNA's, 5-methylcytosine has recently been isolated as the 3′(2′-)-ribonucleotide from the RNA of *E. coli* K 12.[113]

It may be noted that 5-methyldeoxycytidine is a substrate of a purified deoxycytidine deaminase of *E. coli*[114] and by virtue thereof is capable of supporting the growth of the thymine-less mutant, *E. coli* 15T-, at a rate equal to that supported by thymidine.

IV. Biosynthesis of Thymidine Nucleotides

1. Biosynthesis of Thymidine-5′-phosphates

a. Biosynthesis in Whole Cell Systems

Considerable evidence exists pointing to the role of uracil or cytosine derivatives as precursors of polynucleotide thymine. Orotic acid[115] is a known precursor and uracil is utilized for DNA-thymine synthesis in uracil-less mutants of *E. coli*.[116, 117] The incorporation of cytidine and uridine as

[113] H. Amos and M. Korn, *Biochim. et Biophys. Acta.* **29,** 445 (1958).
[114] S. S. Cohen and H. D. Barner, *J. Biol. Chem.* **226,** 631 (1957).
[115] P. Reichard, *Acta Chem. Scand.* **3,** 422 (1949).
[116] A. M. Moore and J. B. Boylen, *Arch. Biochem. Biophys.* **54,** 312 (1955).
[117] M. Green and S. S. Cohen, *J. Biol. Chem.* **225,** 387 (1957).

intact units into DNA-thymidine residues has likewise been repeatedly observed.[99-103, 118]

The 5-methyl group of mammalian DNA would appear to have its origin in a "1-C" unit derivable from formate-C^{14},[119] formaldehyde-C^{14},[120, 121] serine-3-C^{14},[122] glycine-2-C^{14},[119] or methionine-methyl-C^{14}.[123, 124] Elwyn and Sprinson[122] have shown, using L-serine-2,3-D,3-C^{14},N^{15}, that the pathway of incorporation of the β-carbon does not involve a derivative at the oxidation level of formate. Lowy *et al.*[120] have shown that C^{14}, D-labeled formaldehyde (a mixture of $C^{14}H_2O$, CD_2O, and CHDO) is incorporated into the rat DNA methyl group with a considerable loss of D relative to C^{14}. The authors have pointed out, however, that the obvious significance of the results may be vitiated by isotope selection effects (cf. Rachele *et al.*[125] and Abeles[126]). Crosbie[127] has shown that formate-C^{14} is utilized for the "1-C" positions of the purine ring (positions 2 and 8) but not for the thymine methyl group of *E. coli* DNA.

Green and Cohen[117] and Crosbie[127] have shown using methionine-less mutants of *E. coli* that methionine is not a methyl donor in thymine ring biosynthesis. This conclusion is confirmed by the nonutilization of methionine-methyl-C^{14} for DNA-thymine synthesis in a uracil-less mutant of *E. coli*.[117] S-Hydroxymethylhomocysteine does not appear to be a precursor of the methyl group of thymine (or of the 5-hydroxymethyl group of viral 5-hydroxymethylcytosine).[117] It would appear[124] that in systems in which methionine-methyl utilization has been reported the pathway of incorporation involves a prior conversion of the —S—Me group to the active donor, probably a derivative of folic acid (*vide infra*).

A kinetic study of glycine-2-C^{14} and serine-3-C^{14} incorporation into exponentially growing cells of *E. coli* has revealed[127] that glycine and serine do not lie on the pathway of incorporation of serine-3-C^{14} and glycine-2-C^{14}, respectively, into the thymine methyl group.

The evidence of incorporation experiments points to a role of N-hydroxymethyltetrahydrofolic acid (N—CH_2OH—FH_4)—or the N^5,N^{10}-methyl-

[118] H. Amos and B. Magasanik, *J. Biol. Chem.* **229,** 653 (1957).
[119] J. R. Totter, E. Volkin, and C. E. Carter, *J. Am. Chem. Soc.* **73,** 1521 (1951).
[120] B. A. Lowy, G. B. Brown, and J. R. Rachele, *J. Biol. Chem.* **220,** 325 (1956).
[121] R. L. Hamill, R. L. Hermann, R. U. Byerrum, and J. L. Fairley, *Biochim et Biophys. Acta* **21,** 394 (1956).
[122] D. Elwyn and D. B. Sprinson, *J. Biol. Chem.* **207,** 467 (1954).
[123] R. L. Herrmann, J. L. Fairley, and R. U. Byerrum, *J. Am. Chem. Soc.* **77,** 1902 (1955).
[124] S. Kit, C. Beck, O. L. Graham, and A. Gross, *J. Biol. Chem.* **233,** 944 (1958).
[125] J. R. Rachele, E. J. Kuchinskas, F. H. Kratzer, and V. du Vigneaud, *J. Biol. Chem.* **215,** 593 (1955).
[126] R. H. Abeles, *Federation Proc.* **14,** 170 (1955).
[127] G. W. Crosbie, *Biochem. J.* **69,** 1P (1958).

ene-bridge derivative—in the "methylation" reaction [cf. the role of N^{10}-formyltetrahydrofolic acid (N^{10}—CHO—FH_4) in purine biosynthesis, see Greenberg *et al.*,[128] Buchanan *et al.*,[129, 130] and Chapter 35]. Several authors have reported N—CH_2OH—FH_4 (or N^5, N^{10}-methylene-FH_4) as a product (*a*) of serine hydroxymethylase action on serine,[131-133] (*b*) of nonenzymic interaction[132, 134] of HCHO and FH_4, and (*c*) of "*N*-hydroxymethyltetrahydrofolic acid" dehydrogenase action[135, 136] on N^{10}-CHO—FH_4 (or the N^5, N^{10}-methenyl-bridge derivative). The pathway of glycine-2-C^{14} incorporation into the thymine methyl group can be inferred from the work of Nakada and Weinhouse[137] to involve an initial transamination to glyoxylate-2-C^{14} followed by oxidation to formate-C^{14} and then by way of N^{10}—$C^{14}HO$—FH_4 formation and the steps outlined above. The evidence relating to glyoxylate involvement in formate production is based solely on experiments employing the trapping technique. The recent observation by Fleming and Crosbie[138] of the randomization of activity between glycine-2-C^{14} and glyoxylate due to facile nonenzymic transamination thereby renders the proposed details of this pathway suspect. The observation[127] of formate utilization for purine "1-C" positions but not for the thymine methyl group in an organism (*E. coli*) in which glycine-2-C^{14} is utilized for all "1-C" positions also strongly indicates that free formate (and probably N^{10}—CHO—FH_4) is not involved in the glycine incorporation pathway. Consideration must be given to the possible role of δ-aminolevulinic acid or aminoacetone[138a, 138b] as intermediates between glycine and a compound at the oxidation level of formaldehyde.

Experiments designed to illuminate the nature of the "1-C" acceptor molecule in thymine ring synthesis have been described by Reichard.[96] A comparison of the utilization of 5-methyluridine-2-C^{14}, deoxyuridine-2-C^{14}, uridine-2-C^{14}, thymine-2-C^{14}, and thymidine-2-C^{14} for polynucleotide pyrimidine synthesis in regenerating rat liver and intestinal mucosa has

[128] G. R. Greenberg, L. Jaenicke, and M. Silverman, *Biochim. et Biophys. Acta* **17,** 589 (1955).
[129] J. G. Flaks, M. J. Erwin, and J. M. Buchanan, *J. Biol. Chem.* **229,** 603 (1957).
[130] L. Warren, J. G. Flaks, and J. M. Buchanan, *J. Biol. Chem.* **229,** 627 (1957).
[131] L. Jaenicke, *Biochim. et Biophys. Acta* **17,** 588 (1955).
[132] L. Jaenicke, *Federation Proc.* **15,** 281 (1956).
[133] J. M. Huennekens, Y. Hatefi, and L. D. Kay, *J. Biol. Chem.* **224,** 435 (1957).
[134] R. L. Blakley, *Nature* **182,** 1719 (1958).
[135] G. R. Greenberg, L. Jaenicke, and M. Silverman, *Biochim. et Biophys. Acta* **17,** 589 (1955).
[136] Y. Hatefi, M. J. Osborn, L. D. Kay, and F. M. Huennekens, *J. Biol. Chem.* **227,** 637 (1957).
[137] H. I. Nakada and S. Weinhouse, *Arch. Biochem. Biophys.* **42,** 257 (1953).
[138] L. Fleming and G. W. Crosbie, *Biochim. et Biophys. Acta* (in press).
[138a] K. D. Gibson, W. G. Laver, and A. Neuberger, *Biochem. J.* **70,** 71 (1958).
[138b] W. H. Elliot, *Biochem. J.* **74,** 478 (1960).

revealed that deoxyuridine like thymidine is utilized almost exclusively for DNA-thymidine formation. No incorporation of deoxyuridine into DNA-cytosine was noted. Uridine was utilized extensively for polynucleotide uracil, cytosine, and thymine synthesis whereas 5-methyluridine, like thymine, showed a small but significant incorporation into DNA-thymine of regenerating liver (but not of intestinal mucosa). The evidence suggests that deoxyuridine (or a nucleotide derivative) is the primary "1-C" acceptor molecule in thymine ring biosynthesis. Friedkin and Roberts[97] have described a similar aminopterin-inhibited incorporation of deoxyuridine into the DNA-thymine of suspensions of chick embryo and bone marrow cells. Prusoff and co-workers[43, 139] have shown that deoxyuridine, deoxycytidine, and the corresponding ribonucleosides increase the incorporation of formate-C^{14} into DNA-thymine of rabbit bone marrow and Ehrlich ascites carcinoma cells. The cytosine nucleosides are markedly more efficient than the corresponding uracil derivatives. This evidence together with the observation[114] of 5-methyldeoxycytidine deaminase activity in *E. coli* extracts has been interpreted as indicating the possible role of deoxycytidine (or a nucleotide derivative) as a "1-C" acceptor molecule in thymine-ring formation.

The isotopic evidence on which this conclusion is based cannot, however, be considered as unequivocal owing to the difficulties inherent in the interpretation of the results of incorporation studies in whole-cell systems in which permeability, pool size, and kinetic factors of unknown character and magnitude may operate. It may be noted that Kit *et al.*[124] have shown that acid-soluble 5-methyldeoxycytidine and the corresponding 5′-deoxynucleotide are not labeled during the incorporation of formaldehyde-C^{14} into the DNA-thymine of lymphatic-cell suspensions supplemented with 5-methyldeoxycytidine.

The intact incorporation of cytidine-G-C^{14} and uridine-G-C^{14} into the deoxynucleotide residues of mammalian, avian, and bacterial DNA clearly indicates as previously discussed that the conversion of a ribonucleoside (or ribonucleotide) to the corresponding deoxyribonucleoside (or deoxyribonucleotide) can occur without fission of the *N*-glycoside linkage and without effective rupture of the pentose chain.[99-103] These conclusions are amplified by the studies of the distribution of activity in the polynucleotide ribose and deoxyribose chains of *E. coli* grown on a variety of C^{14}-substrates as carbon source. Thus Bernstein and Sweet[140] using lactate-1-C^{14} have found the pattern of activity in C-1 to C-5 of deoxyribose and ribose to be 21, 12, 62, 4, 3 and 10, 15, 69, 1, 1, respectively. These data, more so than those obtained in experiments with specifically labeled glucose, show that

[139] W. H. Prusoff, L. G. Lajtha, and A. D. Welch, *Biochim. et Biophys. Acta* **20**, 209 (1956).

[140] I. A. Bernstein and D. Sweet, *Federation Proc.* **17**, 190 (1958).

deoxyribose is derived from ribose or a precursor thereof. Comparable conclusions have been reached by Bagatell *et al.*[141] using acetate-1-C^{14} as a carbon source.

The exact role of the Racker aldolase enzyme[142] in deoxypentose synthesis is not yet clear. Lanning and Cohen[143] have shown that bacteriophage infection alters the labeling of deoxypentose from glucose-1-C^{14} in a manner tentatively considered to indicate the operation of the Racker aldolase rather than the normal pathway which in *E. coli* is predominantly from phosphogluconate via ribose phosphate.[144]

The role of vitamin B_{12} in nucleic acid synthesis is still obscure. In an attempt to clarify the role of B_{12} in deoxynucleoside synthesis, Downing and Schweigert[145] have studied the incorporation of thymidine-G-C^{14} into *L. leichmanii*, an organism whose B_{12} requirement can be replaced by deoxynucleosides. In the absence of added B_{12} the deoxyribose of the labeled substrate was utilized for DNA-pentose formation without dilution. In the presence of B_{12}, however, considerable dilution occurred. The results strongly suggest a role of B_{12} in deoxynucleoside synthesis, although participation in the Racker aldolase reaction or in the synthesis of the *N*-glycoside linkage does not appear to occur. In contrast to these results Wagle *et al.*[146] have shown using B_{12}-deficient piglets and chicks that the vitamin has no influence either on the incorporation of formate-C^{14}, formaldehyde-C^{14}, glycine-2-C^{14}, serine-3-C^{14}, or methionine-methyl-C^{14} into polynucleotide bases or on the utilization of glucose-C^{14} for polynucleotide pentose formation.

Dinning *et al.*[147] have reported that replacement of the B_{12} requirement of *L. leichmanii* by deoxycytidine considerably reduces the utilization of formate-C^{14} for DNA-thymine synthesis. No influence of B_{12} on the utilization of glycine-2-C^{14}, serine-3-C^{14}, or methionine-methyl-C^{14} was observed. The authors have proposed a role for B_{12} in the reduction of formate during methyl group synthesis. An involvement of B_{12} in "1-C" metabolism has also been suggested recently by Reichard[147a] on the basis of a study of the incorporation of cytidine-G-C^{14}, deoxyuridine-G-C^{14}, and thymidine-G-C^{14} into the pyrimidine nucleoside residues of the polynucleotides of B_{12}-deficient chick embryo minces. In this system, the incorporation of cytidine

[141] F. K. Bagatell, E. M. Wright, and H. Z. Sable, *Federation Proc.* **17**, 184 (1958).
[142] E. Racker, *J. Biol. Chem.* **196**, 347 (1952).
[143] M. C. Lanning and S. S. Cohen, *J. Biol. Chem.* **216**, 413 (1955).
[144] M. C. Lanning and S. S. Cohen, *J. Biol. Chem.* **207**, 193 (1954).
[145] M. Downing and B. S. Schweigert, *J. Biol. Chem.* **220**, 521 (1956).
[146] S. R. Wagle, D. A. Vaughan, S. P. Mistry, and B. Connor Johnson, *J. Biol. Chem.* **230**, 917 (1958).
[147] J. S. Dinning, B. K. Allen, R. S. Young, and P. L. Day, *J. Biol. Chem.* **233**, 674 (1958).
[147a] A. Bolinder and P. Reichard, *J. Biol. Chem.* **234**, 2723 (1959).

and deoxyuridine into DNA-thymidine was depressed as compared with controls. Addition of B_{12} *in vitro* to the deficient preparations stimulated the incorporation. No B_{12} effect on the utilization of cytidine for RNA-pyrimidine nucleoside or DNA-deoxycytidine formation could be detected. Likewise thymidine incorporation into DNA-thymidine was not B_{12} sensitive. The results would appear to demonstrate a role for B_{12} in the reactions concerned with the "methylation" reaction rather than with deoxypentose formation. The results are consistent with an involvement of B_{12} in the synthesis of a hydroxymethylfolic acid derivative (cf. Kisliuk and Woods[147b]). The ability of B_{12} to complex with formaldehyde is of interest in this connection.[147c] Several discussions on the possible relationship between B_{12} and "1-C" metabolism have appeared recently.[147d-147g]

b. Biosynthesis in Cell-Free Systems

The biosynthesis of thymidine-5′-phosphate (TMP) in cell-free systems has recently been described by several groups. Friedkin and Kornberg[98] have described a Dowex-1-(formate)-treated extract of *E. coli* which effects a synthesis of thymidine-5′-triphosphate (TTP) from deoxyuridine-5′-phosphate (dUMP) in the presence of FH_4, ATP, Mg^{++}, and thymidylate kinase. N^{10}-Hydroxymethyltetrahydrofolic acid (N^{10}—CH_2OH—FH_4) could substitute for serine. The TTP formed was assayed as a labeled phosphomonoesterase-resistant charcoal-adsorbable nucleotide. The TTP was also identified by ion-exchange chromatography and by degradation to thymidine.

Flaks and Cohen[112] and Birnie and Crosbie[148] have also studied thymidylate synthetase activity in similar coliform extracts and have described the FH_4-dependent synthesis of TMP from (*a*) dUMP and formaldehyde-C^{14} [112] and (*b*) deoxyuridine, ATP, Mg^{++}, TPNH (or DPNH), and serine-3-C^{14}.[148]

Phear and Greenberg[149] have described a comparable mammalian system [a Dowex-1-(chloride)-treated extract of thymus tissue] which couples deoxyuridine and formaldehyde-C^{14} in the presence of FH_4, ATP, Mg^{++}, and TPNH (rather than DPNH) to give TMP (together with a trace of thymidine formed from TPM through phosphomonoesterase action). The avail-

[147b] R. L. Kisliuk and D. D. Woods, *Abstr. VIIth Intern. Congr. Microbiol.*, Stockholm (1958), p. 135.
[147c] P. Vohra, F. Lantz, and F. H. Kratzer, *Arch. Biochem. Biophys.* **76,** 180 (1958).
[147d] E. Lester Smith, *Nature* **181,** 305 (1958).
[147e] H. R. V. Arnstein, *4th Intern. Congr. Biochem., Vienna,* (1958).
[147f] M. E. Coates and J. W. G. Porter, *Ann. Rev. Biochem.* **28,** 439 (1959).
[147g] J. M. Noronha and A. Sreenivasan, *Biochem. J.* **73,** 732 (1959).
[148] G. D. Birnie and G. W. Crosbie, *Biochem. J.* **69,** 1P (1958).
[149] E. A. Phear and D. M. Greenberg, *J. Am. Chem. Soc.* **79,** 3737 (1957).

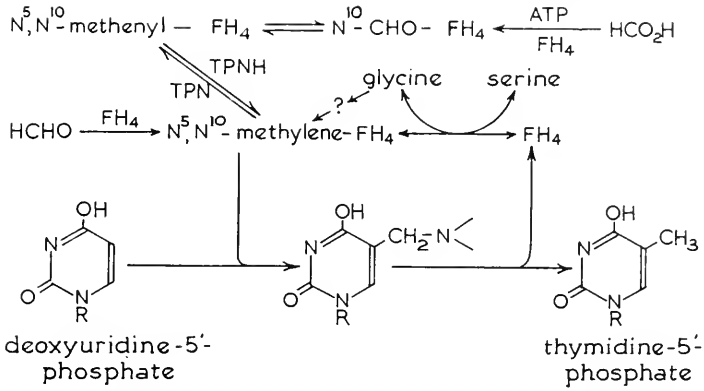

FIG. 4. The thymidine-5′-phosphate synthetase reaction together with certain pathways of "1-C" metabolism.

able evidence indicated deoxyuridine-5′-phosphate to be the primary "1-C" acceptor.

Blakley[150] has similarly studied the methylation of deoxyuridine by Dowex-1-treated soluble enzymes of thymus. In this system, the formation of methyl-labeled thymidine from deoxyuridine and serine-3-C^{14} required the addition of FH_4, ATP, Mg^{++}, and DPNH. Uridine, 4,5-dihydrouridine, and 4,5-dihydrodeoxyuridine were not involved in the methylation reaction (cf. Greenberg and Humphreys[151]). The statement that deoxyuridine-5′-phosphate is not utilized as effectively as deoxyuridine is difficult to reconcile with the observations of the other groups.

The over-all thymidylate synthetase reaction is shown in Fig. 4 together with the pathways of incorporation of methyl group precursors into the thymine ring. The assignation of structure of the immediate "1-C" donor as N^5, N^{10}-methylene-FH_4 may be tentatively made on the basis of the evidence of Blakley[134] and Kisliuk.[152] Kisliuk[152] has shown that formaldehyde binding by FH_4 is dependent on the presence of unsubstituted N^5H- and $N^{10}H$-positions. Blakley[134] has indicated that the primary site of interaction of formaldehyde and FH_4 is at the N^5H-position and that the structure of the adduct contains a N^5,N^{10}-methylene bridge. He has further shown that the only difference between synthetic N^5,N^{10}-methylene-FH_4 and the product of enzymic interaction of serine and FH_4 is in stereoisomeric respects. The data do not, however, rule out the possibility of enzymic involvement of an N-hydroxymethyltetrahydrofolic acid in facile equilibrium with the N^5,N^{10}-methylene-FH_4. The metabolic interrelationships of for-

[150] R. L. Blakley, *Biochim. et Biophys. Acta* **24**, 224 (1957).
[151] D. M. Greenberg and G. K. Humphreys, *Federation Proc.* **17**, 234 (1958).
[152] R. L. Kisliuk, *J. Biol. Chem.* **227**, 805 (1957).

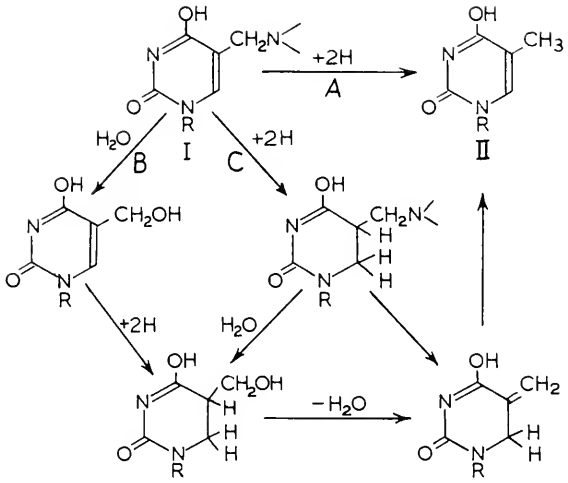

FIG. 5. Possible mechanisms of thymidine-5′-phosphate (II, R = 5-phosphodeoxyribosyl) formation from the postulated intermediate (I, R = 5-phosphodeoxyribosyl) in the thymidine-5′-phosphate synthetase reaction.

mate, N^{10}—CHO—FH_4, N^5,N^{10}-methenyl-FH_4, and N^5,N^{10}-methylene-FH_4 are discussed elsewhere in this volume (Chapter 35).

The mechanism of the "methylation" reaction is not yet clear but several mechanisms have been considered (Fig. 5). Scheme A (Fig. 5) envisages either a reductive fission[98] of the postulated intermediate at the CH_2—N linkage or an elimination reaction to yield TMP and dihydrofolic acid (FH_2), the reduced pyridine nucleotide requirement being in respect of regeneration of FH_4 from FH_2. Evidence bearing on the latter possibility has been provided by the observation by Greenberg and Humphreys[151] of a stoichiometric relationship between thymine methyl group formation and FH_4 concentration in the enzymic system previously described by Phear and Greenberg.[149] DPNH stimulates methyl group synthesis in the presence of suboptimal concentrations of FH_4.

Scheme B (Fig. 5) involves a hydrolysis of the postulated intermediate to yield a 5-hydroxymethylpyrimidine derivative which is successively reduced to the 4,5-dihydro derivative, dehydrated and rearranged.[121, 153] It is of interest to note that Cohen *et al.*[153] have reported that catalytic hydrogenation of 5-hydroxymethyluracil and the corresponding deoxyribonucleoside yields thymine and thymidine, respectively, in addition to 4,5-dihydro derivatives (cf. Fink[154]).

[153] S. S. Cohen, M. Green, and H. D. Barner, *Biochim. et Biophys. Acta* **22**, 210 (1956).
[154] R. M. Fink, R. E. Cline, and K. Fink, *Federation Proc.* **15**, 251 (1956).

Cohen *et al.*[155] have investigated the role of 5-hydroxymethyluracil, 5-hydroxymethylcytosine, and the corresponding deoxyribonucleosides in bacterial and bacteriophage DNA-pyrimidine synthesis using the isotopic competition technique. No evidence for the utilization of any 5-hydroxymethylpyrimidine derivative was obtained. 4,5-Dihydro-5-hydroxymethyluracil, 4,5-dihydro-5-hydroxymethylcytosine, and the corresponding deoxynucleosides do not support[156] the growth of the thymine-less mutant, *E. coli* 15T-. It would appear that any involvement of 5-hydroxymethyl- and 4,5-dihydro-5-hydroxymethylpyrimidine derivatives in thymine ring biosynthesis must occur at the level of the nucleotide.

A possible alternative mechanism not outlined in Fig. 5 involves a reduction of dUMP to the 4,5-dihydro derivative prior to condensation with N^5,N^{10}-methylene-FH_4. There is, however, considerable evidence contraindicating the role of dihydropyrimidine derivatives in TMP synthesis. Thus dihydrodeoxyuridine (or dihydrodeoxycytidine) is not utilized[155] for DNA-thymine synthesis in *E. coli* B, *E. coli* 15T- (a thymine-less mutant), or for viral thymine and 5-hydroxymethylcytosine in $T6r^+$ grown in *E. coli* B or *E. coli* Bu- (a uracil-less mutant). Evidence that dihydrodeoxyuridine-2-C^{14} penetrates the coli cell is provided by the isolation of a phosphorylated derivative (probably the corresponding 5′-nucleotide) from the acid-soluble fraction of *E. coli* 15T-. Blakley[150] has similarly reported the noninvolvement of dihydrodeoxyuridine (and dihydrouridine) in thymidine synthesis in his cell-free thymus system. This evidence is not, however, unequivocal as the "methylation reaction" may well be occurring at the level of the nucleotide in a system possessing no kinase activity towards the dihydrodeoxynucleoside.

E. coli thymidylate synthetase (unlike 5-hydroxymethyldeoxycytidylate synthetase) is inhibited powerfully and irreversibly by 5-fluorouracil deoxyribonucleoside-5′-phosphate.[157]

Integration of the pathway of *de novo* synthesis of UMP with the thymidylate synthetase reaction has been achieved recently by Reichard[100] who has described briefly the formation of dUMP from UMP by a soluble enzyme of chick embryo homogenates. No evidence for a pentose interconversion at the nucleoside level was obtained (cf. Grossman[158] who has recently reported without details that a dithiol-activated soluble enzyme of *Salmonella tymphimurium* LT-2 effects a conversion of uridine to deoxyuri-

[155] S. S. Cohen, J. Lichtenstein, H. D. Barner, and M. Green, *J. Biol. Chem.* **228,** 611 (1957).

[156] M. Green, H. D. Barner, and S. S. Cohen, *J. Biol. Chem.* **228,** 621 (1957).

[157] S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb, and J. Lichtenstein, *Proc. Natl. Acad. Sci. U.S.* **44,** 1004 (1958).

[158] L. Grossman, *Federation Proc.* **17,** 235 (1958).

dine). In view of the speculations regarding the role of cytosine derivatives as "1-C" acceptor molecules in thymine ring biosynthesis, the evaluation of the quantitative significance of the →UMP → dUMP → TMP pathway will be awaited with interest. The mechanism of the reduction at postition 2′ of the ribonucleotide is not yet understood. Brown *et al.*[159] have tentatively speculated on the role of a pyrimidine-O^2,2′-cyclonucleoside structure in deoxypentose synthesis although stereochemical factors would argue against the participation of analogous derivatives in purine deoxynucleotide synthesis. It should be noted that Reichard[159a] could find no evidence for the utilization of O^2,2′-cyclouridine-2-C^{14} for polynucleotide pyrimidine synthesis in a chick embryo mince system which incorporates uridine-2-C^{14} into polynucleotide pyrimidines and deoxyuridine-2-C^{14} into DNA-thymine. Acetone powder extracts of the embryonic cell system possessed, however, no significant kinase activity toward the cyclonucleoside and the evaluation of the role of such cyclic structures in deoxypentose formation must therefore await appropriate experimentation with O^2,2′-cyclouridine-5′-phosphate.

c. Biosynthesis from Preformed Thymine Derivatives

Free thymine, like uracil and cytosine, has generally been considered not to be utilized[39-41] for mammalian DNA synthesis although Reichard[96] in confirmation of earlier work of Holmes *et al.*[160] has observed a small but significant incorporation of thymine-2-C^{14} into regenerating rat liver DNA. Thymidine, like other pyrimidine deoxyribonucleosides is extensively utilized for DNA-thymine formation in a number of avian[161, 162] and mammalian[96, 151-163] tissues. The incorporation of thymidine into the DNA of rat liver homogenates has been reported.[164]

Isotope competition experiments have revealed that both thymine and thymidine are not utilized for *E. coli* DNA synthesis.[165] The relatively insensitive nature of the technique would, however, obscure the small but significant incorporation of thymine and thymidine into *E. coli* DNA reported by Graham and Siminovitch[166] and Kozloff,[167] respectively. It is of

[159] D. M. Brown, D. B. Parihar, C. B. Reese, and A. R. Todd, *J. Chem. Soc.* p. 3035 (1958).

[159a] P. Reichard, *J. Biol. Chem.* **234**, 2719 (1959).

[160] W. L. Holmes, W. H. Prusoff, and A. D. Welch, *J. Biol. Chem.* **209**, 503 (1954).

[161] M. Friedkin and H. Wood, *J. Biol. Chem.* **220**, 639 (1956).

[162] M. Friedkin, D. Tilson, and D. Roberts, *J. Biol. Chem.* **220**, 627 (1956).

[163] P. Reichard and B. Estborn, *J. Biol. Chem.* **188**, 839 (1951).

[164] F. J. Bollum and V. R. Potter, *J. Am. Chem. Soc.* **79**, 3603 (1957).

[165] L. Siminovitch and A. F. Graham, *Can. J. Microbiol.* **1**, 721 (1955).

[166] A. F. Graham and L. Siminovitch, *in* "Canadian Cancer Conference" (R. W. Begg, ed.), Vol. I, p. 358. Academic Press, New York, 1955.

[167] L. M. Kozloff, *Cold Spring Harbor Symposia Quant. Biol.* **18**, 1209 (1953).

interest to note that back-mutation to thymine-independence of the thymine-less mutant, *E. coli* 15T-, does not involve a loss of ability to utilize the free pyrimidine or the deoxynucleoside.[168]

The pathway of utilization of thymidine has been elucidated by the observation of kinase activities in cell-free preparations of mamamlian[95, 169, 170] and bacterial[94] origin which effect the following transphosphorylations:

$$\text{Thymidine} \xrightarrow{\text{ATP}} \text{TMP} \xrightarrow{\text{ATP}} \text{TDP} \xrightarrow{\text{ATP}} \text{TTP}$$

The occurrence of TDP and TTP in extracts of thymus tissue has been reported[93] and the involvement of TTP in DNA synthesis in soluble *in vitro* enzyme systems has also been described.[94, 104, 169, 170]

The coupling of thymidine phosphorylase with thymidine kinase offers a pathway of thymine utilization.

V. Alternate Pathways of Pyrimidine Nucleotide Synthesis

Evidence pertaining to the operation of alternative pathways of pyrimidine nucleotide synthesis has been accumulating in recent years. Consideration has been given to the possible reversal of the reaction sequence[52-56] which effects the degradation of uracil to β-alanine via dihydrouracil and carbamyl-β-alanine (β-ureidopropionic acid). The enzymes involved in this pathway have been extensively investigated.[52, 171] Fritzson[55] has indicated, however, that the only effectively reversible step in rat liver slices is the interconversion of dihydrouracil and carbamyl-β-alanine. As uracil is utilized only to a negligible extent in rat liver for polynucleotide synthesis it would be of interest to study the anabolic potentialities of the degradation pathway in a tissue capable of more extensive uracil utilization. It should be noted in this connection that dihydrouracil and dihydrocytosine (and the corresponding ribonucleosides and deoxyribonucleosides) do not support the growth of a variety of pyrimidine-less mutants of *E. coli*.[155] Lagerkvist *et al.*[172] have also shown that N^{15}-labeled dihydrouracil, carbamyl-β-alanine, and β-alanine, unlike uracil, are not utilized in a significant manner for polynucleotide pyrimidine synthesis in the Ehrlich ascites cell. Heidelberger *et al.*,[47] on the other hand, have reported that dihydrouracil-2-C^{14} is incorporated to a small extent into acid-soluble nucleotides by a high-speed supernatant fraction of homogenates of rat liver or of the Flexner-Jobling carcinoma. The extent of utilization of the dihydropyrimidine was slightly greater than that of uracil. The significance of these observations is not clear. The possibility that the observed incorporation of dihydrouracil-2-

[168] L. V. Crawford, *Biochim. et Biophys. Acta* **30**, 428 (1958).
[169] F. J. Bollum, *Federation Proc.* **17**, 193 (1958).
[170] F. J. Bollum and V. R. Potter, *J. Biol. Chem.* **233**, 478 (1958).
[171] L. L. Campbell, Jr., *J. Biol. Chem.* **227**, 693 (1957).
[172] See ref. 36a, page 328.

C^{14} was due to hydrolysis to carbamyl-β-alanine followed by carbamyl transfer to aspartic acid must be considered.

The possible involvement of β-alanine in pyrimidine synthesis in the pyrimidine-less mutant *N. crassa* 1298 has been proposed by Boyd and Fairley.[173] The mutant is capable of growth on propionic acid or α-aminobutyric acid. The incorporation of these precursors into the pyrimidine ring has been stated without evidence to involve a derivative of β-alanine. It should be noted that certain mutants of *E. coli* capable of growth on dihydrouracil have recently been shown to be pantothenate-less mutants.[174]

The most significant indication of the role of dihydropyrimidines in nucleic acid synthesis derives from the recent work of Mokrasch and Grisolia[175] who have reported that a soluble enzyme system of avian liver is capable of incorporating carbamyl-β-alanine, carbamyl-β-alanine ribonucleoside, carbamyl-β-alanine 5′-ribonucleotide, dihydrouracil, dihydrouridine, and dihydrouridine-5′-phosphate into the RNA of the system by a pathway not involving orotic acid. The more extensive incorporation of the ribonucleotides as compared with the other substrates strongly suggests that the direct reaction sequence involves the ribonucleotide derivatives. The further investigation of these reactions together with an evaluation of their quantitative significance will be awaited with interest. It may be noted that Visser *et al.*[176] have detected negligible incorporation of dihydrouridine-3′(2′-)-phosphate-G-C^{14} into the polynucleotides of rat intestinal mucosa and regenerating liver.

A comprehensive review of pyrimidine nucleotide biosynthesis by Reichard has appeared recently.[177]

[173] M. Boyd and J. L. Fairley, *Federation Proc.* **17,** 193 (1958).
[174] I. J. Slotnick and H. Weinfeld, *J. Bacteriol.* **74,** 122 (1957).
[175] L. C. Mokrasch and S. Grisolia, *Biochim. et Biophys. Acta* **27,** 226 (1958).
[176] D. W. Visser, D. Van Praag, and T. K. Fukuhara, *Arch. Biochem. Biophys.* **70,** 217 (1957).
[177] P. Reichard, *Advances in Enzymol.* **21,** 263 (1959).

CHAPTER 37

The Relationship of Nucleic Acid and Protein Synthesis as Revealed by Studies in Cell-Free Systems* †

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I. Introduction

The unprecedented growth of interest in protein synthesis among biochemists is both a result of, and has contributed to, our rapidly advancing knowledge of the chemistry and metabolism of the nucleic acids. Thus these two lines of biochemical activity have become closely intertwined and it is appropriate that a discussion of protein synthesis should appear in a volume on the nucleic acids. Much has been written on the subject of the relationship of nucleic acids and protein synthesis in the past few years; over and above a vast number of original papers there have been, to the author's knowledge, sixteen published reviews and symposia on the subject in the past four years.[1-16] (General and comprehensive coverage of

* The preparation of this chapter has been aided by grants from the U. S. Public Health Service, the U. S. Atomic Energy Commission, and the American Cancer Society.

† This is publication No. 987 of the Cancer Commission of Harvard University.

[1] J. Brachet, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. II, p. 476. Academic Press, New York, 1955.

[2] H. Borsook, *Proc. 3rd Intern. Congr. Biochem., Brussels, 1955* p. 92 (1956).

[3] G. D. Novelli and J. A. DeMoss, *J. Cellular Comp. Physiol.* **50,** Suppl. 1 (1957).

the literature will be found particularly in the reviews of Brachet,[1] Borsook,[2] and Loftfield,[7] which deal mostly with animal systems; and in the review of Spiegelman[4] which is concerned mainly with bacterial systems. The review of Chantrenne[8] covers both areas and is the most recent. Amino acid activation, and attachment of amino acids to soluble RNA are emphasized in the papers of Novelli and DeMoss,[3] and of Hoagland,[14] and in the symposium.[13] Recent developments in the physicochemistry of the ribosomes is covered in the book "Microsomal Particles and Protein Synthesis."[16] The review by Bonner[12] is chiefly concerned with plant studies, and that of Campbell[11] relates to the biochemistry of cancer. The discussions of Dalgliesh,[9] Monod,[10] and Crick[6] stress theoretical aspects of RNA and protein synthesis). This phenomenon gives the author a degree of freedom and a degree of bondage: he may limit the scope of this treatise with assurance that aspects of secondary interest to his aim will be well covered elsewhere, but he is hard pressed to avoid saying that which has been said better elsewhere. This paper will be limited to a consideration of recent investigations in cell-free systems, particularly those derived from mammalian tissues which bear directly on the role of nucleic acid in protein synthesis. The reader is referred to the reviews cited for details of earlier work, and particularly to the complementary Chapter 38 by Gros concerned with studies on protein synthesis in intact cellular systems, primarily bacterial.

Much of what will be said hereafter, about the role of cellular RNA-containing fractions in protein synthesis, will be predicated on the assumption that the systems used do in fact measure protein synthesis. Therefore, before proceeding further, it would be well to outline briefly the limitations

[4] S. Spiegelman, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 232. Johns Hopkins Press, Baltimore, 1957.

[5] J. L. Simkin and T. S. Work, *Nature* **179**, 1214 (1957).

[6] F. H. C. Crick, *Symposia Soc. Exptl. Biol.* **12**, 138 (1958).

[7] R. B. Loftfield, *Progr. in Biophys. and Biophys. Chem.* **8**, 347 (1957).

[8] H. Chantrenne, *Ann. Revs. Biochem.* **27**, 35 (1958).

[9] C. E. Dalgliesh, *Rec. trav. chim.* **77**, 634 (1958).

[10] J. Monod, *Rec. trav. chim.* **77**, 569 (1958).

[11] P. N. Campbell, *Advances in Cancer Research* **5**, 97 (1958).

[12] J. Bonner, *Fortschr. Chem. org. Naturstoffe* **16**, 139 (1958).

[13] Symposium on Amino Acid Activation, *Proc. Natl. Acad. Sci. U. S.* **44**, 67 (1958).

[14] M. B. Hoagland, *Proc. 4th Intern. Congr. Biochem., Vienna, 1958.*

[15] I. D. Raacke, *Quart. Rev. Biol.* **33**, 245 (1958).

[16] "Microsomal Particles and Protein Synthesis" (R. B. Roberts, ed.). Pergamon, London, 1958. (a) G. E. Palade, p. 36; (b) M. L. Petermann *et al.*, p. 70; (c) J. K. Ashikawa, p. 76; (d) R. B. Roberts *et al.*, p. 84; (e) S. Dagley and J. Sykes, p. 62; (f) J. Wagman and W. Trawick, p. 11; (g) W. C. Gillchriest and R. M. Bock, p. 1; (h) B. D. Hall and P. Doty, p. 27; (i) H. Dintzis *et al.*, p. 95.

of these cell-free systems. These matters have been discussed by Loftfield[7] particularly. Ideally we should like to have a system which would bring about a directly measurable net increase in protein. Some promising results have recently been obtained along these lines which will be discussed later. However, most cell-free systems thus far studied have failed to show a net synthesis and workers in the field have been forced to measure incorporation of tagged amino acids (usually labeled carboxyl-C^{14}) into protein. In the light of our current knowledge, the minimum criteria to be met in equating such incorporation with protein synthesis may be said to be the following: (1) the incorporation of an L-amino acid must be irreversible in the sense that once the amino acid has entered protein the total amount of C^{14}-amino acid in protein in the system cannot subsequently be reduced by a continued incubation in the presence of an excess of the same amino acid, unlabeled. (2) The incorporation should be dependent upon the addition of a metabolic energy source, adenosine triphosphate (ATP) or an ATP-generating system. (3) The incorporated amino acid must be shown to be in true alpha peptide linkage in protein, as evidenced by its appearance in identifiable peptides upon partial hydrolysis of the protein. (4) The amino acid should be located within the peptide chain, not in terminal positions. Other criteria which are desirable but which have not been conclusively demonstrated in all systems are: (5) that the amino acid appear in a single, specific, isolatable protein of the cells of origin, and (6) that incorporation of one C^{14}-amino acid be dependent upon the addition of the other amino acids naturally found in protein.

"Incorporation" refers to an irreversible, energy-dependent inclusion of a monomer in appropriate covalent linkage in a polymer. "Exchange" is a term frequently used in discussing the significance of amino acid incorporation work, describing a process by which a free monomer replaces an interstitial monomer in a polymer chain without breakdown of the latter. An interesting chemical system which will carry out the insertion of an amino acid into a peptide chain has been developed by Brenner.[17] However, with the exception of the special case of the transpeptidation reactions[18] (whose role in amino acid incorporation reactions remains unclear), there is no evidence that such a phenomenon occurs in relationship to protein-amino acid interaction in biological systems. (The often quoted experiments of Gale and Folkes,[19, 20] in which a reversible incorporation of a free amino acid into protein appeared to occur, no longer supports the "exchange"

[17] M. Brenner, *Ciba Foundation Symposium on Amino Acids and Peptides with Antimetabolic Activity* p. 157 (1958).

[18] J. S. Fruton, *Harvey Lectures* **15,** 64 (1957).

[19] E. F. Gale and J. P. Folkes, *Biochem. J.* **59,** 661 (1955).

[20] E. F. Gale and J. P. Folkes, *Biochem. J.* **59,** 675 (1955).

concept: it has since been shown[21] that the amino acid was attached to RNA, not protein.) All evidence available to date appears clearly to support the simple concept that when a free amino acid enters a protein by way of coupling with an endergonic process, it does so by *de novo* synthesis of protein (i.e., a polypeptide chain has increased in length by one amino acid) and when it leaves it does so by degradation of the same chain.

II. Participation of Cellular Nucleic Acid-Containing Fractions in Protein Synthesis

Over the past two decades evidence has accumulated which points to an intimate association between cellular nucleic acid and protein synthetic activity. This evidence has been thoroughly reviewed recently (cf. particularly Brachet[1] and more recently Chantrenne[8]). The highlights along this road should be briefly mentioned. Brachet and Caspersson were the first to point to this association. A large number of studies, stemming from Brachet's early experiments and the more recent work of Gale and Folkes[19, 20] in bacterial systems, have shown that ribonuclease disrupts the cell's protein synthetic machinery and that ribonucleic acid (RNA) can frequently restore it. Studies on bacterial transformation[22, 23] and the discovery of the autonomous infectivity of tobacco mosaic virus RNA[24, 25] unequivocally establish that DNA and RNA alone contained the necessary information in their structure to direct the synthesis of new and genetically specific proteins. A large body of information on the fate of C^{14}-amino acids in whole animals, as we shall see below, demonstrated conclusively that the initial and major site of incorporation of amino acids into protein were the cellular ribonucleoprotein particles or ribosomes. Thus it was clear, before cell-free systems had received much scrutiny, that nucleic acid had some intimate directive role in converting amino acids to protein. We shall now consider in detail the various major nucleic acid-containing cellular components and examine the evidence, based on the more direct *in vitro* studies, for the role of each of these fractions in protein synthesis. In the sections to follow only those experiments which seem to bear directly on the participation of nucleic acids have been considered.

[21] E. F. Gale, *in* "Recent Progress in Microbiology" (G. Tunerall, ed.). Almquist and Wiksel, Stockholm, 1959.

[22] O. T. Avery, C. M. MacLeod, and M. McCarty, *J. Exptl. Med.* **79**, 137 (1944).

[23] R. D. Hotchkiss, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 321. Johns Hopkins Press, Baltimore, 1957.

[24] A. Gierer and G. Schramm, *Nature* **177**, 702 (1956).

[25] H. Fraenkel-Conrat, *J. Am. Chem. Soc.* **78**, 882 (1956).

1. The Ribosomes

The ubiquitous ribonucleoprotein particles, recently rechristened ribosomes,[16] have come to be regarded as the major cellular site of protein synthesis. The evidence that they were the initial site of appearance of new protein came early in the development of our knowledge. However, we shall reverse the chronology and first consider what is known about ribosomes in a physical and chemical context and then discuss the evidence supporting the conclusion that they are in fact the major site of cellular protein synthetic activity.

a. Physicochemical Properties of Ribosomes

(1) *Morphology.* In the electron microscope the ribosomes from a variety of mammalian, bacterial, fungal, and higher plant sources appear as uniform, round electron dense particles having an average diameter of 100 to 200 Å (when unflattened) (cf. the review by Palade[16a]). The ribosomes contain 80–90% of the total cellular RNA. They exist apparently free in the cytoplasm in some tissues (notably bacteria and rapidly growing mammalian cells), perhaps sometimes attached to the limiting membrane of the cell (in bacteria) and frequently, in exocrine tissues, associated with lipoprotein-rich membranous material of the cytoplasm. The ribosomes in natural association with this membranelike material comprise what has been variously named the "cytoplasmic ground substance," "ergastroplasm," or "endoplasmic reticulum" by the electron microscopists. Furthermore, it appears that ribonucleoprotein particles very similar to those found in the cytoplasm may also be found in the nucleus of mammalian cells.[12, 26-28] There is evidence that RNA exists in mitochondria[12, 29] and chloroplasts,[12] suggesting the possibility, at least, that particles may reside in these subcellular structures as well.

Much careful work in correlating electron microscopic observations and biochemical studies[30, 31] (and cf. review by Palade[16a]) has now established the fact that microsomes, the material sedimented by centrifuging the mitochondria-free supernatant fraction of a mammalian tissue homogenate at 100,000 *g* for an hour or more, are in fact ribosomes still attached to

[26] E. de Robertis, *J. Biophys. Biochem. Cytol.* **2,** 785 (1956).
[27] S. Osawa, K. Takata, and Y. Hotta, *Biochim. et Biophys. Acta* **25,** 656 (1957).
[28] P. O. P. Ts'o and C. S. Sato, *J. Biophys. Biochem. Cytol.* **5,** 59 (1959).
[29] H. M. Bates, V. M. Craddock, and M. V. Simpson, *J. Am. Chem. Soc.* **80,** 1000 (1958).
[30] J. W. Littlefield, E. B. Keller, J. Gross, and P. C. Zamecnik, *J. Biol. Chem.* **217,** 111 (1955).
[31] G. E. Palade and P. Siekevitz, *J. Biophys. Biochem. Cytol.* **2,** 171 (1956).

varying amounts of the fragmented membranes of the endoplasmic reticulum. Thus microsomes are centrifugally heterogeneous chiefly because the "particle" size is determined by the extent of damage to the membranous component to which the ribosomes are attached. When measures are taken to remove the lipoprotein membranous component by treatment with a lipid solvent, considerably more uniform material is obtained.

It has been well documented that whole microsomes vary in ultracentrifugal sedimentation characteristics depending both on the tissue of origin and the metabolic activity of the tissue. A valid generalization seems to emerge that cells which are rapidly proliferating (such as tumors, embryonic, and regenerating mammalian cells) yield microsome fractions with a relatively wider and lower average size distribution; while adult differentiated mammalian cells contain more rapidly sedimenting microsomes. The relatively more rapid sedimentation properties of the latter particles is in part due to the fact that a greater proportion of the ribosomes are still attached to the endoplasmic membrane; hence, they are by definition microsomes (cf. Petermann *et al.*[16b]). Accompanying the change to a more self-reproducing synthetic machinery in mammalian tissues is a tendency to find the separation of ribosomes from these membranes resulting in a random dispersion of ribosomes in the cytoplasm.[32-37]

Since the ribosomes are, as we shall see, able to synthesize protein autonomously we may ask—what is the role of the membranous component of the endoplasmic reticulum? Palade has said: "What is known so far about the fine structure of bacterial cells suggests that internal membranous systems, like the endoplasmic reticulum, are not necessary for the organization and function of a simple type of self-sustaining cell. Such membranous systems appear in more elaborate cell forms and could therefore be regarded as superstructures. We do not know what special problems are solved by their introduction, but we may wonder whether they are not connected with an increase in cell volume, subsequent difficulties in diffusion, and relative decrease in available surface."[16a]

Palade[38] and Siekevitz and Palade[39, 40] have obtained preliminary evi-

[32] G. E. Palade, *J. Biophys. Biochem. Cytol.* **1**, 59 (1955).

[33] A. F. Howatson and A. W. Ham, *Cancer Research* **15**, 65 (1955).

[34] M. L. Petermann, N. A. Mizen, and M. G. Hamilton, *Cancer Research* **16**, 620 (1956).

[35] J. W. Littlefield and E. B. Keller, *J. Biol. Chem.* **224**, 13 (1957).

[36] E. Hay, *J. Biophys. Biochem. Cytol.* **4**, 583 (1958).

[37] B. Munger, *Am. J. Anat.* in press.

[38] G. E. Palade, in "Subcellular Particles" (Teru Hayashi, ed.), p. 64. Ronald Press, New York, 1959.

[39] P. Siekevitz and G. E. Palade, *J. Biophys. Biochem. Cytol.* **4**, 557 (1958).

[40] P. Siekevitz and G. E. Palade, *Federation Proc.* **18**, 1282 (1959).

dence that the membrane is apparently an active site for concentrating and storing the protein product of the ribosomes.

A most interesting electron micrographic investigation of the development of the nematocyst in the hydra by Slautterback and Fawcett[41] sheds light on the possible relationship of the membrane and ribosomes in protein synthesis. The nematocyst, an organ consisting of a dartlike projectile contained within a capsule used by the hydra to obtain its living food, is a complex product of certain specialized cells called "cnidoblasts." The undifferentiated interstitial cells from which the cnidoblasts arise have a cytoplasm filled with ribosomes randomly distributed, and there is no evidence of an endoplasmic reticulum. In fact the cytoplasm in this respect resembles that of bacterial and rapidly growing mammalian cells. During the proliferation of these cells the lack of cytoplasmic organization persists, but with the onset of nematocyst construction, there is a striking change in the cytoplasmic organization. This is characterized by the appearance of an endoplasmic membrane system and the organization of the ribosomes along its surface. This system becomes a highly organized network in all respects similar to the endoplasmic reticulum of the cytoplasm of differentiated mammalian cells. In the later stages of this process a continuous tubelike structure appears in the cytoplasm in proximity to the endoplasmic membrane. This is later withdrawn into a capsule and appears to be the shaft of the final nematocyst spear. The implication of these studies is that during the early stages of cell proliferation an endoplasmic reticulum is not essential but when the cells undertake the synthesis of a special product of their differentiated state they require the more complex cytoplasmic organization. It would appear that the product of cytoplasmic synthesis, the nematocyst, accumulates within the membranes to which the synthesizing particles are attached and that this product finally coalesces to form the final structure.

Cytological studies of the regeneration sites of amputated amphibian extremities by Hay[36] show essentially similar results. Here, during the stages of rapid proliferation of cells, there is no evidence of an endoplasmic reticulum but with the onset of differentiation into cartilage cells, with its associated elaboration of extracellular matrix, a typical endoplasmic reticulum appears.

(2) *Ribosomal Subunits.* To return to a consideration of the ribosomes themselves it may be said that they too are obtained from tissues in a variety of molecular sizes depending on the metabolic activity of the tissue, and perhaps other factors. This particle size distribution is to be distinguished from the associated phenomenon, discussed above, of separation from the membrane component.

[41] D. B. Slautterback and D. W. Fawcett, *J. Biophys. Biochem. Cytol.* **5**, 441 (1959).

It is important to note, however, that the ribosomes do not have a continuous size distribution, but rather show a tendency to appear in groups of sizes having sedimentation constants around 80, 40, 20 *S*, and small amounts of even more slowly sedimenting material. Thus Petermann *et al.* found the relative proportion of smaller particles increased in regenerating as opposed to normal rat liver,[42] in leukemic mouse spleen as opposed to normal,[42] and in rat liver hepatomas and cholangiomas versus normal.[34] In ascites tumor cells, Littlefield *et al.*[30] found that the particles obtained were of relatively low average sedimentation constants, 20 and 27*S*. Schachman *et al.*[43] found a wide range of particle sizes in yeast and bacterial cells. Ashikawa[16c] has demonstrated striking changes in the sedimentation patterns of yeast particles in the transition from dormancy to rapid growth; resting cells have a preponderance of 80 *S* particles which disappear and are replaced by more slowly sedimenting particles as the cells enter the log phase of growth.

Petermann suggested early that the smaller ribosomes, and those not attached to membranes, are perhaps concerned with the synthesis of the cell's own protein substance, the larger ones with the synthesis of exportable protein products of the differentiated cell. As we shall see in the following section, however, the bulk of ribosomes, when isolated from tissues and stabilized by Mg^{++}, are remarkably uniform in size. Intracellularly, the particles appear as relatively uniform bodies, also in electron micrographs. One may well ask whether the above-mentioned variety of sizes found in tissues is an indication of the true state of the ribosomes in nature or of a differing lability to isolation procedures. It would seem probable that many of the findings could be explained by dissociation of particles during isolation. Such altered stability of particles reflecting differences in protein synthetic activity of tissues is of considerable interest, but its meaning is unknown as yet.

The uniformity of ribosomes from a wide range of cell types is illustrated in Table I. Ribosomes have now been isolated—and their physical properties studied—from such sources as *Escherichia coli*[44] (cf. also Roberts *et al.*,[16d] Dagley and Sykes,[16e] Wagman and Trawick[16f]), *Azotobacter vinelandii* (cf. Gilchriest and Boch[16g]), yeast,[45] higher plant tissues,[46] liver (cf. Petermann *et al.*,[16b] Hall and Doty[16h]), pancreas,[39] tumor tissue[35] (cf. Petermann

[42] M. L. Petermann, M. G. Hamilton, and N. A. Mizen, *Cancer Research* **14**, 360 (1954).

[43] H. K. Schachman, A. B. Pardee, and R. Y. Stanier, *Arch. Biochem. Biophys.* **38**, 245 (1952).

[44] A. Tissières and J. D. Watson, *Nature* **182**, 778 (1958).

[45] F. C. Chao and H. K. Schachman, *Arch. Biochem. Biophys.* **61**, 220 (1956).

[46] P. O. P. Ts'o, J. Bonner, and J. Vinograd, *Biochim. et Biophys. Acta* **30**, 570 (1958).

TABLE I

A SUMMARY OF SOME PHYSICAL PROPERTIES OF RIBOSOMES

Properties	*E. coli*[a]	*A. vinelandii*[b]	Yeast[c]	Pea Seedlings[d]	Reticulocytes[e]	Liver[f]
Sedimentation constant of major ribosome component in optimum Mg^{++}	70	86	80	80	80	78
Estimated molecular weight	2.8×10^6	—	4.1×10^6	4.5×10^6	4.1×10^6	—
Diameter (Å.)	—	250	240	350	—	150
% RNA	60	—	42	40	50	40
% Protein	40	—	58	60	50	60
Ions binding subunits	Mg	Mg	Mg	Mg + Ca	—	—
Moles ion/mole base	—	—	—	3.7/12	—	—
Reversible dissociation products (S)	51, 32	58, 39	60, 40	60, 40, 26	—	62, 46

[a] A. Tissières and J. D. Watson, *Nature* **182**, 778 (1958).

[b] W. C. Gillchriest and R. M. Bock, *in* "Microsomal Particles and Protein Synthesis" (R. B. Roberts, ed.), p. 1. Pergamon Press, London, 1958.

[c] F. C. Chao and H. K. Schachman, *Arch. Biochem. Biophys.* **61**, 220 (1956).

[d] P. O. P. Ts'o, J. Bonner, and J. Vinograd, *Biochim. et Biophys. Acta* **30**, 570 (1958).

[e] H. M. Dintzis, H. Borsook, and J. Vinograd *in* "Microsomal Particles and Protein Synthesis" (R. B. Roberts, ed.), p. 95. Pergamon Press, London, 1958.

[f] M. L. Petermann, M. G. Hamilton, M. E. Balis, K. Samarth, and P. Pecora, *in* "Microsomal Particles and Protein Synthesis" (R. B. Roberts, ed.), p. 70. Pergamon Press, London, 1958.

et al.[16b]), and reticulocytes (cf. Dintzis *et al.*[16i]). Bacterial ribosomes are discussed further in Chapter 38.

To obtain mammalian ribosomes they must often be freed of the membranous component of the microsomes either by treatment with deoxycholate[35, 39, 47] or a high salt concentration.[35, 48] Bacterial, yeast, and reticulocyte particles may usually be obtained in relatively pure form directly by breaking the cells and centrifuging.

The secret of stabilization of ribosomes seems to lie chiefly in the level of divalent metal ions, particularly Mg^{++}, in the medium. The pH of the medium may also be important.[46] In the presence of adequate Mg^{++} levels it appears that the bulk of the ribosomes from all sources have a sedimentation constant of about 70 to 80 *S*, corresponding to a molecular weight of 3 to 4 $\times 10^6$. In this state they are quite stable. This uniformity extends to the pattern of reversible disaggregation or dissociation which they un-

[47] M. L. Petermann and M. G. Hamilton, *J. Biophys. Biochem. Cytol.* **1**, 469 (1955).

[48] J. L. Simkin and T. S. Work, *Biochem. J.* **65**, 307 (1957).

dergo, as a function of the Mg^{++} concentration of the medium. Thus, in general, when one lowers the Mg^{++} concentration from 0.005 *M* to below 0.001 *M*, the 80 *S* particles in large measure disappear and particles of roughly 60 and 40 *S* appear. The molecular weight of the former is twice that of the latter and the sum of the molecular weights is that of the original 80 *S* particle. Further dissociation of ribosomes occurs if the Mg^{++} concentration is reduced below 0.001 *M*, some of which may be reversible. Clearly irreversible breakdown supervenes if the Mg^{++} concentration falls below 0.0001 *M* with the appearance of a variety of sedimentation peaks of *S* values below 20. In the presence of excessive Mg^{++}, on the other hand, irreversible degradation[44] and precipitation of aggregates (Petermann *et al.*[16b]) have been reported to occur.

(3) *Chemistry of Nucleic Acid and Protein Components.* It has been reported by Ts'o[46] for plant particles and Petermann[49] for mammalian particles, that the divalent ion content of the intact particles is very high—approximately 4 moles of ion per mole of base, with Mg^{++} in excess of Ca^{++} by a factor of 6 or 7.[46] There is some indication[46] that these ions are bound chiefly to the RNA component, rather than to the protein.

The purest ribosomes appear to be formed almost wholly of RNA and protein. Although there are differences in the ratio of one to the other, the RNA content is high—from 40 to 65%. The molecular weight data suggest, as pointed out by Tissières and Watson,[44] that of the two components the RNA may be the invariant of the two, the protein being in greater or lesser amounts in different tissues. It is of interest to note that the subunits obtained by dissociation in low Mg^{++} medium have the same RNA to protein ratio as the parent complex.

It is of basic importance to enquire: what is the minimal subunit of RNA naturally occurring in ribosomes? Studies of this problem are in their very early stages. The work is hampered by the natural occurrence of ribonuclease in close association with most of these particles which may give spuriously low molecular weight values. Preliminary studies indicate, however, that subunits of particulate RNA of molecular weight as low as 250,000 to 120,000 may occur[46] (Hall and Doty[16h]). A minimal value of 500,000 would perhaps be a more cautious estimate. These RNA subunits may represent physiological entities bound to each other or to the particle protein by Mg^{++} and base interaction. However, much more study will be required before this matter is fully clarified. Ts'o and Squires,[50] for example, have isolated RNA from pea seedling and reticulocyte particles in 90% yield using 1% dodecyl sulfate. Ultracentrifugal analysis of this material showed that about half of the material sedimented with $S_{20,w}$ of 28 *S* and half with

[49] M. L. Petermann and N. A. Mizen, *Abstr. Meeting Biophys. Soc.* p. 1 (1959).
[50] P. O. P. Ts'o and R. Squires, *Federation Proc.* **18,** 1351 (1959).

TABLE II

AMINO ACID (AA) COMPOSITION OF MICROSOMAL PARTICLES AND OF PLANT VIRUSES[a]

Amino acid	Rabbit reticulocytes (gm. AA/100 gm. protein)	Pea seedlings (gm. AA/100) gm. protein)	Fraction B (Simkin and Work) (gm. AA/100 gm. AA recovered)	Turnip yellow mosiac (Fraiser and Consentino) (gm. AA/100 gm. protein)	Tobacco mosiac (Knight) (gm. AA/100 gm. AA)
Alanine	5.4	5.4	5.3	5.4	5.1
Aspartic	8.8	9.6	9.5	6.3	9.8
Arginine	11.8	9.2	8.3	2.2	13.5
Cystine	1.1	0.3	—	0	0.69 (cysteine)
Glutamic	11.5	10.7	12.0	7.7	11.3
Glycine	7.1	8.3	4.7	3.8	1.9
Histidine	2.8	2.9	2.5	1.6	0.0
Isoleucine	5.7	6.4	4.2	7.4	6.6
Leucine	8.7	8.2	10.2	8.6	9.3
Lysine	12.7	12.2	9.3	5.0	1.47
Methionine	2.0	2.0	2.0	2.1	0.0
Phenylalanine	4.4	4.8	5.6	3.6	8.4
Proline	4.7	5.2	7.5	11.8	5.8
Serine	1.8 to 10	2.5 to 10	3.9	6.7	7.2
Threonine	4.5	4.9	5.2	12.2	9.9
Tryptophan	1.2	1.5	—	—	2.1
Tyrosine	6.5	7.0	4.0	2.2	3.8
Valine	7.2	7.6	5.8	6.2	9.2
	106.0	106.0	100.0	93.1	100.0

[a] P. O. P. Ts'o, J. Bonner, and H. Dintzis, *Arch. Biochem. Biophys.* **76**, 225 (1958).

18 *S* in a Mg^{++}-free buffer. Whether these large molecules have covalent continuity is not clear, however, although they were analyzed in a Mg^{++}-free medium and their sedimentation properties were unaltered after reprocessing by the detergent procedure.

Another parameter of the particle which shows constancy among different species is the amino acid composition of its protein[51] (Table II). As will be mentioned later, *in vivo* labeling of particles by amino acids has revealed that only a very small fraction of the total protein of the particles is actually in the process of synthesis. The remainder is presumably structural and therefore might be expected to be constant in composition over a wide phylogenetic range. This is suggested by the preliminary data. Note the absence of cysteine in this protein, an observation also made by Roberts *et al.*[16d] Whether this structural protein is made up of subunits of a single molecular species or consists of several proteins is unknown. Roberts *et al.*[16d] have obtained evidence for considerable heterogeneity of *E. coli*

[51] P. O. P. Ts'o, J. Bonner, and H. Dintzis, *Arch. Biochem. Biophys.* **76**, 225 (1958).

TABLE III

BASE COMPOSITION OF SOLUBLE AND RIBOSOMAL RNA

Source	Reference	Adenine	Guanine	Cytosine	Uracil	Odd bases
Soluble						
E. coli	*a*	10	17.2	15.5	8.1	15
E. coli	*b*	10	16.9	15.4	8.7	3.2
E. coli	*c*	10	17.4	14.8	8.7	—
*E. coli**	*c*	10	17.9	13.7	8.9	—
	c	10	11.3	7.1	6.6	—
Baker's yeast	*d*	10	18.3	15.1	9.5	—
Rat liver	*e*	10	17.4	15.4	10.2	—
Rat liver	*f*	10	15.4	9.6	6.7	—
Guinea pig liver	*g*	10	18.3	16.7	11.1	4
Mouse Ehrlich ascites tumor	*h*	10	18.2	13.4	9.6	—
Ribosomal						
E. coli	*b*	10	12.2	8.7	8.0	0
E. coli 30S	*c*	10	13.0	9.7	8.4	—
E. coli 50S	*c*	10	13.2	7.8	6.9	—
A. vinelandii	*h*	10	9.6	7.6	9.6	7
Baker's yeast	*d*	10	10.1	7.1	8.6	—
Rat liver	*e*	10	17.5	15.8	10.1	—
Rabbit liver	*c*	10	15.9	15.5	10.3	—
Mouse Ehrlich ascites tumor	*i*	10	20.6	14.1	10.3	—

* Two RNA fractions produced in presence of chloramphenicol.

References

[a] P. F. Spahr, and A. Tissières, *J. Mol. Biol.*, **1**, 237 (1959).
[b] P. Berg, unpublished data. (1959).
[c] E. T. Bolton, Carnegie Inst. Wash. Yearbook 1958, p. 275.
[d] R. Monier, M. L. Stephenson, P. C. Zamecnik, *Biochim. et Biophys. Acta* in press (1960).
[e] B. Magasanik, *in* "The Nucleic Acids" (E. Chargaff and G. N. Davidson, eds.), Vol. I, p. 373. Academic Press, New York, 1955.
[f] D. A. Goldthwaite, *J. Biol. Chem.* **234**, 3245 (1959).
[g] R. S. Schweet, unpublished data. (1959).
[h] W. C. Gillchriest and R. M. Bock, *in* "Microsomal Particles and Protein Synthesis" (R. B. Roberts, ed.), p. 7. Pergamon Press, London, 1958.
[i] J. F. Scott, unpublished data. (1959).

ribosomal protein by fractionation on DEAE of urea-treated particles. Harris,[52] on the other hand, has obtained evidence, using different techniques, suggesting that the protein may be more uniform.

The base composition of particle RNA from mammalian tissues has been studied (cf. Magasanik[53] and Table III). It shows the usual high guanine

[52] I. Harris, personal communication (1960).
[53] B. Magasanik, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. I, p. 373. Academic Press, New York, 1955.

and cytosine content characteristic of animal tissues. There do not seem to be interspecies differences among animal tissues. A purine-pyrimidine ratio close to unity is also characteristic. Ribosomal RNA from yeast and bacteria is also characterized by near equality of purines and pyrimidines but there is a lack of the high guanine, high cytosine content found in mammalian ribosomes.

(4) *Macromolecular Organization.* It has been pointed out[54] that X-ray powder diagrams of yeast and liver particles, in common with certain viruses, have no resemblance to the diffraction patterns obtained from their isolated RNA or a mixture of their RNA and protein. It would appear that the protein component of particles and viruses can maintain its structure in the absence of RNA, but the "configuration of the RNA in some way closely conforms to that of the protein. Neither the viruses nor the microsomal particles consist of a kind of protein bag inside which lies RNA. This situation may be contrasted with DNA, the *in vivo* and *in vitro* structures of which have been shown to be similar. In at least some of the DNA-protein complexes it is the protein which conforms to the structural configuration of the nucleic acids."[54] Crick and Watson have called attention to certain similarities between the particles and the spherical viruses in which the protein appears to be arranged in subunits having cubic symmetry.[55]

Doty and his associates have used the variation in the optical density of polynucleotides as a function of temperature to study what they consider may be the intramolecular helical content of particle RNA and whole particles.[56] It appears that when base interactions within and between polynucleotides in solution—presumably hydrogen bonding and other noncovalent associations—are weakened by raising the temperature, the increase in optical density of the solution may be used as a direct measure of loss of helical structure. This conclusion is strengthened by concomitant study of changes in optical rotatory properties which would distinguish between lengths of helical continuity along a polymer chain and simple random base pairing. The results of these workers are consistent with the idea that the RNA of these particles and the particles themselves have a substantial content of regular intramolecular hydrogen bonding resulting in helix formation. The whole particles were found to have about two-thirds as much helical content as the RNA isolated from them but this two-thirds was the more strongly interacting type, i.e., the type which

[54] R. E. Franklin, A. Klug, J. T. Finch, and K. C. Holmes, *Discussions Faraday Soc.* **25,** 197 (1958).

[55] F. H. C. Crick and J. D. Watson, *in* "The Nature of Viruses," A Ciba Symposium. Churchill, London, 1956.

[56] P. Doty, H. Boedtker, J. R. Fresco, B. D. Hall, and R. Haselkorn, Ann. N. Y. Acad. Sci. **81,** 693, 1959.

required the higher temperatures to disrupt. It is suggested that the weaker third of the hydrogen bonding capacity of particle RNA may be reserved for interaction with the structural protein. The authors suggest that the particle RNA may consist of areas (totaling about 25% of the total chain length) of helical continuity alternating with nonhelical lengths, the latter being available for interaction with protein, or other molecules. The X-ray diffraction photographs of RNA obtained by Rich and Watson[57] are consistent with this kind of picture. The possible significance of such a structural arrangement of RNA in terms of protein synthetic mechanisms will be discussed more fully below.

We may thus summarize this consideration of ribosomes as physicochemical entities by saying that preliminary studies indicate that they are ubiquitous spherical cellular constituents variously attached to other cellular membrane components or free in the cytoplasm, which undergo physiological changes in molecular size. They consist of equal parts of relatively highly polymerized RNA and protein. Both the RNA and protein would appear to be made up of subunits of undetermined size, most likely bound to each other by Mg^{++} linkages. The protein moiety would appear to function as a structural matrix for the RNA.

b. Enzymic Properties of Ribosomes

Analysis of the enzyme content of particles is in an early stage and very little information is available. A number of considerations make interpretation of results difficult. First, since particles are associated, particularly in mammalian systems, with much other protein material which is known to have enzymic activity, it is hazardous to assume that any enzymic activity is intrinsic to the ribonucleoprotein particle. Secondly, it is reasonable to expect that enzymes intimately involved in protein synthesis might be only loosely bound to the surface of the particles and during rigorous attempts to purify the particles they might be lost. This might, for example, be the case with the amino acid activating enzymes. Finally, since particles are the site of protein synthesis, they are in the process of making enzymes. Indeed, Siekevitz and Palade[40] have reported preliminarily on the finding of synthesized chymotrypsinogen in pancreas particles. A distinction between an intrinsic enzyme necessary to the synthetic machinery and a synthesized enzyme product might be difficult. This is particularly so when one considers that only a small amount of enzyme protein would be necessary to give measurable activity. With these considerations in mind it becomes hazardous to interpret any of the data thus far available.

Glucose-6-phosphatase, nucleotide-cytochrome c reductase, cytochromes m and b_5, and enzymes involved in fatty acid and steroid metabolism,

[57] A. Rich and J. D. Watson, *Proc. Natl. Acad. Sci.* **40**, 759 (1954).

have been found associated with whole mammalian microsomes (cf. Palade[16a]). Only feeble activity remains when the membranous component is removed, however. Cholic acid is activated by microsomes,[58] but the location of the activity in subfractions of microsomes is not stated.

It was observed early that rat liver microsomes catalyze a vigorous pyrophosphate (PP)-ATP exchange reaction.[59] This was not influenced by amino acids and hence could not be said to be due to amino acid activating enzymes. Kenney furthermore reported that microsomes produce PP on incubation with ATP, guanosine triphosphate (GTP), and uridine triphosphate (UTP).[60] The significance and exact anatomical location (i.e., ribosomes or membranes) of these reactions in microsomes remain obscure.

Webster has found that a substantial portion of the total amino acid activating activity in pea seedling extracts is associated with the ribonucleoprotein particles and that it is difficult to remove by various physical and chemical treatments.[61] Cohn,[62] and Rendi and Hultin[63] have found such amino acid activating activity in detergent-prepared animal particles. Peptidase activity has been found in *E. coli* particles.[64] In all these cases the particles are thought to be relatively free of membrane components.

Several workers have reported the presence of ribonuclease in ribosomes.[16, 44, 46, 65] This is particularly interesting since so much of these particles is composed of RNA. The enzyme usually manifests itself as an apparent instability of the particle RNA under certain conditions. Ribosomes derived from reticulocytes,[66] on the other hand, do not appear to have intrinsic ribonuclease activity. The significance of the ribonuclease activity is unknown, but it is, thus far, the only enzyme which appears likely to be an integral part of the ribosomes themselves.

c. Evidence for a Direct Role of Ribosomes in Protein Synthesis

Figure 1 is a schematic summary of the series of reactions thought to be involved in the conversion of free C^{14}-amino acids to peptide linkage in protein. The inclusion of such chemical detail in this scheme has been made possible chiefly as the result of studies on mammalian systems dating back a little more than ten years. Although most of the data upon which the

[58] W. H. Elliott, *Biochem. J.* **65,** 315 (1957).
[59] M. B. Hoagland, E. B. Keller, and P. C. Zamecnik, *J. Biol. Chem.* **218,** 345 (1956).
[60] F. T. Kenney, S. P. Colowick, and E. Barbehenn, *Arch. Biochem. Biophys.* **69,** 617 (1957).
[61] G. C. Webster, *J. Biol. Chem.* **220,** 535 (1957).
[62] P. Cohn, *Biochim. et Biophys. Acta* **33,** 284 (1959).
[63] R. Rendi and T. Hultin, *Exptl. Cell Research* **19,** 253 (1960).
[64] B. J. McCarthy and E. T. Botton, *Abstr. Meeting Biophys. Soc.* p. 10 (1959).
[65] D. Elson, *Biochim. et Biophys. Acta* **27,** 216 (1958).
[66] V. M. Ingram, personal communication (1960).

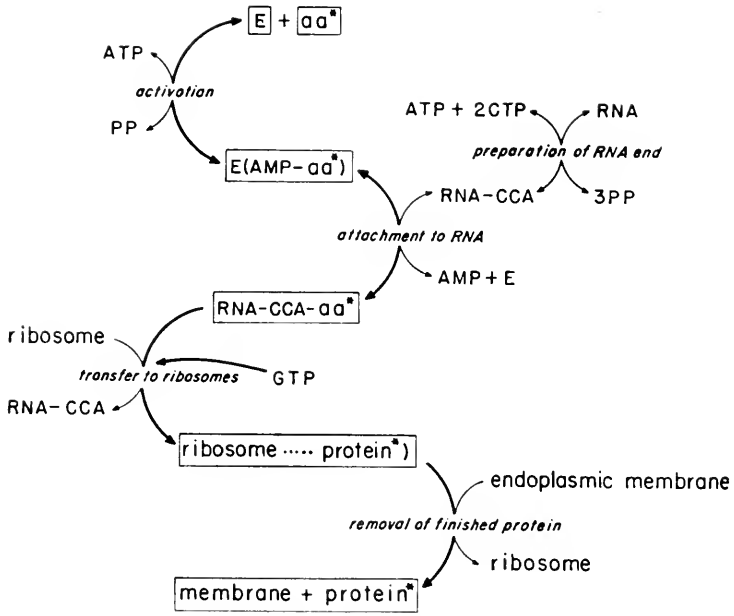

FIG. 1. Steps in protein synthesis.

scheme is based has been derived from *in vitro* cell-free systems, certain basic observations antedated the advent of active cell-free systems and should be mentioned briefly. The pioneering studies of Schoenheimer[67] and Rittenberg[68] indicated that isotopic protein precursors were rapidly incorporated into protein *in vivo* and pointed the way to the later use of C^{14}- and S^{35}-labeled amino acids to study protein synthesis in slices, minces, and dispersed cell systems. Extensive studies were first undertaken by Zamecnik and his associates in rat liver, tumor cells, embryonic tissue, and silk glands,[69-72] by Borsook and his colleagues using bone marrow cells,[73]

[67] R. Schoenheimer, "The Dynamic State of Body Constituents." Harvard Univ. Press, Cambridge, Mass., 1942.

[68] D. Rittenberg, *Harvey Lectures* **44**, 200 (1950).

[69] I. D. Frantz, Jr., R. B. Loftfield, and W. W. Miller, *Science* **106**, 544 (1947).

[70] P. C. Zamecnik, I. D. Frantz, Jr., R. B. Loftfield, and M. L. Stephenson, *J. Biol. Chem.* **175**, 299 (1948).

[71] I. D. Frantz, Jr., P. C. Zamecnik, J. W. Reece, and M. L. Stephenson, *J. Biol. Chem.* **174**, 773 (1948).

[72] P. C. Zamecnik, R. B. Loftfield, M. L. Stephenson, and C. M. Williams, *Science* **109**, 624 (1949).

[73] H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy, *J. Biol. Chem.* **186**, 297 (1950).

and by Winnick *et al.*,[74] Melchior and Tarver,[75] and Melchior *et al.*[76] The key observations among these studies were that the *in vivo* process of protein synthesis was endergonic: that a coupled energy yielding reaction was required to make the synthesis proceed. Such a conclusion had been anticipated by the studies of Borsook and Dubnoff,[77] showing that reversal of proteolysis could hardly account for protein synthesis, and the prediction of Lipmann.[78] Thus, deprivation of oxygen,[69] presence of respiratory inhibitors,[74, 79] or uncoupling agents[71] prevented protein synthesis. The rates of incorporation of C^{14}-amino acids were shown to be commensurate with the rate of total protein synthesis of various tissues,[35, 80] and pure labeled proteins were isolated as products of the incorporation reaction[81, 82] (cf. Dintzis *et al.*[16i]).

Extension of these *in vivo* studies revealed that the protein portion of the microsome fraction was the earliest cellular protein fraction to become labeled in a variety of tissues.[83-88] The actual site of protein labeling within the microsome fraction was shown by Littlefield *et al.*[30, 35] and Simkin and Work[48] to be the ribosome fraction itself:

"When whole tumor cells are incubated in ascitic fluid with a C^{14}-amino acid, the initial incorporation into whole cell protein is at a rate, e.g., 33 μmoles of leucine per gm. of protein per hour, which is more than adequate to account for the known rate of division of such cells *in vivo*. The ribonucleoprotein particles, which are estimated to contain 8 to 9 per cent of the whole cell proteins, are labeled up to 9 times more rapidly than whole cell proteins. This is consistent with the concept that most of the amino acids incorporated into whole cell proteins pass through the ribonucleoprotein particles."[30]

[74] T. Winnick, F. Friedberg, and D. M. Greenberg, *Arch. Biochem.* **15**, 160 (1947).
[75] J. B. Melchior and H. Tarver, *Arch. Biochem.* **12**, 309 (1947).
[76] J. B. Melchior, M. Mellody, and I. M. Klotz, *J. Biol. Chem.* **174**, 81 (1948).
[77] H. Borsook and J. W. Dubnoff, *J. Biol. Chem.* **132**, 307 (1940).
[78] F. Lipmann, *Advances in Enzymol.* **1**, 99 (1941).
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[81] T. Peters, Jr., *J. Biol. Chem.* **200**, 461 (1953).
[82] R. B. Loftfield and E. A. Eigner, *J. Biol. Chem.* **231**, 925 (1958).
[83] H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy, *J. Biol. Chem.* **187**, 839 (1950).
[84] T. Hultin, *Exptl. Cell Research* **1**, 376 (1950).
[85] E. B. Keller, *Federation Proc.* **10**, 206 (1951).
[86] V. G. Allfrey, M. M. Daly, and A. E. Mirsky, *J. Gen. Physiol.* **37**, 157, 1953.
[87] V. G. Allfrey, M. M. Daly, and A. E. Mirsky, *J. Gen. Physiol.* **38**, 415, 1955.
[88] M. Rabinowitz and M. E. Olson, *Exptl. Cell Research* **10**, 747 (1956).

The first success with cell-free mammalian systems was reported about 7 years ago, and although these systems were less active than the *in vivo* incorporating systems, they did appear, qualitatively, to be carrying out the identical process.[30] Thus the energy-dependent nature of the process, and the initial incorporation into microsomal protein was confirmed. Siekevitz and Zamecnik,[89] Siekevitz,[90] and Peterson and Greenberg[91] obtained some C^{14}-amino acid incorporation into protein which was dependent on addition of actively phosphorylating mitochondria. In the absence of mitochondria, glycolysis was also found to serve as an equally effective energy source.[92, 93] Success with the latter cell-free system seemed chiefly to derive from the use of gentle homogenization of the tissues[94] and the use of Mg^{++} in the homogenizing medium.[93] The latter empirical observation is of interest in the light of recent advances in our knowledge of the requirements for maintaining the structural integrity of ribonucleoprotein particles which we have discussed above.

In agreement with the *in vivo* incorporation studies, it was found, as we have said, that the protein of the ribosomes was the initial site of incorporation of the C^{14}-amino acid. The rate of incorporation was considerably slower than that observed *in vivo*; the initial rate of incorporation of leucine into the protein of rat liver, for example, being 0.4% uptake per hour, as compared to a rate of about 2% *in vivo*.[95] Calculations based on the rate and total amount of labeling of microsomal protein *in vivo* indicated that only a small fraction ($\sim$1%) of the total protein of the ribonucleoprotein particles was actually participating in the synthetic process.[30]

Littlefield and Keller[35] were successful in obtaining ribosomes from ascites tumor cells by the use of 0.5 *M* NaCl to separate them from associated protein. These ribosomes, when supplemented with the required soluble enzymes and nucleotides, incorporated amino acids into their protein moiety at an appreciable rate, thus indicating the ability of these particles to synthesize protein in the absence of associated lipoprotein material. Similar observations were made by Simkin and Work.[48]

In company with the development of our understanding of the role of the ribosomes in the mammalian incorporation system, elucidation of the role of the soluble components was also advanced. Thus, as we see from Fig. 1, the participation of GTP in the incorporation reaction, amino acid

[89] P. Siekevitz and P. C. Zamecnik, *Federation Proc.* **10,** 246 (1951).
[90] P. Siekevitz, *J. Biol. Chem.* **195,** 549 (1952).
[91] E. A. Peterson and D. M. Greenberg, *J. Biol. Chem.* **194,** 359 (1952).
[92] P. C. Zamecnik, *Federation Proc.* **12,** 976 (1953).
[93] P. C. Zamecnik, and E. B. Keller, *J. Biol. Chem.* **209,** 337 (1954).
[94] N. L. R. Bucher, *J. Am. Chem. Soc.* **75,** 498 (1953).
[95] E. B. Keller, P. C. Zamecnik, and R. B. Loftfield, *J. Histochem. and Cytochem.* **2,** 378 (1954).

activation, attachment of activated amino acids to a soluble RNA fraction, and the reactions involving the terminal nucleotide end groups of this RNA were brought into focus as integral parts of the over-all scheme. These events will be discussed further in the section dealing with the soluble enzyme-ribonucleic acid system. Zamecnik[96] has recently presented an excellent review of the history of growth of our understanding of the mammalian incorporation systems briefly outlined here.

In microorganisms, it has, thus far, not been possible to effect as complete a separation of the components of a protein synthetic system as in mammalian and higher plant systems, and the role of ribosomes in the process has been assessed in only a preliminary way. We know from the early studies of Gale and Folkes[97] and Spiegelman (cf. review by Spiegelman[4]) that the enzymic removal of RNA from disrupted bacterial cell preparations destroys the protein synthetic process. As we have seen, yeast and bacteria contain these particles; indeed, most of their RNA is found in such particles. Two recent reports strongly point to the ribosomes as the initial site of protein synthesis in bacteria. McQuillen *et al.*,[98] using whole *E. coli* cells, have shown that S^{35} in methionine is rapidly and initially incorporated into the protein of the ribosomes whence it subsequently appears in other fractions. Lamborg[99] has successfully developed an active cell-free incorporation system from *E. coli* cells which is almost identical in its requirements to the cell-free mammalian systems. Thus Mg^{++}, ATP and ATP-generating system, GTP, amino acids, ribosomes, and a soluble enzymatic component are necessary for activity. Initial incorporation is into peptide linkage in the protein of the ribosomes, while the soluble protein acquires label more slowly. The possibility of whole cell contamination has been minimized. However, Spiegelman finds that in *E. coli* the cell membrane material is much more active than the particles plus soluble fraction both *in vivo* and *in vitro*.[100] We shall consider later in somewhat more detail some of the recent observations that implicate the bacterial cell membrane in protein synthesis (see also Chapter 38).

In the cell-free incorporation systems mentioned thus far, it appears that the synthetic machinery is capable of limited but not qualitatively altered protein synthesis. Finished proteins do not appear to accumulate readily in the nonparticulate milieu. This is in contrast to the *in vivo* system where in a short time the soluble proteins are heavily labeled after exposure to

[96] P. C. Zamecnik, *Harvey Lectures* **54**, 256 (1960).

[97] E. F. Gale, *Harvey Lectures* **51**, 25 (1957).

[98] K. McQuillen, R. B. Roberts, and R. J. Britten, *Proc. Natl. Acad. Sci. U. S.* **45**, 1437 (1959).

[99] M. Lamborg, *Federation Proc.* **19**, 346 (1960).

[100] S. Spiegelman, *in* "Recent Progress in Microbiology" (G. Tunevall, ed.), p. 81. Almquist and Wiksell, Stockholm, 1959.

C^{14}-amino acids. It may be that the cell limiting membrane (in bacteria) and the membrane component of the endoplasmic reticulum (in animal tissues) are necessary for the completion of the synthetic sequence by removing the finished proteins from their site of synthesis. By breaking up the cell we may disturb this relationship. This view is supported by the work of Hendler[101] which suggests that lipids may be essential in protein synthesis, of Hokin and Hokin[102] which suggests that transport of protein through endoplasmic membrane may be an energy-requiring process, and of Sachs[103, 104] whose results could be interpreted to mean that newly synthesized protein in ribosomes fails to pass on into the soluble phase when fractionated particles are used.

The incubation of various cell-free tissue preparations with C^{14}-amino acids has led in a few instances to some accumulation of labeled nonmicrosomal protein which has acquired its label later than the microsomal protein in analogy with events in whole cell preparations.[105] These studies do not of course establish that the labeled soluble protein was derived from the ribosomes. Simkin (quoted in Askonas *et al.*[106]) has shown, however, that if one isolates microsomes from an incubation in which their protein has been labeled in the presence of ATP, soluble enzymes, and C^{14}-amino acid, these microsomes will release some labeled protein upon subsequent incubation with unlabeled cell sap, Mg^{++}, and ATP. Such an occurrence might represent a release of newly synthesized soluble protein but much further work is needed to establish this.

We may well ask then whether there is any evidence that truly cell-free preparations containing ribonucleoprotein particles are capable of an unambiguous net synthesis of protein. Encouraging results along these lines have been reported.

Reticulocytes are useful as a protein synthetic system because a large fraction of their synthetic product is in single protein which is relatively easy to isolate and purify. Rabinovitz and Olson had shown[88] that in whole reticulocytes incubated with C^{14}-amino acid there was a rapid rise of radioactivity in ribonucleoprotein particles, followed shortly by an equally precipitous fall and appearance of radioactivity in soluble hemoglobin. These authors, subsequently, reported briefly[107] that this apparent transfer of labeled hemoglobin from the particulate site of synthesis to the soluble

[101] R. Hendler, *Science* **128**, 143 (1958).
[102] L. E. Hokin and M. R. Hokin, *Federation Proc.* **18**, 978 (1959).
[103] H. Sachs, *J. Biol. Chem.* **233**, 643 (1958).
[104] H. Sachs, *J. Biol. Chem.* **233**, 650 (1958).
[105] J. L. Simkin and T. S. Work, *Biochem. J.* **67**, 617 (1957).
[106] B. A. Askonas, J. L. Simkin, and T. S. Work, *Proc. 4th Intern. Congr. Biochem., Vienna, 1958* p. 181. Pergamon Press, London (1960).
[107] M. Rabinovitz and M. E. Olson, *Federation Proc.* **16**, 1011 (1957).

TABLE IV

INCORPORATION OF C^{14}-AMINO ACIDS INTO RABBIT RETICULOCYTE RIBOSOMES COMPARED WITH AMINO ACID COMPOSITION OF RABBIT GLOBIN

Complete system[a]	Ribosomes (c.p.m./mg.)	Ratio	Amino acid	
			Composition (gm/100 gm protein)	Molar ratio
Leucine-C^{14}	748	1.00	11.7	1.00
Isoleucine-C^{14}	90	0.12	1.3	0.11
Valine-C^{14}	538	0.72	8.1	0.77

[a] The complete system contained rabbit reticulocyte ribosomes, guinea pig pH 5 fraction, creatine kinase, creatine phosphate, ATP, GTP, and a complete amino acid mixture. Omission of any one of these components reduced the activity to one-half or more. Data are from R. Schweet, H. Lamfrom, and B. Allen, *Proc. Natl. Acad. Sci. U. S.* **44**, 1029 (1958).

phase could be reproduced in a cell-free system using ribonucleoprotein particles previously labeled in whole reticulocytes which were then incubated with unlabeled soluble fraction, phosphoglycerate, ATP and Mg^{++}. This kind of observation has been amplified and extended by Schweet. He has shown that ribonucleoprotein particles from reticulocytes will synthesize hemoglobin, or a molecule very like it, *in vitro*.[108] Schweet showed that incorporation of labeled amino acids into hemoglobin in a cell-free preparation derived from reticulocytes required ribonucleoprotein particles, ATP and an ATP-generating system, and a soluble fraction containing amino acid activating enzymes. The radioactivity initially appeared in the protein of the particles, but upon further incubation could be shown to appear in soluble protein characterized as hemoglobin by isolation with carrier unlabeled hemoglobin. Using three labeled amino acids it was further shown (see Table IV) that the radioactive protein in the particles contained these amino acids in a ratio characteristic of hemoglobin, and not characteristic of the structural protein of the particles. Furthermore, synthesis of hemoglobin in this cell-free system was possible only in the presence of a mixture of amino acids in the proper proportions. These proportions were those shown by Borsook *et al.*[109] to be optimal for the synthesis of hemoglobin by intact reticulocytes. It seems, therefore, highly probable that new hemoglobin molecules or molecules very closely resembling hemoglobin were synthesized in the particles and subsequently released into the soluble milieu.

Raacke reports that ribonucleoprotein particles isolated from pea seedlings will effect a net increase in protein-like material (approximately 10%

[108] R. Schweet, H. Lamfrom, and E. Allen, *Proc. Natl. Acad. Sci. U. S.* **44**, 1029 (1958).
[109] H. Borsook, E. H. Fischer, and G. Keighley, *J. Biol. Chem.* **229**, 1059, 1957.

of the protein originally present) during prolonged incubation in phosphate buffer and Mg^{++}.[110] The extent of incorporation of phenylalanine-C^{14} into protein in this system coincides with the amount of new protein formed The newly formed material gives a positive test with the Lowry reagent and is precipitable by 5% trichloroacetic acid. The particle preparation is complex, however, apparently containing indigenous activating enzymes, ATP, and amino acids. Perhaps for this reason, it has not been possible thus far, to show a requirement for these adjuvants for the synthesis observed.

Webster[111] also states preliminarily that pea seedling particles will effect a net synthesis of a considerable quantity of protein, such that it can be measured gravimetrically. This system is said to be dependent on addition of amino acid activating enzymes, ATP, GTP, a full complement of amino acids, and an "unidentified fraction," thought to be polynucleotide in nature.

We may ask before concluding this section: what is the minimal size of ribosome capable of incorporating amino acids into its protein? Very little work has been done on this subject in the era since control of particle dissociation by Mg^{++} has been understood, but it would appear from wisps of evidence that ribosomes smaller than the intact 80 *S* particles are able to incorporate amino acids. Thus Littlefield and Keller,[35] and Simkin and Work,[48] using ribonucleoprotein particles from ascites tumor cells, prepared by treatment of cell lysates with 0.5 *M* NaCl, obtained activity in particles with sedimentation constants as low as 20 *S*. Sachs has obtained incorporation in ribonucleoprotein material obtained from rat liver by pyrophosphate treatment (a measure which apparently functions to lower the effective Mg^{++} concentration) in which there was dissociation, though of undetermined extent.[103, 104] Siekevitz and Palade[40] find that a poorly sedimenting small particle fraction has protein-synthetic activity. Webster[61] has treated pea seedling particles by sonic vibration and found that the resulting nonsedimentable material (about 40%) was better able to incorporate glutamic acid into protein than the original particles. However, it should now be possible to repeat this kind of study under carefully controlled conditions to correlate incorporating ability and particle size.

It would seem reasonable, therefore, to conclude that ribosomes play a direct and major role in protein synthesis in most tissues. Whether or not they can account for all protein synthesis is certainly not known, and evidence that other cellular particulates carry out autonomous protein synthesis leads us to be cautious. It has not been convincingly shown, however,

[110] I. D. Raacke, *Biochim. et Biophys. Acta* **34**, 1 (1959).
[111] G. E. Webster, *Federation Proc.* **18**, 1379 (1959).

that intrinsic protein synthetic activity in nuclei, chloroplasts, and mitochondria is not due to ribosome-like particles which might be an integral part of these structures. These matters will be discussed further below.

d. Comment on the Metabolic Origin of Ribosomes

Before concluding this section it might be pertinent to ask: how do ribosomes arise? What machinery is responsible for the synthesis of their RNA and protein? There is still no clear answer to this question but there is a growing body of evidence indicating that the nucleus is capable of carrying out both RNA and protein synthesis, that particles very similar to those we have been describing are found in the nucleus, that cytoplasmic RNA cannot be reconstituted once it has been removed by ribonuclease action unless a nucleus is present, and that cytoplasmic RNA (or its precursors) moves from the nucleus to the cytoplasm but not in the reverse direction. These observations point strongly toward the nucleus as the likely site of ribosome synthesis. Detailed discussion of the evidence in support of these statements may be found in the reviews of Brachet,[1] Bonner,[12] and Chantrenne[8] in particular.

2. The Soluble Enzyme-Transfer Ribonucleic Acid System

Developments in understanding the soluble requirements in the cell-free mammalian amino acid incorporation system illuminated another, and more direct, participation of RNA in protein synthesis (see Fig. 1). It was learned that the soluble, nonparticulate liver cell fraction (earlier referred to as the "pH 5 fraction") found necessary for the over-all incorporation of amino acids into ribosome protein contained enzymes which catalyzed the carboxyl activation of amino acids by formation of enzyme-bound amino acyl adenylate compounds from ATP and amino acids, eliminating PP as a product.[112, 113] Similar enzymes were found in yeast and bacterial preparations[114, 115] and have since been found in all tissues examined (cf. Novelli and De Moss[3] and Hoagland[14]). Although it was gratifying to find amino acid activation reactions in a cellular fraction known to be required for amino acid incorporation into protein, a causal connection between the two processes was not established. Of considerably more pertinence to the subject of this chapter, however, was the finding that this same soluble cell fraction contained a species of RNA uniquely capable of reaction with amino

[112] M. B. Hoagland, *Biochim. et Biophys. Acta* **16**, 288 (1955).
[113] M. B. Hoagland, E. B. Keller, and P. C. Zamecnik, *J. Biol. Chem.* **221**, 45 (1956).
[114] P. Berg, *J. Biol. Chem.* **222**, 1025 (1956).
[115] J. A. DeMoss and G. D. Novelli, *Biochim. et Biophys. Acta* **22**, 49 (1956).

acids in a highly specific manner.[116, 117] Such reactions were independently discovered by Ogata *et al.* in Japan.[118, 119]

That a stable intermediate might be formed on the path between free amino acid and protein was first suggested by Hultin and Beskow.[120] These workers found that incubation of a crude supernatant fraction of rat liver with C^{14}-amino acid and ATP led to a conversion of the C^{14}-amino acid to a state in which it no longer equilibrated with free C^{12}-amino acid. Holley[121] furthermore, had pointed out that a similar crude fraction catalyzed a ribonuclease-sensitive, alanine-dependent incorporation of adenosine monophosphate-C^{14} (AMP-C^{14}) into ATP, suggesting the formation of an RNA-amino acid intermediate.

These matters have now been examined by many investigators and the general picture has emerged that the amino acid activating enzymes not only catalyze the activation of amino acids by the mechanism postulated, but also attach the amino acid so activated through an ester linkage to the terminal nucleotide of specific soluble RNA molecules. These molecules, once charged with amino acid, have been found to be able to transfer the amino acid to the protein of the ribonucleoprotein particles under appropriate conditions.[116, 117] Such soluble RNA-amino acid compounds, which thus satisfy several criteria for intermediates in the incorporation process, have become another strong link in the chain of evidence implicating RNA in protein synthesis. This interesting species of RNA and the reactions in which it participates must now be discussed.

a. Physicochemical Aspects of Transfer RNA

(1) *Occurrence and Methods of Preparation.* "Soluble RNA" (sRNA) or "cell sap RNA" are terms used to designate that fraction of total cellular RNA, usually 10 to 20%, isolatable by methods which depend on the relatively low molecular weight of the material. Usually this means the fraction which remains unsedimented after centrifugation of a tissue homogenate at 100,000 *g* for 1–2 hours. It would also, most likely, include material relatively more soluble in salt than the bulk of the cytoplasmic RNA. Such preparations contain more RNA than that responsible for amino acid binding, due to the presence of degraded microsomal material and perhaps other RNA of unknown significance. It has been suggested by Schweet

[116] M. B. Hoagland, P. C. Zamecnik, and M. L. Stephenson, *Biochim. et Biophys. Acta* **24**, 215 (1957).

[117] M. B. Hoagland, M. L. Stephenson, J. F. Scott, L. I. Hecht, and P. C. Zamecnik, *J. Biol. Chem.* **231**, 241 (1958).

[118] K. Ogata and H. Nohara, *Biochim. et Biophys. Acta* **25**, 660 (1957).

[119] K. Ogata, H. Nohara, and T. Morita, *Biochim. et Biophys. Acta* **26**, 657 (1957).

[120] T. Hultin and G. Beskow, *Exptl. Cell Research* **11**, 664 (1956).

[121] R. W. Holley, *J. Am. Chem. Soc.* **79**, 658 (1957).

that the specific amino acid-binding component of sRNA be called "transfer RNA" because of its role in transferring amino acids from activating enzymes to microsomal protein. These terms—sRNA and transfer RNA, the operational and the functional—are both useful and will be used hereinafter where appropriate.

It has generally been found that most of the sRNA may be almost quantitatively precipitated from the 100,000 *g* supernatant fraction of mammalian tissues along with a considerable fraction of the total soluble protein—by adjusting the pH to about 5. The protein component of this "pH 5 fraction" (earlier called the "pH 5 enzyme"[116]) consists of the bulk of the amino acid activating enzymes, as well as enzymes catalyzing terminal nucleotide additions to transfer RNA, in addition to other unidentified protein material. The remaining supernatant fraction (referred to as the S_4 fraction[122]) in mammalian tissues contains some amino acid activating activity, terminal nucleotide addition enzymes, other enzymic components, and a small amount of sRNA. The association of activating enzymes and sRNA in the pH 5 precipitate is probably fortuitous, as other methods of fractionation give an activating enzyme fraction free of RNA.[123]

Transfer RNA, and reactions catalyzing attachment of amino acids to it, have now been described in bacterial (cf. Berg and Ofengand[13]),[124, 125] yeast,[126] protozoan,[127] mammalian (cf. Schweet *et al.*,[128] see also Hoagland[14]), and avian tissues[129]; indeed in all tissues which have been specifically examined for them. It is remarkable how good the agreement is among various investigators with respect to the details of the nature of transfer RNA and the reactions in which it has been shown to participate. It seems highly likely, therefore, that a common mechanism of amino acid-transfer RNA interaction may exist over a wide phylogenetic range.

Transfer RNA may be obtained in good yield by treating the soluble cell fraction, or the pH 5 precipitable fraction, with phenol, followed by alcohol precipitation after the method of Gierer and Schramm,[24] and Kirby.[130] This product usually contains less than 1 % protein, has a 260/280

[122] L. I. Hecht, P. C. Zamecnik, M. L. Stephenson, and J. F. Scott, *J. Biol. Chem.* **233,** 954 (1958).
[123] E. Herbert, Ann. N. Y. Acad. Sci., **81,** 679 (1959).
[124] S. Lacks and F. Gros, *J. Mol. Biol.* **1,** 301 (1959).
[125] A. Tissières, *J. Mol. Biol.*, **1,** 365 (1959).
[126] R. Monier, M. L. Stephenson, and P. C. Zamecnik, *Biochim. et Biophys. Acta,* in press.
[127] J. Mager and F. Lipmann, *Proc. Natl. Acad. Sci. U. S.* **44,** 305 (1958).
[128] R. Schweet, F. C. Bovard, E. Allen, and E. Glassman, *Proc. Natl. Acad. Sci. U. S.* **44,** 173 (1958).
[129] S. B. Weiss, G. Acs, and F. Lipmann, *Proc. Natl. Acad. Sci. U. S.* **44,** 189 (1958).
[130] K. S. Kirby, *Biochem. J.* **64,** 405 (1956).

ratio of about 1.8, is metabolically active as an amino acid acceptor and as an amino acid donor to protein under appropriate conditions. A very similar preparation may be obtained from whole yeast cells by direct treatment with phenol.[126] Apparently the cell walls are permeable only to the relatively smaller sRNA molecules since ribosomes are not extracted by this method. Methods of isolation which involve very low pH's, or extraction with acidic salts at 100°C. yield metabolically inactive sRNA. These methods may be used, however, for assaying transfer RNA-amino acid compounds in reaction mixtures since they do not cause separation of the amino acid from the RNA. Preiss *et al.*[131] described a method for extracting transfer RNA from *E. coli* cells by the use of hot detergent, followed by salt and alcohol fractionation, and adsorption on and elution from charcoal. This product has general characteristics similar to the material from other sources isolated by the phenol method.

(2) *Molecular Weight and Base Composition.* The only published sedimentation constant for sRNA (from rat liver) is 1.85 $S_{20,w}$ at a concentration of 0.003% in 0.15 *M* NaCl, 0.015 *M* citrate pH 6.8,[117] and this value, with diffusion data, suggested a molecular weight of 15,000 to 20,000. Estimates based on the number of chain ends of mammalian sRNA capable of accepting amino acids or terminal nucleotides, and assuming no branching, give molecular weight values of around 30,000.[117, 122] Direct analysis of nucleosides released on alkaline hydrolysis also yields a similar result.[132] Somewhat higher values, of around 30,000 to 50,000, are estimated for the *E. coli* transfer RNA preparation.[131] Yeast sRNA is reported to have a sedimentation constant of 5,[126] which would be consistent with a molecular weight closer to that of *E. coli* sRNA.

Tissières[125] has obtained a molecular weight of 27,000 for *E. coli* sRNA based on sedimentation and diffusion measurements. The material was obtained by phenol treatment of the upper two-thirds of a 3-hour, 78,000 *g* supernatant fraction from an *E. coli* extract. The RNA gives a single electrophoretic peak and the sedimentation constant is unaltered by versene or salt. It is of considerable interest that the whole supernatant fraction gave an identical sedimentation constant for sRNA ($S_{20,w}$ = 4.0) as was obtained with the phenol extracted material. This suggests that the sRNA in the natural state is not bound to protein and exists free in the cytoplasm.

Some preliminary data on the base composition of sRNA from various tissues are given in Table III.[133-136] Since methods of preparation

[131] J. Preiss, P. Berg, E. J. Ofengand, F. H. Bergmann, and M. Dieckmann, *Proc. Natl. Acad. Sci. U. S.* **45,** 319 (1959).

[132] D. B. Dunn, *Biochim. et Biophys. Acta* **34,** 286 (1959).

[133] P. F. Spahr and A. Tissières, *J. Mol. Biol.* **1,** 237 (1959).

[134] J. F. Scott, unpublished data.

TABLE V

RELATIVE PROPORTIONS[a] OF ADDITIONAL COMPONENTS IN RNA FROM RAT LIVER MICROSOMES AND SOLUBLE FRACTION[b]

Component	Microsome	Soluble
Pseudouridine	7.5	25
5-Methylcytosine	0.4	10
6-Methylaminopurine	0.5	8.1
6-Dimethylaminopurine	0.1	0.1
1-Methylguanine	0.1	3.3
2-Methylamino-6-hydroxypurine	0.1	2.3
2-Dimethylamino-6-hydroxypurine	0.1	3.0

[a] Values are mole/100 mole uridine.

[b] Data are from D. B. Dunn, *Biochim. et Biophys. Acta* **34**, 286 (1959).

vary considerably small differences which may ultimately prove to be significant would not show up here. It will be noted that there is generally a remarkable similarity in the base composition of sRNA from microorganisms and animals. This is in contrast to the clear differences in base composition of ribosomal RNA from these two sources. The possible significance of this finding will be discussed later.

The discovery by Cohn,[137, 138] and Davis and Allen[139, 140] of "pseudouridine" (5-ribosyluracil) has led to an exploration of its location in definable cellular RNA fractions. The occurrence of this compound in yeast, particularly in a relatively salt-soluble fraction perhaps equivalent to sRNA,[139] suggested that it might be a constituent peculiar to sRNA. This was found to be the case by Dunn,[132] whose data appear in Table V. It will be noted that this nucleoside is considerably more abundant in sRNA than in microsomes. It was also observed, as the table shows, that there was a relative excess in sRNA of methylated derivatives of the bases.[132]

(3) *Heterogeneity.* Early ultracentrifugal and electrophoretic analyses of sRNA revealed that the preparations were nonhomogeneous. Furthermore, it became apparent that sRNA must consist of a number of distinct species since amino acids were attached to sRNA additively (thus to specific sites) and terminally. For these reasons much work has been directed toward attempts to fractionate sRNA, both to separate the transfer RNA's from one another and to separate them from contaminating unrelated "junk" RNA.

[135] D. A. Goldthwaite, *Biochim. et Biophys. Acta* **30**, 643 (1958).

[136] R. S. Schweet, unpublished data, (1959).

[137] W. E. Cohn and E. Volkin, *Nature* **167**, 483 (1951).

[138] W. E. Cohn, *Biochim. et Biophys. Acta* **32**, 569 (1959).

[139] F. F. Davis and F. W. Allen, *J. Biol. Chem.* **227**, 907 (1957).

[140] C. Yu and F. W. Allen, *Biochim. et Biophys. Acta* **32**, 393 (1959).

All of this work is in its infancy but a few of the preliminary approaches to the problem may be outlined.

The most direct means of separating and purifying transfer RNA is to centrifuge at 100,000 *g* for a longer period, since transfer RNA is of relatively low molecular weight. Early experiments indicated that a centrifugation at 100,000 *g* for 3 hours instead of 1 hour reduced the RNA content of the supernatant fraction by 50% with no loss in amino acid acceptorability.[117] It is likely that the material sedimented during the prolonged centrifugation was microsomal in nature. Salt fractionation, such as that used by Davis and Allen,[139] may prove to be effective in removing higher molecular weight components. ECTEOLA has been reported[117] to give some separation of transfer RNA from inactive RNA.

With respect to the separation of transfer RNA's one from the other, Smith *et al.*[141] have effected some preliminary fractionation with the use of calcium phosphate gel. Another approach to transfer RNA fractionation has been the use of countercurrent distribution as reported by Holley and Merrill.[142] These workers have obtained partial separation of the transfer RNA which accepts alanine from other transfer RNA's by this means.

Because of the relatively high molecular weight of the specific transfer RNA molecules and the probability that the molecular weight distribution is narrow, fractionation based on size, electrical charge, solubility, difference in base content, etc. may prove to be difficult. For this reason several laboratories have attempted to "pull out" a particular transfer RNA by making use of the chemical properties of the amino acid it specifically binds. Preliminary success has been reported by Brown[143] who has been able to isolate selectively the tyrosine and histidine transfer RNA's by binding the amino acid moiety to a diazotized polydiazostyrene column. A similar approach is to make use of the fact that the amino acid protects its particular RNA from oxidation of the 2′- and 3′-hydroxyl groups of the terminal ribose residue by periodate. The oxidized terminal aldehydic groups could then be utilized for addition reactions to produce RNA molecules sufficiently different from the unaltered ones to permit separation.[96]

b. Reactions Involving the Terminal Nucleotides of Transfer RNA

(1) *Early Studies on Nucleotide Labeling of Soluble RNA and Terminal Attachment of Nucleotides.* Studies in cell-free systems on incorporation of RNA precursors into RNA date back to 1949.[144] The general purpose of

[141] K. C. Smith, E. Cordes, and R. S. Schweet, *Biochim. et Biophys. Acta* **33,** 286 (1959).

[142] R. W. Holley and S. H. Merrill, *Federation Proc.* **18,** 982 (1959).

[143] G. Brown, A. V. W. Brown, and J. Gordon, *Brookhaven Symposia in Biol.* **12,** 47 (1959).

[144] M. Friedkin and A. L. Lehninger, *J. Biol. Chem.* **177,** 775 (1949).

such investigations was, of course, to elucidate the mechanism of RNA synthesis. It has come about, however, that much of this work has tended to illuminate protein synthesis, and has made questionable some of the earlier conclusions about RNA synthesis. This paradox is due to the fact that a large amount of the turnover of base precursors in sRNA has been of a very special and limited sort: the turnover of certain nucleotide end groups of sRNA. This phenomenon seems to be related to the preparation of the end groups of sRNA for amino acid attachment, and not directly to the synthesis of RNA itself.

It was shown by Goldwasser[145] that homogenates of pigeon pancreas incorporated AMP-C^{14} into RNA and that the adenylic acid-2′,3′-C^{14} was recovered on alkaline hydrolysis. If the AMP were labeled instead with P^{32}, alkaline hydrolysis led to the appearance of P^{32} in the 2′3′-nucleotides of all four bases suggesting that the AMP was randomly distributed in the molecules.

Other studies on nucleotide incorporation[146-148] showed similar incorporations into RNA fractions of an unspecified kind, either as to level of phosphorylation of the immediate precursor or the site of incorporation into the RNA molecule. The earliest indication that there was a specific site of incorporation came from the work of Heidelberger *et al.*[149] who, using AMP^{32}, found that diesterase released the incorporated AMP as the 5′-nucleotide and that the P^{32} was associated only with the 2′,3′-cytidylic acid fraction on alkaline hydrolysis. This indicated that a special type of AMP incorporation was proceeding in this particular system—one in which the AMP was preferentially linked to cytidine monophosphate (CMP). This system depended on conditions of oxidative phosphorylation in which the AMP precursor would have been brought to higher levels of phosphorylation prior to incorporation into RNA.

A series of investigations of these phenomena in a number of laboratories unfolded the picture of a predominant terminal attachment of adenine nucleotides to the soluble RNA fraction.[135, 150-154] This was concluded since most of the incorporated adenine was released as the nucleoside on alkaline

[145] E. Goldwasser, *J. Am. Chem. Soc.* **77,** 6083 (1955).
[146] E. Herbert, V. R. Potter, and L. I. Hecht, *J. Biol. Chem.* **225,** 659 (1957).
[147] E. S. Canellakis, *Biochim. et Biophys. Acta* **23,** 217 (1957).
[148] R. Logan, *Biochim. et Biophys. Acta* **26,** 227 (1957).
[149] C. Heidelberger, E. Harbers, K. C. Leibman, Y. Takagi, and V. R. Potter, *Biochim. et Biophys. Acta* **20,** 445 (1956).
[150] P. C. Zamecnik, M. L. Stephenson, J. F. Scott, and M. B. Hoagland, *Federation Proc.* **16,** 197 (1957).
[151] A. R. P. Paterson and G. A. LePage, *Cancer Research* **17,** 409 (1957).
[152] E. S. Canellakis, *Biochim. et Biophys. Acta* **25,** 217 (1957).
[153] M. Edmonds and R. Abrams, *Biochim. et Biophys. Acta* **26,** 226 (1957).
[154] E. Herbert, *J. Biol. Chem.* **231,** 975 (1958).

hydrolysis. In agreement with Heidelberger *et al.*,[149] this AMP appeared to be chiefly incorporated adjacent to CMP. Several of these studies also indicated that the immediate precursor of the end group was the triphosphate.

Detailed analyses of these terminal nucleotide addition reactions have been carried out by Hecht *et al.*,[122, 155] and by Herbert.[123,154]

It is now established that the AMP and cytidine monophosphate moieties of ATP and cytidine triphosphate (CTP) are attached in a specific manner to the ends of the transfer RNA molecules by a specific enzyme system found in the soluble fraction of the cell. The experimental evidence may be conveniently considered under the following headings: the identity of the RNA acceptor, the nucleotide precursors of the end groups, the enzymes catalyzing the reactions, and the mechanism of the reactions. (See Figs. 2 and 3 for the formulas of the reactions discussed under these headings.)

(2) *The Identity of RNA Acceptors of the End Groups.* It has been established that the RNA which participates in the reactions involving the attachment of nucleotide end groups is the soluble RNA of the cell, sRNA. RNA derived from the nucleus and from the ribosomes are essentially inactive in the system. sRNA fractions from widely different species, however, are interchangeable in the system; yeast, bacterial, and mammalian sRNAs are readily labeled with terminal nucleotides in the presence of ascites tumor or rat liver enzymes. It can be stated furthermore, that transfer RNA itself is the acceptor of the terminal grouping of nucleotides for it has been shown that the latter reaction is an obligatory prelude to amino acid attachment.

(3) *The Nucleotide Precursors of the End Groups.* The triphosphates of adenosine, cytidine, and uridine serve as the source of the respective mononucleotide end groups of transfer RNA. The terminal attachment of adenosine and cytidine triphosphates exceeds that of the uridine compound by at least a factor of 4.[122] The significance of the latter reaction (that involving UTP) has yet to be assessed. GTP does not seem to participate significantly in these reactions. In view of the reactions described by Grunberg-Manago *et al.*,[156] and Hilmoe and Heppel[157] in which nucleoside diphosphates served as the substrates for RNA synthesis in bacterial systems, it was important to know what level of phosphorylation of nucleoside was involved in the terminal attachment reactions. The triphosphates are clearly superior to the diphosphates, however, in these reactions. Furthermore, evidence has been presented by Hecht *et al.*,[122, 155] that the

[155] L. I. Hecht, M. L. Stephenson, and P. C. Zamecnik, *Proc. Natl. Acad. Sci. U. S.* **45,** 505 (1959).

[156] M. Grunberg-Manago, P. J. Ortiz, and S. Ochoa, *Science* **122,** 907 (1955).

[157] R. S. Hilmoe and L. A. Heppel, *J. Am. Chem. Soc.* **79,** 4810 (1957).

terminal attachment of CMP is a reversible pyrophosphate splitting reaction: pyrophosphate inhibits the forward reaction and PP^{32} is incorporated into CTP in the back reaction only in the presence of sRNA. Herbert has demonstrated directly that PP accumulates as the product of the terminal attachment of the AMP moiety of ATP to sRNA.[123]

As these workers have pointed out, it is clear that these terminal addition reactions are distinct from the reactions catalyzed by nucleoside phosphorylase not only in that triphosphates are the immediate precursors but also in that the attachment is specific for ATP and CTP and to a lesser extent UTP. The terminal configuration is not altered by the presence of other nucleotide precursors.

(4) *The Enzymes Catalyzing the Reactions.* Although the enzyme system from the rat liver supernatant fraction catalyzing the attachment of terminal nucleotides to sRNA has been studied by many workers, only one report has been concerned with its purification. Herbert has had success in this regard, using ammonium sulfate fractionation and adsorption on a hydroxy apatite column.[123] The extent of purification of activity for both CMP and AMP attachment is parallel at each step, suggesting that a single enzyme may be involved for both nucleotide additions. Furthermore, the pH optima for attachment of both nucleotides are identical. The system is sensitive to trypsin and to heating at 85°C. for a brief period. The fractionation procedure also effects a complete separation of these enzymes from amino acid activating enzymes, thereby permitting the demonstration that the terminal AMP is derived directly from ATP and not from the AMP moiety of the amino acyl adenylate. This point will be discussed further below.

(5) *The Mechanism of the Terminal Nucleotide Attachment.* The evidence available thus far suggests that the reaction may be formulated in the three steps depicted in Fig. 2.

This formulation states that three known nucleotides are attached to the end of a polynucleotide chain of unknown base sequence. (The two last nucleotides, X and Y are shown). The two CMP moieties of two molecules of CTP are first attached sequentially to the 3′-hydroxyl of the ribose of the terminal nucleotide in the chain. Then the resulting terminal CMP is covered by an AMP by a similar pyrophosphoryl cleavage of ATP. The reaction is then complete and proceeds no further. The following evidence supports this formulation[122, 123, 154, 155]: (1) incubation of sRNA with CTP-C^{14} results in the incorporation of C^{14} into the RNA, and upon alkaline hydrolysis approximately 50% of the C^{14} is recovered as cytidine, and 50% as 2′,3′-cytidylic acid. (With the purified enzyme system of Herbert, the results differ: almost all of the incorporated CMP-C^{14} is terminal. The formulation presented here is based on the observations

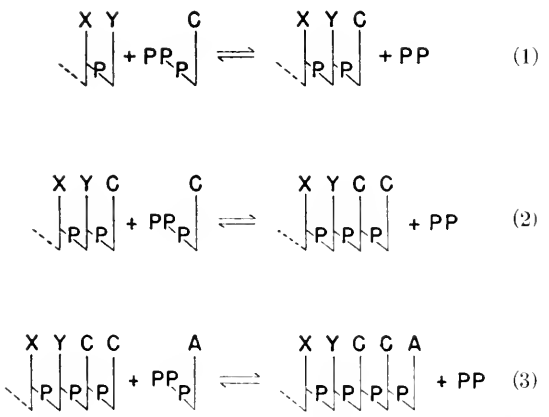

FIG. 2. Terminal nucleotide additions to transfer RNA.

of Hecht *et al.* using a cruder system, and therefore perhaps more representative of *in vivo* events. Furthermore, the RNA used in this system had had the terminal groups removed by preincubation.) These studies do not, however, rule out the unlikely possibility that the innermost CMP is deeper in the polynucleotide chain. (2) When ATP is present in this incubation with the CTP-C^{14}, essentially all of the labeled cytidine is recovered as the 2′,3′-cytidylic acid. (3) Incubation with ATP-C^{14} essentially leads to complete terminal attachment of the AMP-C^{14}. (4) PP inhibits the labeling of RNA by both CTP-C^{14} and ATP-C^{14}; PP^{32} is incorporated into CTP in the presence of RNA and the appropriate enzymes and PP is the product of AMP terminal attachment from ATP. (5) The reversibility of the reactions is further supported by the fact that the enzymic loss of the terminal groups is dependent upon PP, provided measures are taken to remove PP from the enzyme preparation. (6) The addition of other nucleoside triphosphates has no effect on the pattern of labeling obtained with ATP and CTP. (7) Naturally occurring sRNA has only adenosine and cytosine ends upon alkaline hydrolysis.[158]

The foregoing seems to be the mechanism by which transfer RNA is prepared for amino acid attachment; although each molecule would appear to consist of a different series of bases, the common terminal configuration to which the amino acid is to be attached has been prepared. In the next section we shall examine the mechanism of this amino acid-transfer RNA interaction.

c. Enzymic Attachment of Amino Acids to Transfer RNA

Incubation of naturally occurring sRNA, amino acid activating enzymes, ATP, Mg^{++}, and the appropriate C^{14}-amino acids results in the covalent

[158] D. B. Dunn, unpublished data, (1958).

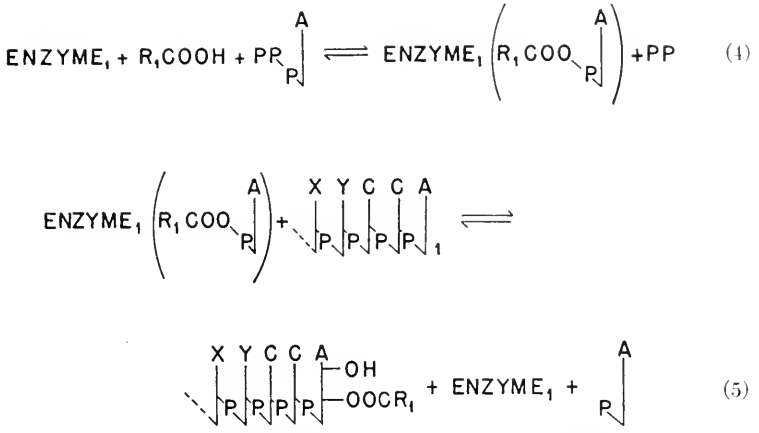

FIG. 3. Amino acid attachment to transfer RNA.

linking of the amino acid to the RNA. Widespread study of this phenomenon in many laboratories has led to the general acceptance of the formulation of the reaction as depicted in Fig. 3.

This formulation states that an amino acid (R_1) is first activated by its specific activating $enzyme_1$, through formation of an enzyme-bound amino acyl adenylate compound. This compound, with associated activating enzyme, then reacts with an RNA molecule specific for the particular amino acyl adenylate-enzyme complex, resulting in the esterification of the amino acid on the 2′- or 3′-hydroxyl of the terminal adenosine of the RNA, and the release of the adenylate moiety of the activated compound. The formulation further states that since, as we have seen, the terminal configuration of nucleotides pCpCpA is common to all RNA's involved in the process, the specificity of attachment must be determined by features of the RNA molecule internal to this terminal grouping. (This is indicated in the figure by the subscript number 1 after the nucleotide sequence of the RNA).

The evidence which supports this formulation must now be examined.

(1) *Amino Acid Activation as a Prelude to Attachment of Amino Acid.* Activation of amino acids by the mechanism depicted in Eq. (4) in Fig. 3 is now well established as the principle pathway of activation of amino acids in all tissues thus far examined. The discovery that animal tissues catalyzed an amino acid dependent incorporation of PP into ATP, and an ATP dependent formation of amino acid hydroxamic acids,[113-115] pointed to the likelihood that amino acids were activated by formation of such amino acyl-AMP compounds. The fact that these compounds were firmly bound to enzymes was suggested by the finding that AMP failed to exchange with ATP in the system, and that no acid soluble product was

formed in the absence of an acceptor for the activated amino acid. It was further apparent that individual enzymes activated individual amino acids[159-163] and that all amino acids were activated by the same mechanism.[164-166] Much work has now established the mechanism on a firm footing.[167-170] Final unequivocal evidence has recently been presented by Kingdon *et al.* who have actually isolated a tryptophan adenylate from large quantities of purified tryptophan activating enzyme.[171]

There are still a number of interesting questions relating to amino acid activation which are being pursued but these do not have relevance to the subject of this chapter. We may, however, emphasize (or reemphasize) three important aspects of the reaction which impinge on our concern with the role of RNA in protein synthesis. First, there is general agreement that amino acid activation—i.e., the formation of the enzyme-bound amino acyl adenylate compounds—is an enzymic reaction entirely independent of RNA. This statement is based on the fact that ribonuclease does not affect the activation of amino acids by crude or purified activating enzyme preparations; and that the more highly purified activating enzymes do not contain measurable RNA and are fully active in the absence of RNA.

[It should be mentioned, however, that Ogata *et al.*[172-174] have obtained evidence that ribonuclease does inhibit activation. Their maximal inhibitory effects (50%) were obtained by preincubating the "pH 5 enzyme" fraction with ribonuclease, followed by reprecipitation of the enzyme at

[159] E. W. Davie, V. V. Koningsberger, and F. Lipmann, *Arch. Biochem. Biophys.* **65,** 21 (1956).

[160] R. D. Cole, J. Coote, and T. S. Work, *Nature* **179,** 199 (1957).

[161] V. V. Koningsberger, A. M. van der Ven, and J. T. G. Overbeek, *Proc. Koninkl. Ned. Akad. Wetenschap.* **B60,** 141 (1957).

[162] R. Schweet, R. W. Holley, and E. Allen, *Arch. Biochem. Biophys.* **71,** 311 (1957).

[163] F. H. Bergmann, P. Berg, J. Preiss, E. J. Ofengand, and M. Dieckmann, *Federation Proc.* **18,** 751 (1959).

[164] B. Nisman, F. H. Bergmann, and P. Berg, *Biochim. et Biophys. Acta* **26,** 639 (1957).

[165] F. Lipmann, *Proc. Natl. Acad. Sci. U. S.* **44,** 67 (1958).

[166] G. C. Webster, *Arch. Biochem. Biophys.* **82,** 125 (1959).

[167] J. A. DeMoss, S. M. Genuth, and G. D. Novelli, *Proc. Natl. Acad. Sci. U. S.* **42,** 325 (1956).

[168] M. B. Hoagland, P. C. Zamecnik, N. Sharon, F. Lipmann, M. P. Stulberg, and P. D. Boyer, *Biochim. et Biophys. Acta* **26,** 215 (1957).

[169] P. Berg, *J. Biol. Chem.* **233,** 601 (1958).

[170] K. Moldave, P. Castelfranco, and A. Meister, *J. Biol. Chem.* **234,** 841 (1959).

[171] H. S. Kingdon, L. T. Webster, Jr., and E. W. Davie, *Proc. Natl. Acad. Sci. U. S.* **44,** 757 (1958).

[172] H. Nohara and K. Ogata, *Biochim. et Biophys. Acta* **31,** 142 (1959).

[173] K. Ogata and H. Nohara, *Biochim. et Biophys. Acta* **31,** 149 (1959).

[174] K. Ogata, H. Nohara, and S. Miyazaki, *Biochim. et Biophys. Acta* **32,** 287 (1959).

pH 5. Van der Decken and Hultin[175] found, however, that the hydrolysis of RNA under these conditions reduced the pH 5 precipitability of the activating enzymes so that the effect was a loss of enzyme. This may be part of the explanation of the findings of the Japanese workers. However, they do obtain inhibition of activation (about 15%) by direct preincubation of the enzyme preparation with ribonuclease (omitting the reprecipitation step) and conclude that the activation of some amino acids may involve RNA. This they further support by preliminary results suggesting that the PP^{32} moiety of ATP becomes linked to sRNA and that an amino acid mixture reduces this labeling. The picture evolved is that there may be an activation pathway in which ATP first pyrophosphorylates sRNA, followed by substitution of the PP group by amino acid.]

Second, it appears highly probable that each amino acid is activated by its own single specific enzyme. Several relatively pure activating enzymes have now been isolated and the work continues. The fact that amino acids do not compete for enzyme sites indicates that in all likelihood the remaining enzymes will be isolated. Third, the activated amino acyl adenylate intermediate is very firmly bound to its specific enzyme and dissociates to only a very small extent in the absence of an acceptor for the amino acid. Thus the activating enzyme and its bound amino acyl adenylate can be thought of as acting effectively as a single unit.

The formulation of Eq. (5) in Fig. 3 indicates that the transfer of amino acid to sRNA is mediated directly by the activating enzyme without the involvement of other enzymes. This conclusion is strongly supported by the work of Berg's group (cf. Symposium[13]) which has shown that during the course of a 100-fold purification of an amino acid activating enzyme, there was a parallel increase in the ability of fractions to catalyze activation and transfer of the amino acid to sRNA. It has also been shown by Berg,[13] and by Schweet,[13] that purified activating enzymes will more readily transfer to sRNA that amino acid which they activate. Furthermore, there is evidence that synthetic amino acyl adenylates may serve as a source of amino acid on transfer RNA provided they are first bound to activating enzymes.[176]

An anomalous situation has been reported by Berg and his associates.[177] They have obtained relatively pure methionine activating enzymes from two sources, *E. coli* and yeast. The former enzyme will attach over two times more methionine than the latter to *E. coli* sRNA. This peculiar result is puzzling and has led to the suggestion that perhaps there are

[175] A. van der Decken and T. Hultin, *Exptl. Cell Research* **17,** 188 (1959).

[176] K. K. Wong, A. Meister, and K. Moldave, *Biochim. Biophys. Acta* **36,** 531 (1959).

[177] F. H. Bergmann, P. Berg, J. Preiss, E. J. Ofengand, and M. Dieckmann, *Federation Proc.* **18,** 751 (1959); and unpublished data, (1959).

more sites than one available on the RNA for methionine attachment, the yeast enzyme may "recognize" only one site while the *E. coli* enzyme can recognize this and another.

We may tentatively conclude that the activating enzymes catalyze a two-step reaction when transfer RNA is present—formation of an acyl adenylate and attachment of the acyl moiety to transfer RNA.

(2) *Individual RNA's as Acceptors of Individual Amino Acids.* Early observations of amino acid-sRNA interaction indicated that amino acids were additively and noncompetitively attached to sRNA. This clearly established that separate sites were available for each amino acid but did not tell us whether separate RNA's were involved. Zachau *et al.*[178] then demonstrated directly that the amino acids were esterified on the terminal adenosine of the RNA. This was done by treatment of leucine labeled sRNA with pancreatic ribonuclease, which resulted in a quantitative yield of leucyladenosine. It had been shown by Hecht *et al.*[155, 179] that the terminal configuration of nucleotides pCpCpA was necessary for amino acid attachment (see below). These workers and Preiss *et al.*[131] also showed that periodate treatment of the sRNA resulted in loss of ability to accept amino acids and that amino acids protected against the action of periodate. Periodate would be expected to oxidize any free 2′-, 3′-hydroxyl groups. Preiss *et al.*[131] carried the periodate experiment a step further by showing that amino acids could protect only their own sites of attachment to sRNA from periodate oxidation.

Hecht *et al.*, furthermore, demonstrated that the ordinarily free reversibility of the reaction by which the terminal pCpCpA grouping was attached to the RNA was markedly inhibited by amino acids.[155] This suggested that the amino acids might, in fact, actually be attached to this terminal grouping, thereby preventing the nucleotide end group from reacting with PP. Thus there was strong indication that the terminal 2′-, 3′-hydroxyl groups of the polynucleotide chains were involved in the link with amino acids.

These experiments supplemented and supported the observations of Schweet's group (cf. Symposium[13,141]) that crude fractionation of sRNA resulted in enrichment of fractions for acceptance of one amino acid over another. This series of observations has, thus, strongly stimulated the search for better means of fractionating sRNA, alluded to earlier, which is likely, ultimately, to result in the isolation of several homogeneous transfer RNA species each capable of reacting with only one amino acid. When this has been accomplished it should then be possible to undertake deg-

[178] H. G. Zachau, G. Acs, and F. Lipmann, *Proc. Natl. Acad. Sci. U. S.* **44**, 885 (1958).
[179] L. I. Hecht, M. L. Stephenson, and P. C. Zamecnik, *Biochim. et Biophys. Acta* **29**, 460 (1958).

radative studies to reveal the structural characteristics of the RNA molecules which underlie their specificity.

(3) *The Terminal Nucleotide Grouping as a Requirement for Amino Acid Attachment.* Because of the reversibility of attachment of the terminal nucleotide groups to transfer RNA it became possible to "strip" the RNA of these nucleotides by incubating it with the terminal nucleotide system and PP. This "stripped" RNA could then be tested for its ability to accept amino acids in the presence of ATP, CTP, or both.

Careful exploitation of this technique by Hecht *et al.*[155, 179] has revealed that both ATP and CTP are necessary to relabel such "stripped" RNA with C^{14}-amino acids. That the added requirement for CTP is due to the reattachment of this nucleotide's CMP moiety to the end of the RNA was shown by preincubating the RNA with CTP and the enzyme system which attaches CMP to the end of the RNA. This RNA, when reisolated, was then able to accept amino acids, like the naturally occurring material, in the presence of ATP alone. The requirement for both CTP and ATP in the labeling of "stripped" RNA was shown to be common to all amino acids. It therefore seems highly probable that the same type of terminal configuration of nucleotides is required for attachment of all amino acids to their specific RNA molecules.

It should perhaps be stated explicitly that the terminal AMP grouping of the RNA to which the amino acid becomes attached is not derived from the AMP moiety of the amino acyl adenylate which furnishes the amino acid. This was considered a possibility at one time but is ruled out by the facts that (1) terminal AMP attachment to RNA is clearly catalyzed by enzymes other than those which activate amino acids[123]; (2) in crude systems containing both activities terminal AMP attachment is not dependent on amino acids[155]; (3) the optimal ATP concentration for AMP attachment is lower, by a factor of 100, than the optimal ATP concentration for amino acid attachment.[155]

(The anomalous observation of Lipmann,[180] that in the presence of tryptophane,-activating enzyme and ATP, tryptophan-C^{14} could esterify the ribose-2′(3′)-hydroxyl of ATP suggested a mechanism by which both amino acid and AMP could be attached to transfer RNA in a single step. This seems improbable in the light of the above evidence and the reaction probably represents an unphysiological phenomenon resulting from the excess of ATP present and the absence of a natural acceptor for the amino acid.)

(4) *The Site of Amino Acid Attachment.* It was apparent, early, that the chemical behavior of the RNA-amino acid link seemed to preclude its being a phosphoanhydride type of bond. The bond was quite stable

[180] S. B. Weiss, H. G. Zachau, and F. Lipmann, *Arch. Biochem. Biophys.* **83**, 101 (1959).

at pH's below neutrality even at a high temperature, and in alkali it hydrolyzed at a rate considerably slower than amino acyl phosphoanhydrides. It was also much less reactive with hydroxylamine and ammonia. The periodate inactivation studies mentioned above were also strongly indicative of an ester bond between amino acid carboxyl and ribose-2′- or 3′-hydroxyl group. As was mentioned above, the nature of the linkage has now been established in both mammalian[178] and bacterial[131] systems by direct removal by ribonuclease of the terminal adenosine-amino acid ester. Thus, the configuration of the terminal grouping illustrated in Eq. (5) appears to be correct.

This particular type of linkage is of special interest because it is in equilibrium with the AMP pyrophosphoryl bond of ATP via the steps in Eqs. (4) and (5). For example, Schweet[181] has obtained an equilibrium constant of 0.4 for the reaction

$$\text{ATP} + \text{RNA} + \text{threonine} \rightleftharpoons \text{threonine} - \text{RNA} + \text{AMP} + \text{PP}$$

Similarly Berg's group found a value of 0.32 for valine RNA formation.[177] A simple ester bond would certainly not be expected to have such a high energy content and we must therefore assume that the presence of the adjacent hydroxyl group on the ribose confers special reactivity properties to the linkage.

Naturally occurring sRNA derived from mammalian pancreas[178] and liver[14, 182] and *E. coli* (cf. Chapter 38) has been found to have amino acids bound to it. Analysis indicates that nearly all of the amino acids are represented,[182] and that the sRNA is actually saturated with amino acids.[14] Preliminary studies indicate that this is also true for yeast transfer RNA.[126] The *E. coli* transfer RNA preparation of Berg's group, on the other hand, is reported to be free of amino acids.[131] This may be due to loss during the preparative procedure. The "pressure head" of ATP in cells would serve to saturate the transfer RNA pool with amino acids as soon as they entered the cell. Thus, transfer RNA is an activated amino acid pool of not inconsiderable dimensions.

d. Evidence for a Direct Role of Transfer RNA in Protein Synthesis

The evidence that a particular cellular RNA fraction binds amino acids specifically suggests the likelihood that it may be involved in the mechanism by which these amino acids ultimately find their way into protein. The existence of these RNA-amino acid reactions in a tissue component required for incorporation of amino acids into protein further supports

[181] R. S. Schweet, unpublished data, (1959).

[182] G. Acs, G. Hartmann, H. G. Boman, and F. Lipmann, *Federation Proc.* **18**, 700 (1959).

this view. To establish the fact that RNA-amino acid compounds are true intermediates in protein synthesis, however, it is necessary to show, *in vivo*, that they satisfy the kinetic requirements for an intermediate and to show, *in vitro*, that they may serve directly as the source of amino acid in specific newly synthesized protein molecules. Some approaches to satisfying these criteria have been made.

Because of the rapidity of labeling of ribosomal protein in *in vivo* systems it was necessary to slow down the process in order to investigate events which might precede the appearance of amino acid in protein. This was accomplished by incubating ascites tumor cells in ascitic fluid at a reduced temperature. By this means, it was shown that following the exposure of the cells to a C^{14}-amino acid, the radioactivity appeared initially in the sRNA fraction, rising rapidly to a plateau value. At a slower rate the amino acid found its way into particle RNA where it also quickly reached a low but definite plateau value. The particle protein accumulated label even more slowly but progressively and finally label appeared in soluble protein.[117] Furthermore, as we see in Fig. 4, from the recent work of Stephenson and Zamecnik (cf. Zamecnik[96]), when maximal activity has been introduced into the RNA fractions, the addition of a quenching dose of C^{12}-amino acid resulted in a rapid loss of label from sRNA. Con-

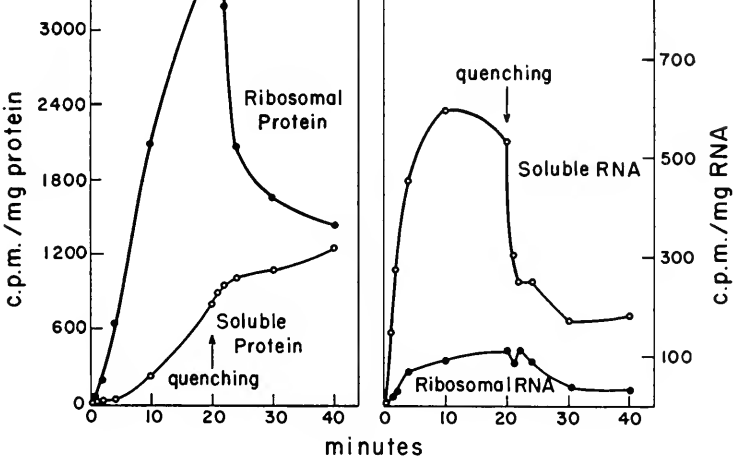

FIG. 4. The incorporation of valine-C^{14} into the RNA and protein of whole Ehrlich ascites tumor cells incubated in their own ascitic fluid at 20°C. At 20 minutes valine-C^{12} was added to the incubation medium such that the intracellular valine-C^{14} specific activity was reduced to about a third of its previous activity. [From P. C. Zamecnik, *Harvey Lectures* **54**, 256 (1960).]

siderably more detailed studies on the *in vivo* interrelationship of these fractions have been carried out by Lacks and Gros.[124] These investigators have confirmed the mammalian results in an *E. coli* system and have elegantly established two further points: (1) the rate of attachment of amino acids to transfer RNA is a function of the rate of protein synthesis. Inhibition of protein synthesis slows the rate of equilibration of a C^{14}-amino acid with the transfer RNA pool but does not alter the final equilibrium level. Amino acids of which the cells have been deprived equilibrate much more rapidly with the pool. These findings suggest that the availability of sites on transfer RNA is a direct function of the rate at which the sites are made available by subsequent steps in protein synthesis. (2) Removal of the inhibition of protein synthesis results in a transfer of the labeled amino acid from the RNA pool to protein. These matters are considered more fully in Chapter 38.

Brachet[183] has recently contributed to the growing body of evidence supporting the role of transfer RNA in protein synthesis. Extending his earlier studies on the ribonuclease inhibition of protein synthesis in onion root tips, he has shown that in the inhibited system, it is the sRNA that is depleted, while ribosomal RNA is relatively stable. It would be safe to conclude that the *in vivo* kinetic behavior of transfer RNA suggests that it could be an intermediate in protein synthesis.

When we turn to the cell-free systems available to us we find we are only on the verge of discovery, but what has come to light, thus far, further supports the role of transfer RNA amino acid as an intermediate.

It was found early that transfer RNA labeled with amino acids in the whole pH 5 fraction would transfer the amino acids to microsome protein in the presence of ATP, GTP, and a nucleoside triphosphate generating system[117] (see Fig. 5). Furthermore, transfer RNA, properly labeled with C^{14}-amino acids by activating enzymes and ATP, may be isolated by the phenol method and alcohol precipitation as a fairly pure group of compounds, free of protein and adsorbed amino acids. Incubation of this material with microsomes, Mg^{++}, ATP, an ATP generating system, GTP, and an enzymic component of the soluble cell fraction, [the S_4 fraction described in Section II, 2, *a*, (1)], resulted in a rapid and irreversible transfer of the amino acid to ribosomal protein[117] (see Fig. 6). The omission of any of these components gave low activity. The amino acid in this protein was not found in terminal positions but within the peptide chain.[96] (This is in contrast to the more recent results of Webster[166] who finds, starting with glutamyl-C^{14} RNA and methionyl-C^{14} RNA, that the amino acids are reversibly incorporated into pea seedling ribosomal protein and are in *N*-terminal positions.) It could be demonstrated that when transfer

[183] J. Brachet, *Biochim. et Biophys. Acta* **35**, 580 (1959).

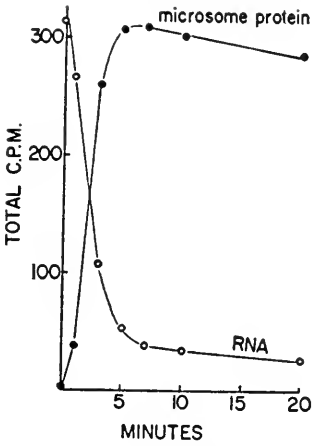

FIG. 5. The transfer of leucine-C^{14} from whole leucine-C^{14} labeled pH 5 fraction to microsomal protein. The open circles are the c.p.m. in transfer RNA, the closed the c.p.m. in particle protein. [From M. B. Hoagland, M. L. Stephenson, J. F. Scott, L. I. Hecht, and P. C. Zamecnik, *Biochim. et Biophys. Acta* **24**, 215 (1957).]

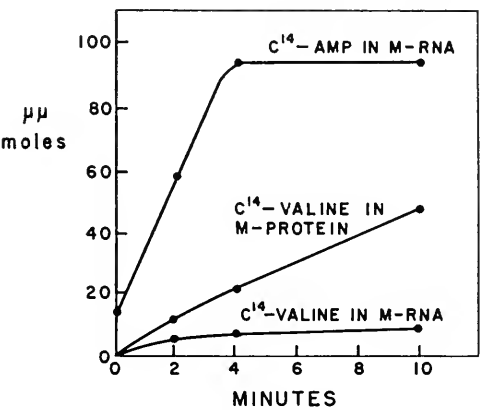

FIG. 6. Transfer of amino acid and terminal adenine from transfer RNA to ribosomal RNA, and of amino acid to ribosomal protein. The system consisted of yeast soluble RNA, dialyzed ascites tumor S_4 fraction, rat liver microsomes, ATP, GTP, and a nucleoside triphosphate generating system. The yeast soluble RNA was labeled in one case (the two lower curves) with valine-C^{14} and in the other (upper curve) with AMP-C^{14} in the terminal position. After incubation at 21°C. the microsomes were reisolated centrifugally and their RNA and protein isolated and counted. [Data are from P. C. Zamecnik, *Harvey Lectures* **54**, 256 (1960).]

RNA amino acid was limiting, all of the labeled amino acid was transferred to protein.[96] This means that all of the sites on RNA occupied by amino acids are metabolically competent in the sense that they are able to participate in the reactions which bring about the transfer of their

amino acid to peptide linkage. The C^{14}-amino acid, in its transit to protein, did not pass through a free state since the addition of its C^{12}-homolog in excess had no effect on the transfer.[117]

A more careful examination of the *in vitro* system reveals that during the course of incubation the microsomal RNA acquires a small amount of attached amino acid which accumulates rapidly and remains at the same level during the period in which the amino acid is accumulating in protein. This confirms the *in vivo* observation mentioned above. That this amino acid accumulation in microsomal RNA is significant is supported by the finding that it is stimulated by GTP.[96, 184]

We are prompted next to inquire: does the transfer RNA molecule accompany the amino acid into the ribosome? This question could be answered by labeling different parts of the transfer RNA molecule and studying the appearance of label in the RNA subsequently sedimentable as ribosomal RNA. Presumably, however, once the transfer RNA has completed its mission of giving up its amino acid to protein in the ribosome it would return again to the soluble milieu. Thus, one might expect to find a steady state of labeling of ribosomal RNA by transfer RNA in parallel with the behavior of the amino acid. Preliminary studies are encouraging in that they indicate that sRNA labeled by using orotic acid-C^{14} as a precursor, does in fact become incorporated into microsomal RNA.[14, 184] This incorporation is increased twofold or more by GTP, an essential criterion for assessing the significance of such a labeling. In more critical time studies, as Fig. 6 shows, sRNA labeled in its terminal adenine moiety rapidly reaches a steady state on the microsomal RNA, as did the amino acid, and this is again GTP dependent.[96] On a molar basis, approximately 12 times as much terminal adenine enters the microsomal RNA as does a single amino acid valine (Fig. 6). This ratio agrees well with the ratio of total terminal adenine to valine in the original sRNA used and indicates that all of the adenosine ends, with their attached unlabeled amino acids, are accompanying the valine-C^{14} into the microsomal RNA.

What may prove to be the *in vivo* counterpart of this event has been demonstrated by Lacks and Gros.[124] They have shown that the exposure of whole *E. coli* cells to adenine-C^{14} results in a rapid equilibration of this base with the sRNA. The subsequent addition of nonradioactive adenine results in a decrease in the specific radioactivity of sRNA but a continued increase in radioactivity of particulate RNA. Of course, in this case, it is hard to distinguish phenomena related to RNA synthesis from those related to the terminal group of sRNA.

It will be most interesting to examine the fate of the other parts of the transfer molecule: in particular, the whole terminal pCpCpA grouping

[184] M. B. Hoagland, *Rec. trav. chim.* **77**, 623 (1958).

and the remaining polynucleotide exclusive of the terminal group. One, thus far, obtains the tentative picture that at least part of and perhaps all of the molecule enters the microsome with its attached amino acid. This is consistent with the theoretical considerations dealt with at the end of this chapter.

Let us now consider the other components required in these transfer reactions. The continued requirement for ATP in the absence of a need to activate the amino acids is still unexplained. It could be argued that since the system is crude and is not entirely free of activating enzymes it is necessary to add ATP to prevent, by mass action, the reversal of the reaction by which the RNA amino acid was originally formed. This possibility seems unlikely since C^{12}-amino acid does not interfere with transfer. Hokin and Hokin[102] have obtained evidence that the transport of protein across the microsomal lipoprotein membrane may be an ATP dependent reaction. This might also be examined as an explanation of the ATP requirement. Thus far, however, it remains a mystery.

Even more puzzling is the system's requirement for GTP. This nucleotide was found early by Keller and Zamecnik,[185] to be an obligatory component of the over-all incorporation system. It was shown neither to be involved in the activation of amino acids[113] nor in the attachment of amino acids to sRNA.[117] It is, however, clearly a requirement in the rat liver and ascites tumor sRNA amino acid to protein transfer systems, as we have seen. The nucleotide does appear to be involved directly in the transfer reaction itself and not in some later step, for it has been found to be a requirement in the transfer to isolated ribosomes, free of membrane component.[35] An interesting sidelight on the GTP requirement is the finding of Chantrenne[186] that azaguanine is an effective inhibitor of protein synthesis under conditions where there is no striking effect on over-all RNA metabolism. Chantrenne suggests the possibility that such action may be concerned with a step in the protein-synthesizing process involving GTP. At any rate, the GTP requirement of the system remains one of its most beguiling mysteries!

The soluble enzymic component—the S_4 fraction—of the sRNA amino acid to the protein transfer system is still another mystery. As we have said, this is the supernatant fluid obtained after a pH 5 precipitation of a 100,000 *g* supernatant fraction of an ascites tumor cell lysate.[122] This fraction contains amino acid activating enzymes (the bulk of which are precipitated at pH 5), the terminal nucleotide addition system, and relatively little RNA. Whether these known enzymic components are the explanation for its requirement in the amino acid transfer to protein, or

[185] E. B. Keller and P. C. Zamecnik, *J. Biol. Chem.* **221,** 45 (1956).
[186] H. Chantrenne, *Rec. trav. chim.* **77,** 586 (1958).

whether some specific transfer enzyme may reside in this fraction is unknown at present.

Acs[187] has performed an experiment which sheds light on a hitherto puzzling aspect of amino acid incorporation studies. As was mentioned earlier, transfer RNA in the natural state contains amino acids; it is, in essence, a pool of activated amino acids. These workers showed that if this natural complement of amino acids is first stripped from the RNA (by treatment in dilute alkali) and then this RNA is labeled with a single amino acid (threonine-C^{14}), the resulting compound is less effective in donating its amino acid to microsomal protein. This would appear to mean that the other unlabeled amino acids naturally bound to transfer RNA are required for the incorporation of the single labeled amino acid, and most likely are incorporated into protein with it. This conclusion would be supported by the finding described in Section II, 2, *d*, that all of the transfer RNA ends accompany a single amino acid into the ribosomes during transfer. Amino acid incorporation studies have long embarrassed their practitioners by failing to show a requirement for other free amino acids in the incorporation of a single radioactive one. The explanation may be that there is a sufficient pool of transfer RNA-bound amino acids to account for the small quantity of C^{14}-amino acid which enters protein in these *in vitro* experiments.

An encouraging advance in the study of protein synthesis has been reported by Webster,[111] as was mentioned in Section II, 1, *c*. He reports that ribonucleoprotein particles from pea seedlings similar to those characterized by Ts'o *et al.*[46] will carry out the net synthesis of considerable quantities of soluble protein when supplemented with Mn^{++}, ATP, GTP, a nucleoside triphosphate generating system, and a full complement of amino acids. Omission of any one of these components prevents synthesis. The particles possess indigenous amino acid activating activity. The system is said further to require the addition of a "polynucleotide" fraction, as yet not fully characterized. The unequivocal demonstration that transfer RNA is an obligatory requirement of such a highly active protein synthetic system will go far toward removing any lingering doubts about its role.

This section cannot be concluded without reference to a number of investigations which are claimed not to support the role of transfer RNA in protein synthesis.

Beljanski and Ochoa[188-190] have isolated and purified from *Alcaligenes*

[187] G. Acs, unpublished data, (1959).

[188] M. Beljanski and S. Ochoa, *Proc. Natl. Acad. Sci.* **44**, 494 (1958).

[189] M. Beljanski and S. Ochoa, *Proc. Natl. Acad. Sci.* **44**, 1157 (1958).

[190] M. Beljanski, *Compt. rend. acad. sci.* **248**, 1446 (1959).

faecalis cells an enzyme, free of amino acid activation activity and apparently of sRNA, which will stimulate the incorporation of amino acids into "washed" rat liver microsomes in the presence of ATP. It is as effective as the pH 5 fraction in this respect. The only other catalytic activity of the preparation is an incorporation of all four C^{14}-ribonucleoside diphosphates into the corresponding triphosphates, an activity which parallels the amino acid incorporating activity during purification.

An enzyme preparation derived from the soluble protein fraction of rat liver has been found by Sachs[191] to stimulate incorporation of amino acids into liver microsomal protein. This enzyme ("S-protein") differs from the pH 5 enzyme fraction, or whole supernatant fraction, in that it requires glutathione for activity, and is assumed to be free of sRNA. Cohn[62] has described a method for preparing microsomal particles by use of the non-ionic detergent "Lubrol W." These particles are said to be capable of incorporating amino acids into their proteins almost as well in the absence of soluble enzymes as in their presence.

Rendi and Hultin[63] have combined the use of the "S-protein" of Sachs and Lubrol W treated particles of Cohn (after a further extraction of the latter with KCl to remove indigenous activating enzymes) to obtain an active incorporation system which appears to have no sRNA component. (In neither the Sachs, nor the Rendi and Hultin experiments was the RNA content of the soluble fraction measured, however.) The system is, nevertheless, dependent on both of these enzymic components as well as ATP, GTP, and glutathione. These authors conclude that incorporation may occur via pathways not involving sRNA-amino acid intermediates.

It may prove to be that there are, in fact, alternate pathways of protein synthesis. Certainly the results on studies of protein synthesis in other cell fractions point to this possibility. However, this conclusion is not justified from the data presented in any of the above experiments. These experiments may, in fact, be taken as examples of the principle that the failure to demonstrate the requirement for an intermediate does not establish its inessentiality. Microsomes and particles derived therefrom are grossly "contaminated" with activating enzymes, and presumable transfer RNA and other tissue components may also be present. Experiments of Stephenson in our laboratory have shown that extremely small quantities of sRNA (50 μg.) are required for the transfer of activated amino acids to relatively large quantities of microsomal protein (8 mg.).[96] Each system undoubtedly has a different limiting component; if this is sRNA-amino acid, as in the experiments which use it as the only source for amino acid in protein, then its essentiality is apparent. If, on the other hand, the limiting factor is an enzyme required, for example, in the trans-

[191] H. Sachs, *J. Biol. Chem.* **228**, 23 (1957).

fer of amino acid from sRNA to protein, then this enzyme will be found to be the essential missing link. The "S-protein" of Sachs and the incorporation enzyme of Beljanski and Ochoa may be such an enzyme which has been fortuitously separated from the other components of the system. Indeed, in none of the experiments quoted were these enzymes tested in addition to sRNA and activating enzymes. Only the advent of a vigorous protein synthesizing system, each of whose components may be clearly separated one from the other, will give us the final answer to these questions.

3. Note on the Occurrence of Certain Nucleotide-Peptide Compounds

During the past few years a variety of amino acid or peptide-containing nucleotide compounds have been described in the literature. Reith[192] has found in ascites cells an aspartic acid-uridylic acid compound. Hansen and Hageman have found in chicken liver a compound tentatively identified as adenosine diphosphoglutamic acid.[193] Hase *et al.*[194] have found in yeast and chlorella cells peptides containing several amino acids chemically associated with oligonucleotides containing adenine, uridine, and cytidine. Brown[195] has reported the occurrence of peptides containing several amino acids associated with AMP in *S. faecalis*. Habermann,[196] and Keil and Hrubešová[197] have found that RNA prepared by the phenol method from yeast, ascites tumor cells, mouse liver, and brain contain strongly bound peptides released by ribonuclease. They refer to other instances of occurrence of such peptides. In most of these cases the acidic amino acids are prominent, and the bond between peptide and nucleotide is relatively alkali-resistant. In none has the nature of the linkage between amino acids and nucleotides been identified. A different situation has been reported by Koningsberger *et al.*[198] who have found carboxyl activated amino acid-oligonucleotide compounds in extracts of baker's yeast. The bond between nucleotide and amino acid has the lability characteristics of transfer RNA-amino acid compounds but the material is dialyzable. The relationship of such compounds to those apparently involved as intermediates in cell wall synthesis[199-201] is not known.

[192] W. S. Rieth, *Nature* **178**, 1393 (1956).
[193] R. G. Hansen and E. Hageman, *Arch. Biochem. Biophys.* **62**, 511 (1956).
[194] E. Hase, S. Mihara, H. Otsuka, and H. Tamiya, *Biochim. et Biophys. Acta* **32**, 298 (1959).
[195] A. D. Brown, *Biochem. J.* **71**, 5P (1959).
[196] V. Habermann, *Biochim. et Biophys. Acta* **32**, 297 (1959).
[197] B. Keil and M. Hrubešová, *Chem. listy* **49**, 274 (1955).
[198] V. V. Koningsberger, C. O. Van der Grinten, and J. T. G. Overbeek, *Biochim. et Biophys. Acta* **26**, 483 (1957).
[199] J. Mandelstam and H. J. Rogers, *Nature* **181**, 956 (1958).
[200] R. Hancock and J. T. Park, *Nature* **181**, 1050 (1958).

4. The Mitochondria and Chloroplasts

A number of investigations have made it clear that the ribosome in its usual anatomical situation is not the only cellular site of protein synthesis. *In vivo* and *in vitro* studies have shown that nuclei, cell membrane material in bacteria, plant chloroplasts, and plant and animal mitochondria are all capable of incorporating amino acids into their proteins. We shall briefly consider these possible other sites of protein synthesis insofar as they shed light on RNA-protein synthesis relations.

In none of these experiments has the evidence for autonomous protein synthesis been so convincingly marshaled as in the studies of Simpson and co-workers.[29, 202] These workers have shown that calf heart mitochondria are capable of net synthesis of an intrinsic protein–cytochrome c—when an energy source and an amino acid mixture are supplied. They have established that there is a net increase in new enzyme protein gravimetrically, by isotope dilution technique and by isolating peptide fragments of the protein into which a C^{14}-amino acid has become incorporated. (It is of interest in respect to this latter study that valine-C^{14} differs in specific activities in two peptides derived from different parts of cytochrome c.) Pertinent to our subject is the finding that an extract of sonically disrupted mitochondria is still capable of incorporating labeled amino acids into cytochrome c, and this reaction is sensitive to ribonuclease.[203] The extract contains RNA and it will be of great interest to follow developments in this story to see whether reaction pathways similar to those found in the transfer RNA-ribosome system are found.

Greengard and Campbell[204] have studied the effect of added soluble components on incorporation reactions in mitochondria. They have found, in agreement with an earlier report of McLean *et al.*,[205] that ribonuclease had no effect on amino acid incorporation in intact mitochondria. The addition of "pH 5 fraction" (containing transfer RNA) to mitochondria does produce some stimulation of incorporation which is ribonuclease sensitive. This suggests the possibility that transfer RNA might be diffusible into the mitochondria and participate in the synthetic reaction.

Although it is known that chloroplasts contain RNA[12] and that they incorporate amino acids into protein,[206] little is yet known about the chemical events involved.

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[201] J. T. Park and J. L. Strominger, *Science* **125**, 99 (1957).

[202] H. M. Bates and M. V. Simpson, *Biochim. et Biophys. Acta* **32**, 597 (1959).

[203] M. V. Simpson, unpublished data, (1959).

[204] O. Greengard and P. N. Campbell, *Biochem. J.* **72**, 305 (1959).

[205] J. R. McLean, G. L. Cohn, I. K. Brandt, and M. V. Simpson, *J. Biol. Chem.* **233**, 657 (1958).

[206] M. L. Stephenson, K. V. Thimann, and P. C. Zamecnik, *Arch. Biochem. Biophys.* **65**, 194 (1956).

5. The Nuclei

The most extensive studies on protein synthesis in isolated calf thymus cell nuclei have been carried out by Allfrey, Mirsky, and Osawa.[207] Nuclei are generally difficult to isolate uncontaminated with other cell constituents and whole cells, and in thymus cells in particular they fill a large fraction of the total cell volume. It would appear to be uncertain that such preparations contain only nuclei. These workers have found that such preparations, which microscopically appear to be nuclei only, carry on an active energy-dependent incorporation of C^{14}-amino acids into protein and adenine-C^{14} into RNA. Treatment of these nuclei with deoxyribonuclease, which removes 70–80% of the DNA, completely inactivates these incorporation abilities. Ribonuclease, on the other hand, has no such effect, although it was not possible to remove more than half of the RNA. Logan *et al.*[208] have confirmed these findings using other techniques and have found similar effects using rat liver nuclei.

Another activity of thymus nuclei is the ability to carry out the oxidative synthesis of ATP.[209] Also, this activity is lost upon deoxyribonuclease treatment. A surprising finding, however, is that although the addition of DNA to deoxyribonuclease-treated nuclei can restore all three of these lost activities, this capacity of DNA is not specific. DNA's from other sources, RNA, polyadenylic acid, and non-polynucleotides such as heparin, polyethylene sulfonate, and chondroitin sulfate were all able to restore activity. No explanation of this phenomenon is immediately apparent. If it were possible to follow the synthesis of specific proteins in this system it would be intriguing to determine whether the specificity of the process was altered in the presence of these polyanions. For further consideration of the synthesis of protein in nuclei and of the relationship of DNA thereto, the reader is referred to the recent review by Chantrenne.[8] Further elucidation of protein and RNA synthesis in nuclei is much to be desired since it should shed much light on the role of DNA in directing the synthesis of specific templates which will in turn carry out the extranuclear (ribosomal) synthesis of protein.

6. Bacterial Cell Membranes

Cell-free bacterial preparations should be an excellent source of information about the mechanism of protein synthesis. Not only are bacteria homogeneous in cell type, but they make protein vigorously and offer an ideal system to observe the events which occur when a cell population un-

[207] V. G. Allfrey, A. E. Mirsky, and S. Osawa, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 200. Johns Hopkins Press, Baltimore, 1957.
[208] R. Logan, A. Ficq, and M. Errara, *Biochim. et Biophys. Acta* **31**, 402 (1959).
[209] V. G. Allfrey and A. E. Mirsky, *Proc. Natl. Acad. Sci. U. S.* **44**, 981 (1958).

dertakes synthesis of a new protein (adaptive enzyme formation). Thus far, however, it has been found difficult to obtain active cell-free preparations which can be dissected as extensively as has been possible with the mammalian systems. The experimental problem is complicated further by the danger of contamination of "subcellular" fractions by whole bacteria. This danger is potentiated by the fact that thus far, the most active protein synthesizing preparations from bacteria have been associated with cell membranes which sediment at forces close to those which sediment whole bacterial cells. Nor is it enough to reduce whole-cell contamination of fractions to numbers which by themselves will give negligible synthesis, for whole cells may function more efficiently in the presence of the particular cell fraction one is examining by virtue of being exposed to an ideal culture medium.[210]

Earlier studies, notably by Gale and Folkes, and by Spiegelman (cf. review by Spiegelman[4]) showed that various sonically disrupted or osmotically lysed preparations of bacterial cells or protoplasts were capable of protein synthesis, and that removal of a large part of the RNA of the preparations by ribonuclease led to a cessation of this synthesis. The activity could be restored by RNA or, where RNA synthesis was possible, by appropriate RNA precursors. Similar relationships were shown for DNA *vis-à-vis* protein synthesis by these workers. The extent to which these results were influenced by whole cells in the preparation is not clear.

Gale[21] has obtained evidence that in sonically disrupted staphylococcal preparations under conditions where protein synthesis is inhibited by chloramphenicol, or by withholding other amino acids essential for protein synthesis, C^{14}-amino acids accumulate on a material tentatively identified as polynucleotide in nature. Release of the inhibition results in the disappearance of the amino acids from this fraction and their appearance in protein. This result resembles that of Gros, quoted above, in whole *E. coli* cells, and fits agreeably with the general picture of protein synthesis in mammalian systems developed in the earlier sections of this chapter.

Attempts by several workers to fractionate various disrupted bacterial cell preparations[100, 188, 189, 211-213] have led to one tentative generalization: that unequivocal protein synthesis (requiring all amino acids and resulting in enzyme increase as opposed to simple amino acid incorporation) seems to be more closely associated with the fragmented cell membrane material than with the ribosomes or other less easily sedimentable fractions of the cell.

[210] P. Rogers and G. D. Novelli, *Federation Proc.* **18,** 1232 (1959).
[211] B. Nisman, *Biochim. et Biophys. Acta* **32,** 18 (1959).
[212] P. Brookes, A. R. Crathorn, and G. D. Hunter, *Biochem. J.* **71,** 31P (1959).
[213] G. E. Connell, P. Lengyel, and R. C. Warner, *Biochim. et Biophys. Acta* **31,** 391 (1959).

Nevertheless, using the techniques and conditions found to be effective in evolving active mammalian incorporation systems, Lamborg and Zamecnik[214] have obtained active incorporation of C^{14}-amino acids into protein which is clearly dependent on ribosomes, soluble enzymes, ATP, GTP, and an amino acid mixture. Thus it is possible to obtain a system from bacteria which has all the properties of the more clearly defined mammalian systems, and it may be that further study of such systems will clarify the apparent discrepancies.

It has been stressed earlier that bacteria possess amino acid activating enzymes, transfer RNA, and ribosomes in all respects very similar to mammalian cells. A question which remains unanswered, therefore, is the extent to which this membrane material contains bound particles. The material certainly contains RNA and it is possible that only those ribonucleoprotein particles physiologically associated with the cell membranes are capable of completing the protein synthetic sequence. One is reminded that in mammalian cells the particles are frequently associated with the membranous component of the endoplasmic reticulum and the most active incorporation occurs in microsomes which are, in essence, particles with attached membranes. There is growing evidence that the membranous component is a saclike storage compartment for protein synthesized in particles and that the removal of the protein from the particle into this compartment is an active, energy-requiring process. Thus, it might be that in bacteria the cell membrane serves in a capacity similar to the membrane of the endoplasmic reticulum. The disruption of the bacterial cell may bring about an irreversible separation of particles from the cell membrane with resultant loss of apparent synthetic ability; the particles do incorporate amino acids but a net increase in protein cannot be observed because the finished protein molecules cannot be removed from the site of synthesis. Be that as it may, we shall expect that further work on the bacterial systems will do much to shed light on the general mechanism.

III. Theoretical Considerations

1. The Role of Ribonucleic Acid

It can no longer be doubted that ribonucleic acid is intimately involved in the protein synthetic mechanism. Although much of our information is derived from systems of limited synthetic capacity, we have seen that peptide-bond condensation occurs in ribonucleoprotein particles and that amino acids are brought to these particles in an activated form by virtue of having been first attached to ribonucleic acid molecules. The essential riddle which the newer knowledge poses for us is: why are two distinct kinds of ribonu-

[214] M. Lamborg and P. C. Zamecnik, *Biochim. et Biophys. Acta*, in press.

cleic acids involved in protein synthesis, and how does deoxyribonucleic acid exert its ultimate control when it does not participate in the act of synthesis itself? In this section we shall briefly consider the first part of this problem. An able discussion of theoretical aspects of protein synthesis in the light of our newer knowledge has recently been presented by Crick.[6] The author and his associates have also presented a brief discussion of these matters.[215] Implicit or explicit in these statements are the following working assumptions: (1) the essential task before us is to understand how the primary structure of protein is determined, i.e., how the amino acids are arranged in a specific sequence. The determination of secondary and tertiary structure may be either an active process or a spontaneous result of the order of amino acids. In either case it does not alter the argument since the genetic specificity must reside in the amino acid sequence. (2) We assume that the sequence is determined by a template: that some cellular polymer contains information in linear order which may be translated directly into a linear sequence of amino acids. Arguments in favor of template mechanisms have been reviewed recently by Dounce,[216, 217] Spiegelman,[4] Dalgliesh,[9] and Crick[6] and will not be presented here. (3) We assume that the template is, in fact, some form of ribonucleic acid and that the linear order of its four bases is somehow directly translatable into the linear order of twenty amino acids in the protein. The mathematical arguments behind the use of four symbols (bases) to code for twenty amino acids have been set forth in detail by Gamow *et al.*,[218] Brenner,[219] Crick and associates,[6, 220] and by Delbrück and associates.[221] We are concerned here, not with the manner of coding but with the biological mechanism of information transfer, i.e., how the cell makes use of its coded genetic information. (4) We assume that the linear sequence of bases in RNA must be fairly directly related to the linear sequence of bases in DNA—the ultimate repository of the cell's total hereditary constitution. In respect to this latter point, the elegant studies of Benzer[222] and of Jacob[223] have established the linearity

[215] M. B. Hoagland, P. C. Zamecnik, and M. L. Stephenson, *in* "A Symposium on Molecular Biology" (R. E. Zirkle, ed.), p. 105. Univ. of Chicago Press, Chicago, Ill., 1959.

[216] A. L. Dounce, *Enzymologia* **15**, 251 (1952).

[217] A. L. Dounce, *J. Cellular Comp. Physiol.* **47**, Suppl. 1, p. 103 (1956).

[218] G. Gamow, A. Rich, and M. Yčas, *Advances in Biol. and Med. Phys.* **4**, 23 (1955).

[219] S. Brenner, *Proc. Natl. Acad. Sci. U. S.* **43**, 687 (1957).

[220] F. H. C. Crick, J. S. Griffith, and L. E. Orgel, *Proc. Natl. Acad. Sci. U. S.* **43**, 416 (1957).

[221] S. W. Golomb, L. R. Welch, and M. Delbrück, *Kgl. Danske Videnskat. Selskab, Biol. Medd.* **23**, (9) (1958).

[222] S. Benzer, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 70. Johns Hopkins Press, Baltimore, 1957.

[223] F. Jacob and E. L. Wollman, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 468. Johns Hopkins Press, Baltimore, 1957.

of information in DNA, and the work of Ingram[224] has shown us that a change in genetic constitution results in corresponding small changes in amino acid sequence. The autonomy of DNA with respect to its ability to induce specific changes in genetic constitution of organisms (i.e., their enzyme content) has been amply and elegantly shown by Hershey,[225] by Avery *et al.*,[22] and by Hotchkiss.[23]

Our assumptions are then, that information passes in only one direction in cells: from DNA to RNA to protein; and that the information resides in the linear arrangement of the monomer units of each of these polymers. (Crick has referred to these concepts as the "Central Dogma" and the "Sequence Hypothesis" respectively.[6])

Theoretical contributions to our understanding of chemical mechanisms of protein synthesis, based on modern concepts of energy-transfer mechanisms have been made in particular by Lipmann,[78] Chantrenne,[226, 227] Dounce,[217] and Koningsberger.[228] All of these hypotheses in common envisioned a direct reaction between amino acid and template: either by amino acid acylation or amination of nucleic acid phosphate groups, the positioning of the amino acid being somehow due to noncovalent interaction between an amino acid R-group and template surface (cf. also Loftfield[7]). There was chemical precedent for the reactions suggested but the mechanism by which the amino acids were directed into the proper order remained vague.

As the result of the discovery of the role of transfer RNA in protein synthesis, the author and his associates suggested[14, 215] that it might react with the ribosomes by hydrogen bonding between its bases and complementary bases on ribosomal RNA, thereby affording a specific mechanism for locating the amino acids. This is depicted schematically in Fig. 7. Such a concept had been arrived at independently by Crick on theoretical grounds. Crick stated:

"I cannot conceive of *any* structure (RNA or DNA) acting as a direct template for amino acids, or at least as a specific template. In other words, if one considers the physico-chemical nature of the amino acid side chains we do not find complimentary features on the nucleic acid. Where are the knobby hydrophobic surfaces to distinguish valine from leucine and isoleucine? Where are the charged groups, in specific positions, to go with the acidic and basic amino acids? It is true that a 'Teller' scheme, in which the amino acids already condensed act effectively as part of the template, might be a little easier, but a study of sequences from this point of view is not encouraging.

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[224] V. M. Ingram, *Nature* **180**, 326 (1957).
[225] A. D. Hershey, *Advances in Virus Research* **4**, 25 (1957).
[226] H. Chantrenne, *Biochim. et Biophys. Acta* **2**, 286 (1948).
[227] H. Chantrenne, *Pubbl. staz. zool. Napoli* **23**, Suppl. 70 (1951).
[228] V. V. Koningsberger, Thesis, University of Utrecht (1955).

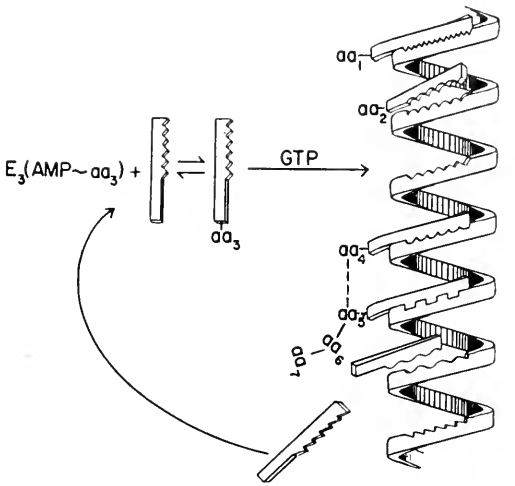

FIG. 7. A schematic representation of the "adaptor hypothesis." [From M. B. Hoagland, P. C. Zamecnik, and M. L. Stephenson, *in* "A Symposium on Molecular Biology" (R. E. Zirkle, ed.), p. 105. Univ. of Chicago Press, Chicago, Ill., 1959.] The contours of the transfer RNA molecules represent a specific base sequence, for each molecule (upper part) and a common terminal nucleotide sequence (lower part) to which the specific amino acids (aa_1–aa_7) are attached. Ribosomal RNA is depicted as a helix but this is only for convenience of exposition. The complementary nature of the interaction of transfer RNA and ribosomal RNA is suggested by the complementarity of their contours.

"I don't think that anybody looking at DNA or RNA would think of them as templates for amino acids were it not for other indirect evidence.

"What the DNA structure *does* show (and probably RNA will do the same) is a specific pattern of *hydrogen bonds*, and very little else. It seems to me, therefore, that we should widen our thinking to embrace this obvious fact." . . . "Each amino acid would combine chemically, at a special enzyme, with a small molecule which, having a specific hydrogen-bonding surface, would combine specifically with the nucleic acid template. This combination would also supply the energy necessary for polymerization. In its simplest form there would be 20 different kinds of adaptor molecule, one for each amino acid, and 20 different enzymes to join the amino acid to their adaptors. Sydney Brenner, with whom I have discussed this idea, calls this the 'adaptor hypothesis,' since each amino acid is fitted with an adaptor to go on to the template."[229]

These thoughts had value in focusing attention on the problem of the specificity of sequence determination. However, we now know that it is not the free amino acid which would in all probability be required to find its place on the template, but the amino acyl adenylate enzyme complex. This complex might well be expected to be much better able to "recognize" the correct sequence of bases on the template. We suspect that it can recognize

[229] F. H. C. Crick, "A Note for the RNA Tie Club," 1955.

the correct sequence of bases on transfer RNA for it is able to deposit its amino acid on only one kind of transfer RNA molecule. Thus the *a priori* argument for the adaptor hypothesis loses some of its impact. But an important feature of an adaptor would be its ability chemically to bind an amino acid in such a way as to maintain it in a reasonably stable, and yet a still activated state. The most likely candidate for a molecule with "a specific hydrogen-bonding surface," the adaptor, would of course, as Crick suggested, be one containing nucleotides.[230] An oligoribonucleotide itself would serve ideally as such an adaptor, having both acylatable groups for amino acid attachment, and at the same time a specific pattern of hydrogen bonds (a specific base sequence) which could react directly with a complementary sequence on the RNA template. Such base pairing would be in all respects analogous to that found in DNA and to that most likely existing in RNA. Such hydrogen-bonding interaction between bases in a polynucleotide would have the specificity demanded by what is known thus far about protein structure.

The adaptor hypothesis may be stated explicitly as follows: "Amino acids, before entering the ribonucleoprotein particles first react with small polynucleotide molecules. These adaptor molecules accompany the amino acids into the particles and are responsible for properly locating them on the particle RNA. This is accomplished by pairing of the adaptors' bases with complementary base sequences on the particle RNA. Having completed their mission the adaptors then return to the soluble milieu.[231]"

This new concept has important qualitative differences from earlier suggested mechanisms of amino acid-template interaction. It predicts: (1) that each amino acid must initially be attached to an oligonucleotide, and each of these oligonucleotides will be specific for a particular amino acid. (2) As a consequence of this first prediction, separate enzymes would be necessary to attach each amino acid to its specific adaptor. One could hardly visualize a single enzyme being capable of distinguishing between twenty different oligonucleotides. (3) It predicts that the amino acid would arrive at the template in company with this nucleotide component. This means that the amino acid would be found transiently associated with ribosomal RNA before it appears in protein. (4) It implies that the amino acid need never have any direct chemical contact with the template—that the adaptor alone makes the contact with the specific site on the template. The amino acid might of course then be transferred to the template itself but this step is not a requirement of the theory. At any rate the mechanism by which the amino acid is brought to the proper locus on the template does not require that the template ever "see" the amino acid. (5) The theory

[230] F. H. C. Crick, *Biochem. Soc. Symposia* **14,** 25 (1957).
[231] M. B. Hoagland, *Brookhaven Symposia in Biol.* **12,** 40 (1959).

implicitly states that the adaptor molecules, and the enzymic reactions catalyzing attachment of amino acids thereto, need have no species specificity; they could be common to all organisms having the same twenty amino acids to deal with. Indeed, the reactions by which the amino acids are attached to the adaptors and those by which the amino acid adaptors are conveyed to the template need not differ from species to species. Only in the template itself—in the ordering of the specific hydrogen bonding, adaptor-reacting sites—would the genetic constitution of the organism be manifest. (6) Finally, the theory states that the association of adaptor and ribosome is transitory; the adaptor constantly cycling through the ribosome bearing its charge of amino acid.

It will already be clear that the discovery that two kinds of RNA are involved in protein synthesis made necessary a revision of conventional concepts and found the adaptor hypothesis a valuable hypothetical framework. Let us look at the data in the light of the concept.

It seems highly probable that the RNA of the ribosomes is the cytoplasmic template for protein synthesis. *A priori*, one would expect that a template might have to be particulate, thus permitting a reasonably rigid spatial arrangement of the RNA. Experimentally, we have seen that the ribosome is the chief site of peptide bond condensation. There are no discernible stages in the synthetic process prior to the admission of the amino acids to the particles in which they could be arranged in sequence: i.e., peptides do not appear to be formed as intermediates in protein synthesis (cf. Loftfield[7]).

We have seen that amino acids are first activated by formation of amino acyl adenylate compounds. Nature could conceivably have arranged that this step be catalyzed by a single species of enzyme but we find that a specific enzyme is required for each amino acid. An explanation of this becomes clear when we observe that the same enzyme which activates the amino acid must also transfer it to a specific RNA molecule. Thus twenty amino acids use twenty enzymes to convey them to twenty specific RNA molecules.

The versatility of the amino acid activating enzymes is impressive. They have in common a site able to recognize ATP, each one has a specific site for an amino acid R-group, and each is further able precisely to discern differences in base sequence of twenty closely related RNA molecules; this base sequence in each case being removed, by at least three nucleotides, from the site at which the enzyme deposits its amino acid.

We have seen that the transfer RNA is uniquely able to serve as the source of new amino acid appearing in protein in the particles. We have seen further that during the course of this reaction the RNA itself, or at least part of it, accompanies the amino acid into the particle where it be-

comes indistinguishable from particle RNA in sedimentation properties. Thus, it must become bound to microsomal RNA in some reasonably firm way. Further, it was clear that the amino acid also became associated with particle RNA, and the kinetics of this reaction were similar to the kinetics of attachment of the transfer RNA to particles.

These observations are consistent with the adaptor hypothesis in most respects. We should expect to find that amino acid bound to soluble adaptor would appear at least in a transitory way on particle RNA in company with the adaptor. This intermediate state would be the template or particle RNA hydrogen bonded, by complementary base pairing, to the adaptor RNA, the amino acid still being attached to the latter. This stage would be transitory, for the amino acid would condense with its neighbors, be removed from the adaptor, and the latter would then be expected to return to the soluble milieu, thus completing a catalytic cycle. It should be said, however, that although the association of amino acid and transfer RNA (or part of it) with the particle RNA appears to be transitory and in a steady state situation, this has not yet been proved.

We have observed that there is apparently little species specificity in the reactions we have been discussing. Particles from one animal source are able to accept amino acids from transfer RNA derived from other species. This is consistent with the theory. We should predict, however, that the proteins synthesized would be those characteristic of the organism from which the particles had been derived, and this should soon be experimentally attackable. Indeed, Schweet has already shown that soluble enzymes from guinea pig assist rabbit reticulocyte ribosomes in the synthesis of hemoglobin.[108] Brachet[183] found that protein synthesis in whole onion root tips depleted of their sRNA by ribonuclease could be restored by adding back RNA from either yeast or onion root. However, there is, as we have mentioned, evidence[177] of species differences in the extent to which activating enzymes can attach amino acids to a given sample of sRNA. Thus the picture is still far from clear.

If the hypothesis is correct, we should expect that at least part of the particle RNA would be free to react with the adaptor, i.e., its hydrogen bonds should be unoccupied. As we have mentioned, Doty and his associates have obtained tentative evidence that a large fraction of the particle RNA is not involved in internal hydrogen bonding and could thus be available for reactions with adaptors.

Ts'o and Lubell[232] have made a calculation based on data from rabbit reticulocytes, to determine whether the frequency of collisions of transfer RNA molecules with ribosomes would be high enough to account for the rate at which these cells synthesize hemoglobin. They estimate that a vol-

[232] P. O. P. Ts'o and A. R. Lubell, *Abstr. Am. Chem. Soc., 135th Meeting, Boston* (1959).

ume of (830 Å^3) within a reticulocyte would contain twenty to thirty sRNA molecules and one ribosome capable of producing one peptide bond per second. Collision frequency estimates (based on an equation for coagulation of colloids, as well as the kinetic theory of gases and Brownian movement) lead to the conclusion that even if the collision efficiency were 0.1 % there would be enough collisions to account for the rate of synthesis.

Thus, we find that experimental observation is in good agreement with the broad idea of an adaptor serving as an intermediate. However, there are two matters which cause some perplexity. One is the fact that transfer RNA is a larger molecule, by a factor of at least 10, than one would require on the basis of current coding concepts. Perhaps these concepts are totally wrong. It certainly seems difficult to conceive, however, of a reason why such a large molecule should accompany each amino acid into the particle. Perhaps a "coding piece" is split off the transfer RNA during the transfer reaction and only this piece accompanies the amino acid into the particle. The remainder of the molecule would only serve as a polymerized carrier for the end group. An answer to this question can come from quantitative studies on how much of the transfer RNA accompanies the amino acid into the particles. These studies are in their infancy as we have seen and do not give us a clear answer to this question. That acid soluble oligonucleotides (possibly corresponding to fragments of transfer RNA) are produced and consumed during the course of protein synthesis is suggested by interesting observations of Gobert (cf. Chantrenne[186]). It was found that immediately after maltase induction into resting yeast cells the acid soluble nucleotide pool rapidly increased, apparently by breakdown of existing RNA; and the increase was due chiefly to material tentatively identified as oligonucleotide in character. This was followed by a sharp fall in the pool as new enzymes were synthesized. Such events might be indicative of the release and subsequent consumption of coding fragments from transfer RNA. Another experimental approach to this problem would be to look for acid-soluble oligonucleotides with attached amino acids when transfer RNA-amino acids are incubated with microsomes in the absence of GTP or incubated in the presence of microsomes rendered incapable of incorporation for some other reason. Thus far, these experiments have shown that oligonucleotide-like material is in fact enzymically produced from sRNA by microsomes but it has not been possible to show that these fragments contain amino acids, are able to bind amino acids, or are able to be used by the ribosomes for protein synthesis.[233]

Perhaps the content of "pseudouridine" in sRNA has some significance in such reactions. It has been suggested, also, that the high content of unusual bases might be an indication that the noncoding end of the molecule

[233] M. B. Hoagland, unpublished data, (1960).

is simply "junk" RNA used to carry the coding end. Such RNA would, thus, not itself be required to have specific genetic structure and hence might be synthesized by nonspecific means from any available nucleotides.

A second problem in respect to the adaptor hypothesis is less easy to answer experimentally at this juncture. How does the hypothesis envision the arrangement of template RNA and adaptors such that amino acids would be brought into the proper contiguity for condensation with their neighbors? This question is mentioned only to emphasize the importance of learning more about the actual physical state of the RNA in the particles. Presumably a variety of methods could be used by nature to make the necessary spatial arrangements. Man has thus far tried few models and this will become possible as our knowledge of particle structure grows. The common pCpCpA end may also have some significance in the arrangement in giving the amino acid the proper "reach" to join its neighbor, as has been suggested by S. Brenner.

Is the adaptor hypothesis adequate to explain the known discriminatory precision of the protein synthetic process; can it, for example, account for the precision with which the system rejects the wrong amino acids? If an amino acid analog can be activated and attached to transfer RNA, it would be expected to become incorporated into protein because the hypothesis makes no provision for a further exclusion step after attachment to the RNA.

It has been shown by Sharon and Lipmann[234] that certain amino acid analogs are activated by amino acid activating enzymes and that in general those which can ultimately be incorporated into protein are found to be the more readily activated. Loftfield and his associates[235, 236] have examined this question more critically and have concluded that the exclusion of an analog from protein cannot entirely be explained by the discrimination of the activation step plus the attachment to transfer RNA. This is based on the finding that alloisoleucine competes with valine and isoleucine for sites on the corresponding activating enzymes and to a lesser extent for sites on transfer RNA. The analog is utilized for the over-all process of protein synthesis only $^1/_{2000}$ as well as the natural amino acids, however. This question will require further critical examination, particularly under conditions which simulate as closely as possible the *in vivo* situation.

However, the occurrence of reactions between RNA and amino acids for the first time permits a direct experimental attack on the coding problem. It is of fundamental importance to determine the minimal structural re-

[234] N. Sharon and F. Lipmann, *Arch. Biochem. Biophys.* **69,** 219 (1957).

[235] R. B. Loftfield, *Proc. 4th Intern. Congr. Biochem., Vienna, 1958* Symposium 8 (1958).

[236] R. B. Loftfield, L. I. Hecht, and E. A. Eigner, *Federation Proc.* **18,** 1090 (1959).

quirements of transfer RNA underlying the specificity with which it reacts with amino acids. Presumably the secret lies in the sequence of bases in some part of the molecule. With the refinement of techniques for fractionating RNA, and of techniques for sequential degradation of nucleic acids, we may hope to find the answer.

2. The Role of Deoxyribonucleic Acid

Cell-free systems have shed little light on the role of DNA in protein synthesis. Just as we can now be assured that RNA participates actively in protein synthesis, we can state with equal certainty that DNA must in some way ultimately control the process. The *in vitro* systems we have discussed and other experiments in whole cells (see particularly the review by Chantrenne[8] and Chapter 38 by Gros) make it clear that protein synthesis can proceed in the absence of DNA. It seems obvious, therefore, that DNA must somehow be able to convey a stable pattern of information to the site of protein synthesis. We have reviewed the evidence that the ribonucleoprotein particle is the site of protein synthesis and have expressed the view that RNA is the most likely candidate for the template. It, therefore, seems highly probable that DNA must be involved in the manufacture of this RNA template. This activity would most likely occur in the nucleus and as we have seen there is accumulating evidence that RNA and probably ribosomes are synthesized in the nucleus. DNA can replicate itself and it is not unreasonable to expect that it might also be able to produce a molecule of RNA, thus conferring to the latter a complementary copy of its base sequence.

We visualize particle RNA as relatively highly polymerized. The total molecular weight of the RNA of the 80 *S* particle may be about 2×10^6. However, we have seen that such particles can be dissociated reversibly into smaller units simply by lowering the Mg^{++} concentration and we are tempted to wonder what the true covalently continuous size of particle RNA subunits really is. This has relevance to the template theory since we conventionally picture genetic information residing in large molecules. If it is found that particle RNA consists of relatively small subunits, then they must be arranged in some kind of rigid structure by the protein of the particles. This would be all the more reason to suspect that the whole particle, not just the RNA, is synthesized in the nucleus.

The origin of transfer RNA then becomes an intriguing problem. Two possibilities might be considered. Perhaps once the ribosomal (template) RNA is synthesized, it in turn is able to synthesize strands of RNA complementary to segments of itself which, under the proper conditions, would be released as soluble RNA into the cytoplasm. Perhaps the indigenous ribonuclease activity of particles has some role in releasing such copies

from the particles. Such a mechanism would be a convenient way of deriving the exact proportion of transfer RNA molecules required to react with the amino acids required for the synthesis of the protein that particular ribosome is making. However, since transfer RNA alone appears to contain no sequence information, only information permitting recognition of amino acids, some other synthetic mechanism for producing the twenty or so transfer RNA molecules, could be used. It is clear, for example, that the enzymes which attach the terminal grouping of nucleotides to transfer RNA reside in the soluble phase and perhaps other enzymes can synthesize the proper nucleotide sequences specific for the amino acids. Or alternatively, such synthesis might occur in the nucleus.

However these matters may be, the understanding of the mechanism of synthesis of transfer RNA and ribosomal RNA is clearly of very basic importance to the unraveling of the cellular mechanism by which the genetic information in DNA is translated into the specific proteins of the living organism.

Acknowledgment

I should like to express my thanks to my colleagues Dr. Zamecnik, Dr. Stephenson, and Dr. Loftfield for helpful criticism during the preparation of this chapter. I am also in debt to many colleagues who permitted me to read their manuscripts before publication.

CHAPTER 38

Biosynthesis of Proteins in Intact Bacterial Cells

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I. Introduction

It might appear paradoxical to justify the use of intact cells as biological material. Is it not the main goal of biology to study the behavior of the cell?

Nevertheless in biochemical studies, at present, there is a growing tendency to fractionate cells into subcellular components or to extract from them soluble enzymes, in order to determine the systems catalyzing the biosynthesis of the macromolecules. Great progress has been made in this

direction; for instance, in the discovery of enzymes involved in the synthesis of polynucleotides[1, 2] or in activating amino acids.[3] This is well illustrated in Chapter 37 on protein synthesis in *in vitro* systems.

Now the time has come to wonder whether the enzymic reactions, the existence of which has been demonstrated *in vitro*, represent the mechanisms by which a cell actually synthesizes its essential constituents. As long as this question remains unanswered it would be wise to consider the *in vitro* synthesizing systems only as possible models for the cellular mechanisms of biosynthesis.

Thus, since *in vitro* studies on amino acid activation and the formation of RNA amino acid complexes implicate these reactions as the initial step in protein synthesis, a part of the present review will be concerned with the question of whether amino acid activation operates *in vivo* in a manner which makes it essential to the synthesis of protein by the cell. The review will also deal with those problems of protein and nucleic acid biosynthesis which have proved as yet to be too complex to be thoroughly studied in *in vitro* systems. For instance, the problem of the role of nucleic acids as the stable and specific templates for protein synthesis has been approached in most cases by comparing the capacity of the cell to synthesize macromolecules under various conditions. Consequently a discussion of this problem will be particularly pertinent.

For studies with intact cells, the bacterial cell is the one of choice: its growth rate is very high; biochemical mutants with a precise metabolic block can be easily selected; finally, enzyme induction which has proved to be an important tool for studying the synthesis of specific proteins has been thoroughly explored in bacteria.

II. Organization of the Bacterial Cell

Before considering mechanisms of protein biosynthesis, it is desirable to survey briefly what is known about the chemical and cytochemical structure of the bacterial cell in the hope of discerning what cellular elements are the important parameters in protein or nucleic acid synthesis.

1. The Pool of "Free" Metabolites

The cells of both prototrophic bacteria, which are able to synthesize all their constituents from single nitrogen and carbon sources, and of heterotrophic bacteria, always contain a pool of metabolites (amino acids, bases etc.) in the "free" form. The size of this pool varies considerably with the specific nutritional requirements of the strain and with the external concentration of the metabolites. The pool is larger

[1] M. Grunberg-Manago and S. Ochoa, *J. Am. Chem. Soc.* **77**, 3165 (1955).

[2] A. Kornberg, *in* "Chemical Basis of Heredity," p. 579. Johns Hopkins Press, Baltimore, 1957.

[3] M. B. Hoagland, *Biochim. et Biophys. Acta* **16**, 288 (1955).

in heterotrophs than in prototrophs such as *Escherichia coli*.[4, 5] The existence of an active transport mechanism for the metabolites is not *a priori* evident in bacteria, since the possibility exists of passive diffusion of organic salts and metabolites across the cell membrane. Gale's early studies with *Staphylococcus aureus*[6] showed that amino acid transport is energy dependent. From the work of Cohen, Rickenberg *et al.*[7, 8] and of Cohen and Monod with β-galactosides,[9] and that of Cohen *et al.*[10] and Britten *et al.*[11] with amino acids, it is evident that *E. coli* contains systems (permeases) for concentrating these metabolites several hundred-fold within the intracellular space. These systems also possess a high degree of specificity for the metabolites.[9-11] Ingenious experiments by Sistrom[12] have demonstrated that β-galactosides, after their uptake by the permease, exist in a free state inside the cell, and more precisely as shown by Rickenberg,[13] inside the cytoplasmic region limited by the cell membrane.

The ability of *E. coli* to actively concentrate a given amino acid can be dissociated from its ability to synthesize protein.[10, 11] Therefore, when amino acid incorporation into protein is measured, the first limiting factor is the system carrying out the specific concentration of this particular amino acid. For example, the valine permease in *E. coli* becomes saturated when the concentration of valine in the medium reaches 2×10^{-5} *M*.[10] If radioactive valine is present at lower concentrations, its rate of incorporation during growth would give a false measure of the absolute rate of protein synthesis. Similarly, when protein synthesis is measured by incorporation of a radioactive amino acid in a culture of *E. coli* growing on simple mineral medium, inhibition of amino acid transport by a drug or a competitive inhibitor would be falsely interpreted as an inhibition of protein synthesis.

Interest in the intracellular pool of metabolites is not restricted to the methodology for studying the rate of protein synthesis. Chemical analysis should lead, in principle, to the recognition of some intermediates in protein and nucleic acid synthesis (either during normal growth or after inhibiting selectively the formation of a macromolecular component to allow the corresponding intermediates to accumulate).

In fact, except for glutathione, free peptides are seldom found in large quantities in the bacterial pool. There are a few interesting exceptions: for instance, after treatment with one of several drugs (including penicillin) gram-positive bacteria accumulate considerable quantities of a mucopeptide.[14-16] However, this peptide (which contains alanine, glutamic acid, and lysine residues, and is bound to *N*-acetylhexosa-

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[4] E. F. Gale, *Symposia Soc. Exptl. Biol.* 242 (1954).

[5] J. Mandelstam, *Intern. Rev. Cytol.* **5**, 51 (1956).

[6] E. F., Gale, *J. Gen. Microbiol.* **1**, 53 (1947).

[7] G. N. Cohen and H. V. Rickenberg, *Compt. rend. acad. sci.* **240**, 466 (1955).

[8] H. V. Rickenberg, G. N. Cohen, G. Buttin, and J. Monod, *Ann. inst. Pasteur* **91**, 829 (1956).

[9] G. N. Cohen and J. Monod, *Bacteriol. Revs.* **21**(3), 169 (1957).

[10] G. N. Cohen and H. V. Rickenberg, *Compt. rend. acad. sci.* **240**, 2086 (1955).

[11] R. J. Britten, R. B. Roberts, and E. F. French, *Proc. Natl. Acad. Sci. U. S.* **41**, 863 (1955).

[12] W. R. Sistrom, *Biochim. et Biophys. Acta* **29**, 579 (1958).

[13] H. V. Rickenberg, *Biochim. et Biophys. Acta* **25**, 206 (1957).

[14] J. T. Park, *2nd Intern. Congr. Biochem., Paris* p. 31 (1952).

[15] J. L. Strominger, *J. Biol. Chem.* **224**, 509 (1957).

[16] J. L. Strominger and R. H. Threm, *Biochim. et Biophys. Acta* **33**, 280, (1959).

mine and to uridylic acid) is in fact a component of the cell wall, the synthesis of which is prevented by the inhibitor.

In regard to nucleic acid synthesis, the compounds related to ribonucleic acid (RNA) most frequently found are ribonucleoside triphosphates or ribonucleoside diphosphates[17, 18]; those related to deoxyribonucleic acid (DNA) are deoxynucleoside diphosphates, or deoxyribosides.[19] No oligonucleotide, of a size bigger than a dinucleotide and smaller than the soluble RNA (sRNA) has been found in the pool of any bacterium. Very recently, however, bacteria, fungi, and algae, have been shown to contain nucleotide derivatives of a new type such as mononucleotide-peptide complexes.[20-25] Their concentration in the cell is small and their role is unknown. They may possibly originate from an RNA peptide complex[26] and may be common precursors of protein and RNA,[27, 28] or products of amino acid activation by nucleoside triphosphates.

2. Amino Acid Activation Systems—Soluble RNA

Since the discovery of activating mechanisms for amino acids in animal cells[3, 29] and the isolation of RNA amino acid complexes[30] similar systems have been sought in intact bacteria. Adenylamino acids have never been found in the free state, a fact which is probably accounted for by the extreme affinity of such products for their specific enzymes.[29] However, the existence of amino acid-activating systems has been widely demonstrated in bacteria. Crude bacterial extracts can activate all the amino acids[31] in spite of previous reports to the contrary.[32] Furthermore, many activating

[17] R. B. Hurlbert, *in* "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. III, p. 785. Academic Press, New York, 1957.
[18] J. Baddiley and A. P. Mathias, *J. Chem. Soc.* p. 2733 (1954).
[19] E. Hoff-Jorgensen, *Biochem. J.* **50,** 400 (1951).
[20] V. V. Koningsberger, C. O. van der Grinten, and J. T. C. Overbeek, *Biochim. et Biophys. Acta* **26,** 483 (1957).
[21] J. H. Weil, G. Dirheimer, and J. P. Ebel, *4th Intern. Cong. Biochem., Vienna, Abstr.* p. 21 (1958).
[22] A. P. Brown, *Biochim. et Biophys. Acta* **30,** 447 (1958).
[23] G. Haris, J. W. Davies, and R. Parsons, *Nature* **182,** 1565 (1958).
[24] R. Bergkvist, *Acta Chem. Scand.* **12,** 364 (1958).
[25] E. Hase, S. Mihara, H. Otsuka, and H. Tamiya, *Biochim. et Biophys. Acta* **32,** 298 (1959).
[26] V. Habermann, *Biochim. et Biophys. Acta* **32,** 297 (1959).
[27] A. B. Pardee and L. S. Prestige, *J. Bacteriol.* **71,** 677 (1956).
[28] F. Gros and Françoise Gros, *Biochim. et Biophys. Acta,* **22,** 200, (1956).
[29] M. B. Hoagland, *4th Intern. Congr. Biochem., Vienna* Symposium No. 8, (1958).
[30] M. B. Hoagland, P. C. Zamecnik, and M. L. Stephenson, *Biochim. et Biophys. Acta* **24,** 215 (1957).
[31] B. Nisman, F. Bergman, and P. Berg, *Biochim. et Biophys. Acta* **26,** 639 (1957).
[32] J. A. De Moss and D. Novelli, *Biochim. et Biophys. Acta* **18,** 592 (1955).

TABLE I

COMPOSITION OF THE RNA AMINO ACID POOL AT SATURATION AFTER ADDITION OF VARIOUS AMINO ACIDS TO A GROWING CULTURE OF *E. coli*

Amino Acids	RNA (mμmoles/mg.)
Proline	0.8
Tyrosine	6.0
Phenylalanine	0.12
Methionine	0.15
Valine	0.20
Isoleucine	0.27
Leucine	0.19

enzymes have been isolated in pure form, each of them being specific for a single amino acid.[33-38] The cytological distribution of these enzymes is not known as they are not found specifically bound to any subcellular fractions.

The bacterial species studied so far seem to contain a "soluble" or "noncentrifugable" fraction of RNA, which functions as an amino acid acceptor *in vitro*[39, 40] as well as *in vivo*.[41]

In *E. coli*, this sRNA represents approximately 10 to 20% of the total RNA, the remainder being particulate.[42, 43] Its base composition differs very much from that of the "bulk RNA." It contains large quantities of cytosine; and about 15% of its bases are atypical. When single radioactive amino acids are added to cultures of *E. coli*, a complex is formed between the corresponding amino acid and RNA[41] as occurs also in animal material[30] (Chapter 37).

The kinetics of formation will be considered later (see Section III). The composition at saturation for seven amino acids is represented in Table I.

[33] E. W. Davie, V. V. Koningsberger, and F. Lipmann, *Arch. Biochem. Biophys.* **65,** 21 (1956).
[34] R. S. Schweet, *Federation Proc.*, **16,** 244 (1957).
[35] R. D. Cole, J. Coote, and T. S. Work, *Nature* **179,** 199 (1957).
[36] R. W. Holley, *Federation Proc.*, **17,** 964 (1958).
[37] P. Berg, *J. Biol. Chem.* **222,** 1025 (1956).
[38] R. S. Schweet, R. W. Holley, and E. H. Allen, *Arch. Biochem. Biophys.* **71,** 311 (1957).
[39] P. Berg and E. J. Ofengand, *Proc. Natl. Acad. Sci. U. S.* **44,** 78 (1958).
[40] T. Erdös and A. Ullmann, *Nature* **183,** 618 (1959).
[41] S. Lacks and F. Gros, *J. Mol. Biol.* **1,** No. 4.5, p. 301 (1959).
[42] A. B. Pardee, K. Paigen, and L. S. Prestige, *Biochim. et Biophys. Acta* **23,** 162 (1957).
[43] M. Nomura and J. D. Watson, *J. Mol. Biol.* **1,** 204 (1959).

TABLE II

NATURE OF THE RADIOACTIVITY BOUND TO THE RNA FRACTION AFTER INCORPORATION OF A RADIOACTIVE AMINO ACID[a]

Property of RNA fraction	Amount of fraction (%)
Precipitable by TCA (5% in the cold)	90
Extractable in hot TCA (20 minutes at 100° C.)	95
Dialyzable against water or urea (12 hours at 4° C.)	5–10
Dialyzable after ribonuclease treatment (digestion for 3 hours at 30° C. with 0.1 mg./ml.)	70
Lability in the presence of alkali (0.05 *N* at 20° C. for 20 minutes)	80
Electrophoretic mobility in agar	As for RNA

[a] Bacteria are labeled during growth for a few minutes in the presence of a radioactive amino acid. The pool of the free amino acids is removed by extensive washing with 5% TCA in the cold. Bacterial residues are then washed in alcohol and in alcohol plus ether. RNA is extracted by two successive treatments in 10% NaCl at 100°C. for 20 minutes, and precipitated from the extracts by 60% alcohol at −10°C.

In these experiments the bulk of the RNA was extracted together with the RNA amino acid complex. This explains the relatively low content of amino acids. For six amino acids (valine, phenylalanine, arginine, methionine, leucine, and isoleucine) the average value is 0.2 mμmole per milligram RNA; this indicates one amino acid for every fifty nucleotides if all the twenty amino acids are represented in the RNA amino acid pool. There are a few important exceptions: the RNA proline, and the RNA tyrosine pools are much higher in amino acid content than the other amino acid RNA pools. Either these two amino acids are linked to RNA fractions other than sRNA itself, or more than one molecule of these amino acids is bound (specifically or not) per molecule of sRNA. Work on this question is in progress.

Some general properties of the amino acid RNA complexes of *E. coli* are presented in Table II. These properties are very similar to those of the amino acid RNA complex from liver tissue.[29] The question arises as to whether the RNA amino acid complex isolated after incorporation of a radioactive amino acid by intact bacteria is merely a biological artifact. It might, for instance, be formed only when the intracellular concentration of free amino acid is very high due to the functioning of the permease. It is doubtful in fact whether the sRNA of *E. coli* growing in the absence of external amino acid contains amino acids. An experiment by Preiss *et al.*[44] suggests that it does not: sRNA was isolated from *E. coli* which had grown in a simple mineral medium unsupplemented with amino acids. All the biological sites of this RNA could be inactivated by periodate. Since a bound

[44] J. Preiss, P. Berg, E. J. Ofengand, F. H. Bergmann, and M. Dieckmann, *Proc. Natl. Acad. Sci. U. S.* **45**, 319 (1959).

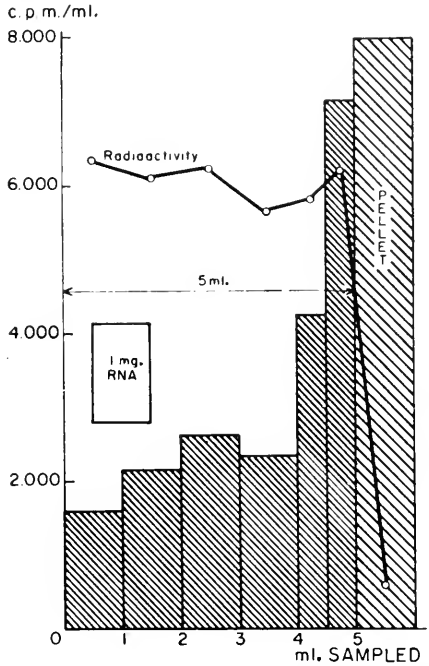

FIG. 1. Distribution of the "RNA bound" radioactivity after ultracentrifugation of an extract of *E. coli* K12 labeled with S^{35}. S^{35}-labeled bacteria are converted to protoplasts by treatment with lysozyme in the presence of sucrose. The extract obtained after osmotic shock is freed of DNA and ultracentrifuged for 2 hours at 100,000 *g*. Aliquots are drawn off separately with a syringe, and the "RNA bound" radioactivity is determined according to the principle described in Table II.

amino acid protects its corresponding specific site from such an inactivation, a possible interpretation of this result is that the sRNA of *E. coli* does not naturally contain any amino acid attached to it. Preiss *et al.* consider rather that the amino acid may have been split off during the process of isolation and purification, since sRNA from other sources usually contains amino acids when isolated in the "native form."[45]

Moreover, if *E. coli* is cultivated in an "amino acid free" medium plus S^{35}, which is converted into radioactive methionine and cystine, the total RNA extracted from the bacteria is highly labeled. By ultracentrifugation (Fig. 1) it can be shown that most of the radioactivity is bound to the nonsedimentable fraction of the bacterial RNA. Therefore in *E. coli* a fraction of the endogenously formed amino acids is attached to the sRNA. Furthermore, only a very low concentration of amino acid is required to

[45] G. Acs, G. Hartmann, H. G. Boman, and F. Lipmann, *Federation Proc.* **18**, 178 (1959).

saturate the sRNA. A rough estimate shows that the total amount of amino acid bound to the sRNA (proline and tyrosine excepted) corresponds to 0.05% of the dry weight of the bacterium, that is $^1/_{20}$ to $^1/_{50}$ of the amino acid pool originating from glucose.[46]

3. State of the Ribonucleoprotein in *E. coli*

In bacterial cells most of the RNA is organized into particles whose properties are very similar to those of the microsomes of animal cells. A few years ago, Schachmann *et al.*[47] examined several bacterial species and found that most of the RNA is present in the form of a nucleoprotein which is easily sedimentable (40 *S*). Similar observations were made by Weibull.[48, 49] At that time, the role of divalent cations as stabilizers of the particles was still unknown. The work of Tissières and Watson,[50] and that of Roberts *et al.*[51] has now established that if the concentration of Mg^{++} ions is high enough, a whole series of particles can be isolated from exponentially growing cells of *E. coli*. These particles vary in size: 30 *S*, 50 *S*, 70 *S*, 100 *S*. Their possible relationship is:

$$2(30\ S) + 2(50\ S) \rightleftharpoons 2(70\ S) \rightleftharpoons 100\ S$$

The molecular weight of 30 *S* is about 0.75×10^6, that of 50 *S* is 1.8×10^6. The 70 *S* particle is composed of one 30 *S* plus one 50 *S*; 100 *S* is a dimer of 70 *S*. When the Mg^{++} concentration exceeds 10^{-3} *M* there is a tendency for 30 *S* and 50 *S* to aggregate (70 *S*). When the concentration is between 10^{-4} *M* and 10^{-3} *M*, 30 *S* and 50 *S* are predominant in the crude extract. Below 10^{-4} *M*, the 30 *S* and 50 *S* particles irreversibly decompose into smaller subunits.

Fractionation of the RNA of *E. coli* can also be achieved by starch electrophoresis.[42, 43] The fastest peak corresponds to the sRNA, the slowest peak (sometimes split into two peaks) contains the "bulk RNA" and is a mixture of 30 *S* and 50 *S*.

Roberts *et al.*,[51] taking advantage of the high resolving power of activated cellulose, have chromatographed crude extracts of *E. coli* and have obtained a large number of protein peaks. The elution pattern is characteristic of *E. coli* and is reproducible. A particular fraction, to which they applied the term "ribosome," contains almost all the RNA of the cell. The ribosomes

[46] J. Mandelstam, *Biochem. J.* **64,** 55 (1956).
[47] H. K. Schachmann, A. B. Pardee, and R. Y. Stanier, *Arch. Biochem. Biophys.* **38,** 245 (1952).
[48] C. Weibull, *J. Bacteriol.* **66,** 688 (1953).
[49] C. Weibull, *Exptl. Cell Research* **9,** 139 (1955).
[50] A. Tissières and J. D. Watson, *Nature* **182,** 778 (1958).
[51] R. B. Roberts, R. J. Britten, and E. T. Bolton, *in* "Microcomal Particles and Protein Synthesis" (Washington Acad. Sci.) p. 84. Pergamon Press, London, 1958.

are in fact the "microsome" fraction free of cytoplasmic proteins and phospholipids.

The chemical composition of the particles in *E. coli*, prepared by differential ultracentrifugation is that of a pure ribonucleoprotein containing 63% of RNA and 37% of protein. The "RNA particle" is highly polymerized with a molecular weight between 500,000 and 1,000,000. Its properties have been studied by Doty[52, 53] and more recently by Watson *et al.*[54]

The protein fraction of the particle constitutes a chemically well-defined class or entity distinct from other proteins. It contains very little cystine,[51] and its content in methionine and aspartic acid is lower than in most of the other proteins of *E. coli*, whereas its content of glutamic acid, alanine, glycine, and lysine is higher.

No enzymic activity other than ribonuclease[55] can be detected in purified particles from *E. coli*,[50, 51] in contrast to the situation usually found in cytoplasmic granules of bacteria which contain respiratory enzymes and the systems involved in oxidative phosphorylation.[56-58]

Such granules probably contaminate the preparations of cell membranes made by osmotic lysis of bacterial protoplasts,[48, 49] in which various anabolic processes can be shown to occur.

4. State of the DNA in Bacteria

Little is known about the organization of the DNA in the bacterial cell. Bacteria contain a "nuclear material" ("nuclear bodies," "chromatinic bodies") inside which all the DNA is localized as was shown by the early work of Piekarski,[59] Robinow[60] and Boivin *et al.*[61] The question whether this nuclear material is a true nucleus or a set of chromosomes is still under discussion by cytologists.[62]

E. coli and *Bacillus megaterium* during exponential growth have an average number of 3 haploid chromosomes (or nuclei) per cell. This number

[52] B. D. Hall and P. Doty, *in* "Microsomal Particles and Protein Synthesis" (Washington Acad. Sci.), p. 27. Pergamon Press, London, 1958.

[53] P. Doty, H. Boedtker, J. R. Fresco, R. Haselkorn, and M. Litt, *Proc. Natl. Acad. Sci. U. S.* **45**, 482 (1959).

[54] J. D. Watson, Personal communication (1959).

[55] D. Elson, *Biochim. et Biophys. Acta* **27**, 216 (1958).

[56] A. Tissières, *Nature* **174**, 183 (1954).

[57] A. Tissières and E. C. Slater, quoted by E. C. Slater, *Proc. 3rd Intern. Congr. Biochem., Brussels, 1955* p. 264 (1956).

[58] R. Y. Stanier, I. C. Gunsalus, and C. F. Gunsalus, *J. Bacteriol.* **66**, 543 (1955).

[59] G. Piekarski, *Arch. Mikrobiol* **8**, 428 (1937).

[60] C. F. Robinow, *J. Hyg.* **43**, 413 (1944).

[61] A. Boivin, R. Vendrely, and R. Tulasne, *Bull. acad. natl. méd. (Paris)* [3] **131**, 2 (1947).

[62] E. T. Spooner and B. A. D. Stocker, "Bacterial Anatomy," 6th Symposium Soc. Gen. Microbiol. Cambridge Univ. Press, London and New York, 1956.

varies according to the physiological state of the bacteria. When cultivated in a low phosphate content medium, *E. coli* becomes uninucleated.[63]

Especially since the discovery of bacterial transformations,[64] bacterial DNA has been thoroughly studied with respect to its chemical[65] and physicochemical properties,[66] and its biological activity,[67-71] but little is known about the nature of the bacterial protein associated with the DNA.[42]

Contrary to what seems to be the rule for other kinds of cells which synthesize DNA only during a certain period of the mitotic cycle (late interphase[72-74]), it appears that bacteria synthesize DNA in a continuous manner. This interpretation can be derived from the rate of decay of P^{32} in unsynchronized cultures of bacteria labeled for a very short time,[75] or from the rate of uptake of tritiated thymidine by individual bacteria.[76]

5. The Cell Wall and the Cytoplasmic Membrane

Consideration of the chemical structure and of the mode of biosynthesis of the cell wall may be of interest in a study of incorporation of radioactive amino acids into bacteria, since, in some cases, the observed incorporation may represent the exclusive synthesis of the mucopolypeptides of the cell wall rather than the formation of true cytoplasmic protein.

In gram-negative bacteria the cell wall is composed of two layers. The outside layer is a lipoprotein, and the inner is a complex of lipopolysaccharides with a mucopolypeptide. In the peptide moiety, diaminopimelic acid, glutamic acid, and alanine are always present. In addition, lysine, serine, and glycine have sometimes been found.[77-79] The cell wall of gram-positive bacteria is simpler and contains no lipoprotein, but a mucopolypeptide containing glutamic acid and alanine residues together with either diaminopimelic acid or lysine. The bacterial membrane can be prepared by osmotic lysis of protoplasts, that is, the bacterial cell stripped of its

[63] E. McFall, A. B. Pardee, and G. S. Stent, *Biochim. et Biophys. Acta* **27**, 282 (1958).
[64] O. T. Avery, C. M. MacLeod, and McCarty, *J. Exptl. Med.* **89**, 137 (1944).
[65] E. Chargaff, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. I, p. 307. Academic Press, New York, 1955.
[66] M. Meselson and F. W. Stahl, *Proc. Natl. Acad. Sci. U. S.* **44**, 671 (1958).
[67] R. D. Hotchkiss, *Cold Spring Harbor. Symposia Quant. Biol.* **16**, 457 (1951).
[68] R. D. Hotchkiss, *in* "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. II, Chapter 27. Academic Press, New York, 1955.
[69] R. D. Hotchkiss, *Harvey Lectures* **49**, 124 (1955).
[70] H. Ephrussi-Taylor, *Cold Spring Harbor Symposia Quant. Biol.* **16**, 445 (1951).
[71] H. Ephrussi-Taylor, *Exptl. Cell Research* **2**, 589 (1951).
[72] J. Pasteels and L. Lison, *Arch. biol. (Liège)* **61**, 445 (1950).
[73] H. H. Swift, *Physiol. Zoöl.* **23**, 169 (1950).
[74] J. H. Taylor and R. D. McMaster, *Chromosoma* **6**, 489 (1954).
[75] E. McFall and G. S. Stent, quoted in 178.
[76] M. Schaechter, M. W. Bentzon, and O. Maaløe, *Nature* **183**, 1207 (1959).
[77] E. Work, *Biochem. J.* **49**, 17 (1951).
[78] E. Work, *Nature* **179**, 841 (1957).
[79] M. R. J. Salton, *in* "Bacterial Anatomy," p. 81. Cambridge Univ. Press, London and New York, 1956.

wall by the action of lysozyme,[48, 49] or by penicillin,[80] or by treatment with alkali.[81] The membrane is a lipoprotein, the structure of which has been studied by Mandelstam *et al.*[82] The wall or the pure membrane contains no nucleic acids but it includes pentose-containing substances such as ribityl phosphates.[83, 84]

The synthesis of the mucopeptides of the wall and the synthesis of cytoplasmic protein are two completely independent and dissociable processes in bacteria. Thus, on the one hand, the synthesis of cytoplasmic protein, can take place in bacteria lacking their cell walls (protoplasts),[85] or in bacteria in which synthesis of the cell wall is blocked by penicillin.[86] The converse is also true; the formation of mucopeptides can be observed in the absence of synthesis of cytoplasmic proteins.[87]

III. Synthesis of Macromolecular Components during Normal Bacterial Growth

Since each metabolic process in the cell is integrated in a very precise manner, it is difficult to obtain information about the relationship between nucleic acid and protein synthesis from a study of the normal cell. To obtain such information one has to interfere specifically with the synthesis of a particular component by the use of an inhibitor or by selective modification of the medium; this type of experiment will be considered later in this review in the study of the "uncoupled synthesis."

However, the study of the behavior of normal bacteria does reveal some important aspects of the biosynthesis of macromolecules, such as the degree of metabolic renewal of the protein and of the nucleic acids, the rate of their formation, and the nature of the intermediate steps.

1. Metabolic Stability of Proteins and Nucleic Acids

Before considering the kinetics of protein and nucleic acid biosynthesis in bacteria, it is necessary to discuss the question of "turnover" in these macromolecules (see also Chapter 37).

Monod and Cohn[88] and Hogness, Cohn, and Monod[89] showed a few years ago that when bacteria are first labeled in their protein by growing in the presence of S^{35}, then washed and transferred to a nonradioactive medium

[80] J. Lederberg, *Proc. Natl. Acad. Sci. U. S.* **42,** 574 (1956).
[81] N. D. Zinder and W. F. Arndt, *Proc. Natl. Acad. Sci. U. S.* **42,** 586 (1956).
[82] J. Mandelstam and H. J. Rogers, *Nature* **181,** 956 (1958).
[83] J. J. Armstrong, J. Baddiley, J. G. Buchanan, B. Carss, and G. R. Greenberg, *J. Chem. Soc.* p. 4344 (1958).
[84] J. J. Armstrong, J. Baddiley, J. G. Buchanan, and B. Carss, *Nature* **181,** 1692 (1958).
[85] K. McQuillen, *in* "Bacterial Anatomy," p. 127. Cambridge Univ. Press, London and New York, 1956.
[86] F. Gros and M. Macheboeuf, *6th Intern. Congr. Microbiol., Rome* p. 38 (1953).
[87] A. R. Crathorn and G. D. Hunter, *Biochem. J.* **69,** 47 (1958).
[88] J. Monod and M. Cohn, *6th Intern. Congr. Microbiol., Rome* p. 4 (1953).
[89] D. S. Hogness, M. Cohn, and J. Monod, *Biochim. et Biophys. Acta* **16,** 99, (1955).

containing an inducer for β-galactosidase, the newly formed enzyme, after extensive purification, shows no radioactivity. This experiment and some variants of it, led to the conclusion that in normally growing *E. coli*, the proteins are metabolically stable. A similar conclusion was reached by Rotman and Spiegelman[90] in studying β-galactosidase formation in *E. coli* previously labeled by incorporation of lactate-C^{14}.

The lack of turnover in microbial proteins has also been illustrated by Koch and Levy's experiments,[91] based on quite a different principle. Glycine is the precursor both of the protein glycine and of the purines of nucleic acids. Bacteria were therefore grown in the presence of glycine-C^{14} plus a mixture of nonradioactive purines in order to prevent labeling of the RNA fraction. Such cells with labeled proteins were transferred to a simple mineral medium without any radioactive tracer or purines and allowed to grow for a few generations. If protein turnover takes place, the glycine liberated by the breakdown of the protein would lead to labeling in the purines of the nucleic acid. The specific radioactivities of the isolated purines showed however, that the protein turnover was quite negligible.

During the last few years[92-94] doubt has been thrown on these conclusions regarding the stability of bacterial protein. It has, for example, been observed that amino acid-requiring mutants of *E. coli*, when starved of their essential metabolites, still incorporate into their proteins radioactivity from other amino acids[92] or from H_2O^{18}.[94] Moreover, the pool of free amino acids in *E. coli* rises appreciably above the normal value when bacteria are allowed to metabolize glucose in a medium where net protein synthesis is not possible. Finally Rickenberg[95] interprets his finding of preferential synthesis of enzymes as suggesting a metabolic instability of the protein in *E. coli*; when grown in the presence of a mixture of glucose and lactose, *E. coli* utilizes glucose first and lactose only after glucose exhaustion.[96] During the lag period which separates growth on glucose from growth on lactose, the bacteria show no increase in total protein but they begin to synthesize β-galactosidase very rapidly. Rickenberg's interpretation of these results is that preexisting protein could undergo turnover and liberate endogenous products which would be reutilized for the formation of β-galactosidase.

These arguments which might appear to disprove the concept of meta-

[90] B. Rotman and S. Spiegelman, *J. Bacteriol.* **68**, 419 (1954).
[91] A. L. Koch and H. R. Levy, *J. Biol. Chem.* **217**, 947 (1955).
[92] J. Mandelstam, *Nature* **179**, 179 (1957).
[93] J. Mandelstam, *Biochem. J.* **69**, 110 (1958).
[94] E. Borek, L. Ponticorvo, and D. Rittenberg, *Proc. Natl. Acad. Sci. U. S.* **44**, 369 (1958).
[95] H. V. Rickenberg and C. T. Lester, *J. Gen. Microbiol.* **13**, 279 (1955).
[96] J. Monod, "Recherches sur la croissance des cultures bactériennes." Herman et Cie. Paris, 1942.

bolic stability in bacterial proteins, must, however, be examined critically. In relation to the incorporation of radioactive amino acids or of O^{18} into the protein fraction of bacteria in the absence of net protein synthesis, it must be kept in mind that such incorporation may involve only a certain class of proteins (for example the proteins of the wall, the alcohol-soluble fraction of Cowie *et al.*[97] etc.). In any case the rate of this incorporation is slow.

In connection with the phenomenon of preferential synthesis, Naono's recent experiments,[98] carried out to test Rickenberg's hypothesis of protein removal during diauxic growth, are important to consider. *E. coli* was first labeled in its protein fraction with S^{35} following the method of Hogness *et al.*[89] Cells were washed and incubated in a medium containing glucose plus lactose, under the conditions described by Rickenberg.[95] β-Galactosidase was then isolated in the lag phase and in the phase of growth on lactose. The enzyme was highly purified by ammonium sulfate fractionation, by zone electrophoresis on starch columns, and finally by precipitation with antiserum. Determinations of radioactivity show that the rate of breakdown of pre-existing protein during preferential formation of enzyme is less than 1% of the rate of net synthesis.

Accordingly, even if the proteins are capable of some renewal, this phenomenon has been detected only under conditions where bacterial growth is inhibited,[92, 94] and it may be inherent in this particular condition. The observation by Halvorson[99] that some turnover can be observed in nonproliferating yeasts whereas the protein of the actively growing cells shows no turnover at all, strengthens this point of view. It is possible that malnutrition leads to a certain degree of protein degradation. Indeed this has been shown to be the case with the alcohol-soluble protein of *E. coli* which breaks down completely under sulfur starvation while it is stable under normal conditions.[97]

In tissue cultures, on the contrary, protein turnover can be shown to occur, both in the resting and in the growing state.[100] Therefore it can be concluded that, if any turnover of protein occurs, its rate is negligible compared with the over-all rate of protein synthesis during normal growth of bacteria.

Proteins are not the only macromolecular components of the cell which are metabolically stable under normal conditions, this is also true of the

[97] R. B. Roberts, P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten; in "Studies of Biosynthesis in *Escherichia coli*" Carnegie Inst. Wash. Publ. **607**, p. 318 (1957).

[98] S. Naono, personal communication (1959).

[99] H. Halvorson, *Biochim. et Biophys. Acta* **27**, 255 (1958).

[100] H. Eagle, K. A. Piez, R. Fleischman, and V. Oyama, *J. Biol. Chem.* **234**(3), 592 (1959).

nucleic acids. Evidence of the stability of the nucleic acids was obtained from Hershey's early experiments with *E. coli*[101] and more recently from those of Manson,[102] of Siminovitch and Graham,[103] and of Koch and Levy.[91]

Nevertheless, several observations indicate that under suitable conditions some of the cellular RNA can undergo partial or complete turnover. For instance, in a mutant of *E. coli* requiring both thymine and uracil, significant incorporation of radioactive precursors takes place in RNA in the absence of the two pyrimidines.[104] The same observation has been made with another uracil-dependent mutant of *E. coli*.[105] Another possible example of RNA turnover comes from the fact that the purine analog, 8-azaguanine, is incorporated either into a fraction of the RNA or on to an appropriate site on the RNA in an apparently reversible manner, since the incorporated analog can be rapidly displaced by the corresponding natural base[106-108] and released in the medium.

These few cases of metabolic renewal of the RNA may concern only the soluble fraction of this nucleic acid, since it is known that the sRNA can reversibly fix nucleotides in the terminal position.[109] Moreover, during incorporation of adenine-C^{14} or P^{32} by growing cultures of *E. coli*, the sRNA becomes labeled much earlier than do the RNA particles.

Finally, a few special cases, in which bacterial RNA manifests an extremely active renewal, must be mentioned. For instance, the RNA which is synthesized in the presence of chloramphenicol, undergoes very rapid destruction after the removal of the inhibitor.[110, 111] Also, after infection of *E. coli* by phage T2 or T6 no net RNA synthesis occurs, but P^{32} and radioactive bases are still actively incorporated into the "RNA pool." The relative rates of incorporation into the nucleotides of the RNA suggest the reversible formation of a new RNA species, the base composition of which is specific for the infecting phage.[112]

It has recently been shown that bacteria grown in a medium of very low

[101] A. D. Hershey, *J. Gen. Physiol.* **38**, 145 (1954).
[102] A. L. Manson, *J. Bacteriol.* **66**, 703 (1953).
[103] L. Siminovitch and A. F. Graham, *J. Histochem. and Cytochem.* **4**, 508 (1956).
[104] H. D. Barner and S. S. Cohen, *Biochim. et Biophys. Acta* **30**, 12 (1958).
[105] A. B. Pardee, *J. Bacteriol.* **69**, 233 (1955).
[106] H. G. Mandel, *J. Biol. Chem.* **225**, 137 (1957).
[107] R. E. F. Matthews and J. D. Smith, *Nature* **177**, 271 (1956).
[108] H. Chantrenne and S. Devreux, *Exptl. Cell Research*, Suppl. **6**, 152 (1958).
[109] L. I. Hecht, P. C. Zamecknik, M. L. Stephenson, and J. F. Scott, *J. Biol. Chem.* **235**, 954 (1958).
[110] F. C. Neidhardt and F. Gros, *Biochim. et Biophys. Acta* **25**, 513 (1957).
[111] F. E. Hahn, M. Schaechter, W. S. Ceglowski, H. E. Hopps, and J. Ciak, *Biochim. et Biophys. Acta* **26**, 469 (1957).
[112] E. Volkin and L. Astrachan, *Virology* **2**, 149 (1956).

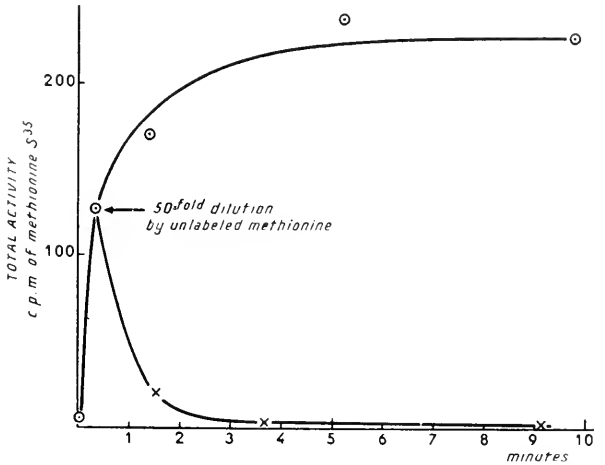

FIG. 2. Incorporation of methionine-S^{35} into the RNA of *E. coli* K12 growing exponentially. Dilution by unlabeled methionine. Temperature 20°C.; DL-methionine-S^{35} (2.10^{-4} *M*), specific radioactivity (2 μc./μmole).

phosphate content, can degrade up to 30% of their RNA, whereas DNA synthesis continues.[113]

2. Rate of Incorporation of Radioactive Precursors into Proteins and Nucleic Acids

Since the incorporation of single radioactive precursors into proteins and nucleic acids can under certain circumstances be an irreversible process, the use of radioactive tracers should reveal not only the absolute rate of synthesis of these macromolecules but also the nature of possible intermediates in their synthesis.

a. Amino Acid Incorporation into the "Intracellular Pool" and into the RNA

By measuring the comparative rates at which radioactive amino acids label the pool, the RNA, and the protein, one should be able to determine whether amino acids are fixed to an acceptor RNA before being built into a peptide chain. Figure 2 presents a typical example of the kinetics of this type of incorporation obtained by adding a radioactive amino acid to a growing culture of *E. coli*. The results are compatible with the idea that the pool of free amino acids and the amino acid-RNA complexes are protein precursors. These two fractions become labeled according to a typical saturation curve while the label in the protein increases exponentially.

[113] T. Horiuchi, S. Horiuchi, and D. Mizuno, *Biochim. et Biophys. Acta* **31**, 570 (1959).

Hoagland[29] has obtained similar results in measuring the kinetics of amino acid incorporation in the whole animal. It must be stressed, however, that in the experiment described here the amino acid-RNA pool includes amino acids attached to *both* the sRNA and the RNA particulate.

The RNA amino acid pool is saturated very rapidly (in 4 to 5 minutes at 20°C.) and a little earlier than the "free" pool which is saturated in about 10 minutes. This may be accounted for by the extremely small size of the pool of the RNA bound amino acids; a low internal concentration of free amino acids is sufficient to saturate the activating enzymes. The free amino acids, and those bound to the RNA, undergo very fast renewal. Thus, if bacteria are labeled with a radioactive amino acid, dilution of this tracer by an excess of the corresponding unlabeled amino acid quantitatively displaces the radioactive amino acid in both the "free" pool and in the amino acid-RNA complex.

In the case of the "free" pool, this renewal should depend on both the activity of the permease,[9] and the utilization of the free amino acids to make protein. However, if the external concentration of the radioactive amino acid is sufficient to saturate the permease, the rate of its uptake for protein synthesis is very small compared with the rate of its penetration into the bacterial cell. Therefore, the renewal of the free amino acid pool is practically independent of the rate of protein synthesis.[10] In order to explain the renewal of the RNA amino acid pool, one might be led to various interpretations following that one considers this pool as composed *mostly* of the sRNA bound amino acids or of the RNA particulate bound amino acids. In the first case, the factors involved in the renewal of the bulk of the RNA amino acid pool can be of at least two types:

(1) A reversible fixation of the activated amino acids in the sRNA

$$\text{Adenyl amino acid} + \text{sRNA} \underset{b}{\overset{a}{\rightleftharpoons}} \text{sRNA amino acid} + \text{AMP}$$

(2) A reaction or a series of reactions leading irreversibly to the use of these RNA bound amino acids for protein synthesis

$$\text{sRNA amino acids} \xrightarrow{c} \text{protein} + \text{sRNA}$$

Experiments show that when protein synthesis is inhibited (reaction *c*) for instance, by chloramphenicol, the rate of renewal of the RNA bound amino acids considerably diminishes as the rate of incorporation of a radioactive amino acid into this fraction (Fig. 3) and the rate of its displacement by a nonradioactive amino acid are both reduced by about 80%. Consequently, the extent of protein synthesis could be a limiting factor in the renewal of the amino acids bound to the sRNA, contrary to the situation for the free amino acids.

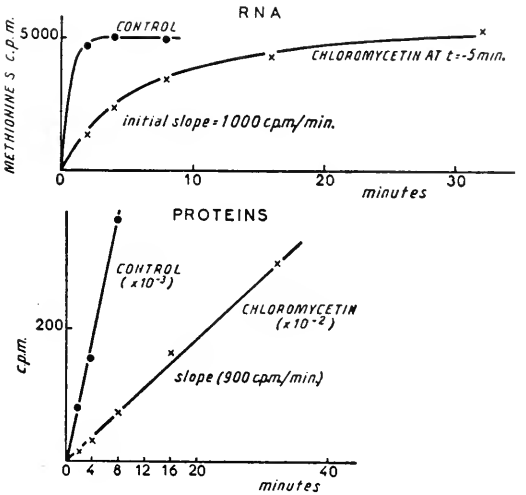

FIG. 3. Effect of chloramphenicol (Chloromycetin) on the incorporation of methionine-S^{35} into the RNA and protein of *E. coli* ML 30 chloramphenicol (21 μg./ml.); DL-methionine-S^{35} (4.10^{-5} *M*), specific radioactivity (2 μc./μmole).

If the kinetics of renewal of amino acids bound to the total RNA (Figs. 2 and 3) represent, in fact, the rate at which both the sRNA and the RNA particulate are simultaneously or sequentially labeled, (rather than the exclusive labeling of the sRNA), another entirely different interpretation of data in Fig. 3, is possible. They could suggest that Chloromycetin slows down the rate of incorporation of amino acids into the RNA particulate while the size of the sRNA amino acid pool would slowly increase above normal values, due to the fact that acceptor sites on the sRNA would not be entirely saturated and that Chloromycetin would inhibit transfer of amino acids from the sRNA to the RNA particulate. The choice between these interpretations has to wait for studies on amino acid incorporation into separated fractions of RNA. Whatever the correct interpretations may be, displacement of a radioactive amino acid fixed on RNA by an excess of the corresponding unlabeled amino acid gives rise to an almost equivalent increase in the radioactivity of the protein fraction. The results of such a "transfer experiment" are given in Table III. Gale[114] obtained similar results using preparations of disrupted *Staphylococcus aureus*.

The question as to whether all the proteins are formed via the "activating" systems and the sRNA, or whether other pathways for amino acid incorporation into protein exist will be discussed later.

[114] E. F. Gale, *7th Intern. Congr. Microbiol., Stockholm* Symposium 2, p. 104 (1958).

TABLE III

TRANSFER OF A RADIOACTIVE AMINO ACID FROM THE RNA AMINO ACID POOL TO THE PROTEIN FRACTION[a]

Experiment	Loss of radioactivity from the "RNA pool" in 8 minutes (c.p.m.)	Increase in radioactivity of the proteins in 8 minutes (c.p.m.)
No. 1		
Control	2,700	2,400
Chloramphenicol	1,600	1,000
No. 2	2,140	1,500
No. 3	3,100	2,100

[a] A culture of the auxotroph *E. coli* M-191 (methionine-histidine) is starved for 4 hours at 32°C. Methionine-S^{35} ($2 \times 10^{-5}M$) is then added and allowed to label the "RNA pool" for 5 minutes in the absence of histidine; at the end of this period, a large excess of nonradioactive methionine ($4 \times 10^{-3}M$) plus some histidine are added to the culture. The total radioactivity of the "RNA pool" and of the proteins is measured at the time of addition of the unlabeled amino acid and 8 minutes later.

b. Amino Acid Incorporation into "Soluble" Protein and into the Ribosomes

Many experiments with whole animals or tissue slices have established that after incorporation of a radioactive amino acid, the label first appears in the proteins of the microsomal fraction (see Chapter 37 and the review by Loftfield[115]). When labeled microsomes are fractionated, the highest specific radioactivity is associated with the fraction insoluble in deoxycholate, that is the ribonucleoprotein. These observations have often been quoted as showing that the protein-forming site is a ribonucleoprotein.

This question has been examined in bacteria by Roberts *et al.*[51] The results show that if individual variations in amino acid content are taken into consideration, no protein fraction, not even that of the ribosomes, exhibits a higher specific radioactivity than any other. Two explanations are possible:

(1) The first highly radioactive proteins formed have been stripped off the ribosome during the fractionation procedure. This is improbable however since even the crude microsome pellet does not contain any one protein of high specific radioactivity.

(2) The time of exposure to the tracer (4 minutes) may still be too long because of the very high growth rate of *E. coli*. A calculation shows[51] that if ribosomes are specific templates for protein synthesis, and if polypeptide chains of 150 residues are formed, each of the 10,000 ribosomes present in a cell of *E. coli* must produce a polypeptide chain every 10 seconds according to the observed growth rate of the organism. Therefore, if only one poly-

[115] R. B. Loftfield, *Progr. in Biophys. and Biophys. Chem.* **8**, 347 (1958).

peptide chain can adhere to each ribosome, only $^1/_{24}$ of the newly synthesized peptides will be found to be still adhering to the ribosomes after 4 minutes of exposure to the radioactive amino acid.

Accordingly, the negative results of Roberts *et al.*[51] are not conclusive, and new experiments with shorter times of exposure to the tracer are required.

c. The Cell Membrane as the Protein-Forming Site

It has been observed[116, 117] that during growth of *B. megaterium* or *E. coli*, radioactive amino acids are incorporated more rapidly into the protein fraction of the membrane than into the cytoplasmic proteins. The fraction considered here as cytoplasmic membranes corresponds actually to the "ghosts" described originally by Weibull,[48] i.e., the insoluble fraction found after osmotic lysis of protoplasts. These experiments have led to the conclusion that cytoplasmic membranes could be the active site of protein synthesis.

In addition to these *in vivo* experiments it has been shown that amongst the subcellular fractions of bacteria, membrane preparations are by far the most active in incorporating amino acids into protein.[117-120] Enzyme and RNA synthesis have also been reported in the membranes.[117, 118]

Butler *et al.*[116] observe that the proteins manufactured in the membrane diffuse very rapidly into the cytoplasm. Thus, labeled preparations of membranes, when mixed with unlabeled cytoplasmic fractions, transfer in 1 minute 60% of their radioactive proteins to the cytoplasm. No transfer occurs in the absence of cytoplasm.

Although the incorporation of amino acid into the protein fraction of isolated membranes is stimulated by ribonuclease and not by the sRNA and the activating enzymes,[117, 120] it must be remembered that the preparations still contain appreciable amounts of RNA and some activating systems. For instance, in Spiegelman's experiments, the amino acid incorporation *in vitro* depends on the presence of adenosine triphosphate (ATP)[117]; furthermore, formation of amino acid RNA complexes has been observed in the preparations used by the British authors.[121]

The hypothesis that bacterial membranes could be the primary site of protein synthesis is perhaps premature since they still contain ribonucleo-

[116] J. A. V. Butler, A. R. Crathorn, and G. D. Hunter, *Biochem. J.* **69,** 544 (1958).
[117] S. Spiegelman, *7th Intern. Congr. Microbiol., Stockholm* Symposium No. 2, p. 81 (1958).
[118] E. F. Gale and J. P. Folkes, *Biochem. J.* **59,** 661 (1955).
[119] B. Nisman, *Biochim. et Biophys. Acta* **32,** 18 (1959).
[120] B. Nisman and H. Fukuhara, *Compt. rend. acad. sci.* **248,** 1438 (1959).
[121] G. D. Hunter, P. Bzookes, A. R. Crathorn, and J. A. V. Butler, *Biochem. J.* **73,** 369 (1959).

protein and are probably associated with particles or granules of high intrinsic metabolic activity.

d. Incorporation of Radioactive Precursors into the Nucleic Acids

Although this chapter is concerned with protein formation, the integration of nucleic acid and protein synthesis is of such an importance that some problems dealing with the mechanism of RNA formation deserve particular discussion. These problems relate to the role of the "free" amino acids in the synthesis of RNA, and to the possible nature of the intermediates in the formation of the RNA particles. The first problem will be considered later (see Section IV). In a consideration of the mechanism of formation of the particulate RNA three possible precursors must be examined: (1) the sRNA itself, (2) the RNA fraction associated with the membranes, and (3) the free RNA of high molecular weight.

e. sRNA as a Precursor of the Stable RNA?

When adenine-C^{14} is added to an exponentially growing culture of *E. coli*, radioactivity appears much earlier in the sRNA than in the "RNA particles (pRNA)" (Fig. 4).[122] Similar observations have been made using P^{32}.[123]

If to the bacteria labeled in the steady state, an excess of adenine-C^{12} is added, the specific radioactivity in the sRNA is appreciably diminished while it rises in the RNA of the particles.

Several mechanisms could account for these observations:

(1) Fixation of nucleotides to the terminal position of the sRNA (BTP = nucleoside triphosphate; BMP = nucleoside monophosphate), according to the reaction first described by Hecht *et al.*[109] which prepares the sRNA for its combination with activated amino acids

$$\text{BTP} + \text{sRNA} \rightleftharpoons \text{BMP-sRNA} + \text{PP}$$

$$\text{BMP-sRNA} + \text{ENZ-adenyl-AA} \rightarrow \text{AA-BMP-sRNA} + \text{AMP}$$

(2) Conversion of the sRNA to the stable "RNA particles"

$$n\ \text{sRNA} \rightarrow \text{pRNA}$$

or

$$n\ \text{sRNA} \rightarrow n'\ \text{nucleotides} \rightarrow \text{pRNA}$$

(3) Fixation of nucleotides onto the end group of the sRNA, followed by their incorporation into the "RNA particle"

$$n\ \text{sRNA} + n\ \text{BTP} \rightarrow n\ \text{BMP-sRNA} + n\ \text{PP}$$

$$n\ \text{BMP} - \text{sRNA} \rightarrow \text{pRNA} + \text{sRNA}$$

[122] S. Naono and F. Gros, unpublished data (1959).
[123] S. Naono, unpublished (1958).

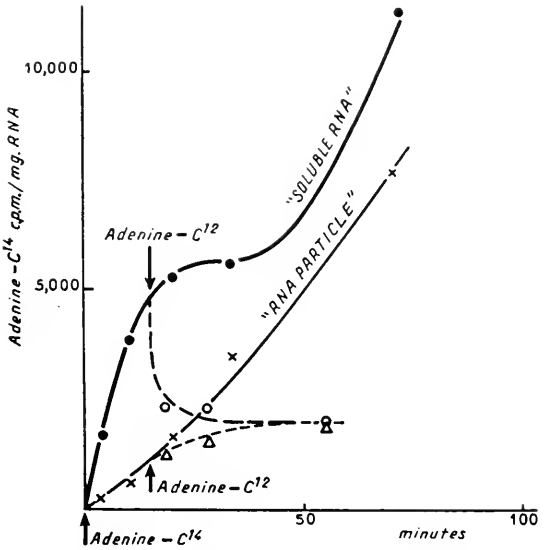

FIG. 4. Rates of incorporation of adenine-C^{14} into the "soluble" and "particulate" RNA; *E. coli* ML 308, growing at 18°C. in mineral medium plus glucose, at a given time adenine-C^{14} (10 μg./ml. and 300 c.p.m./μg.) is added. Samples of the culture are taken at intervals, and cooled rapidly at 0°C. Bacteria are washed in dilute Tris buffer (0.005 *M*) pH 7.6 and ground with alumina in 0.002 *M* Tris + $MgCl_2$ 5×10^{-3} *M* at pH 7.4. The extract is freed of DNA and the sRNA is separated from the "RNA particles" by ultracentrifugation for 2 hours at 100,000 *g*.

The direct or indirect conversion of sRNA to pRNA [mechanism (2)] is an unlikely process, since after starvation of uracil in a particular mutant of *E. coli*, the sRNA does not disappear as would be expected since its resynthesis would be prevented.[41]

Mechanisms (1) or (3) are more probable and the possibility of their existence is strengthened by the observation[122] that part of the radioactivity found in the sRNA after adenine-C^{14} incorporation by growing bacteria, can be released as radioactive adenosine on submitting the labeled sRNA to mild alkaline hydrolysis.

On the other hand, since mechanism (1) has been demonstrated to occur *in vitro*, the possibility of its occurrence *in vivo* is not unreasonable. It can be assumed therefore, that the sRNA is not a precursor of the pRNA and is not involved in its synthesis.

f. The Cell Membrane as the Site of RNA Synthesis

Spiegelman[117] found that when intact protoplasts of *E. coli* are incubated with uridine-C^{14} in a medium supporting growth, the most rapidly labeled fraction of the RNA was associated with the membranes, followed by the

sRNA and finally the pRNA. Similar results have been obtained with *E. coli* labeled with P^{32}.[124]

The kinetics of incorporation of the precursors in the steady state and the way the radioactivity in the RNA is distributed after "pulse" experiments, suggest that the membrane RNA could be a precursor of the other fractions of the RNA in the cell.

Unfortunately it is not known with certainty whether the RNA which is found in membrane preparations is a chemically distinct entity and whether the relationship between the "RNA forming system" and the cytoplasmic membrane is fortuitous or not.

g. Free Polymerized RNA as a Precursor of the Ribonucleoproteins

After addition of P^{32} to a growing culture of *E. coli* the RNA fraction labeled most rapidly corresponds to an RNA of high molecular weight, free or combined with a small amount of protein. This "free" RNA can be distinguished from the microsomal RNA of the bacteria, since the radioactivity precipitable from this fraction with trichloroacetic acid sediments half as rapidly as the bulk of the ribonucleoprotein (80 *S* particles).[51] Kinetic studies suggest that this special RNA fraction could be a precursor of the ribosomes. Indeed, synthesis of RNA by *E. coli* in the presence of chloramphenicol might merely represent an accumulation of this precursor.

It is therefore probable that bacterial RNA is first synthesized in a free form, before being combined to protein to constitute the normal particles of the cell.

IV. Uncoupled Synthesis of Macromolecules in Bacteria

So far consideration has been given only to bacteria under normal conditions of growth in order to analyze some intermediary steps in protein biosynthesis. The relationship between nucleic acid and protein synthesis, which is one of the main problems of this chapter can be studied more appropriately in bacteria altered in their chemical composition or in their ability to synthesize one particular constituent.

This explains the large amount of work which has been devoted to the uncoupled synthesis of macromolecules, or to the metabolic capacities of bacteria after partial or complete destruction of their nucleic acids.

The various conditions under which protein and nucleic acid synthesis can be uncoupled will be examined first.

1. Protein and RNA Synthesis in the Absence of DNA Synthesis

It has been known for a long time that growth can take place without cytoplasmic or nuclear division. For instance, bacteria after short exposure

[124] E. Volkin and L. Astrachan, *Virology* **2**, 433 (1956).

to ultraviolet irradiation may lose their capacity to give colonies on solid media while still retaining their ability to increase in size. Investigations have therefore been made to determine whether protein synthesis is possible in the absence of DNA synthesis.

The most useful system for this purpose is probably the mutant of *E. coli* (strain 15T) discovered by S. S. Cohen[125] which exhibits a specific requirement for thymine. When maintained in a minimal mineral medium containing a source of energy, this organism does not synthesize DNA to a significant extent (3% of the normal rate) but still produces adequate amounts of protein and RNA. In addition, thymine starvation does not prevent the induction of various enzymes (xylose isomerase[126] and β-galactosidase[127]). It appears, therefore, that the organization of polypeptides into a specific protein does not require DNA replication.

Similar conclusions have been obtained by the use of several inhibitors (proflavine,[127] nitrogen mustard,[127-130] ultraviolet- or X-irradiation[131, 132]). In all cases the system of DNA replication appears to be more sensitive than that involved in protein or enzyme formation.

Protein and RNA synthesis without concomitant DNA synthesis (a situation depicted by S. S. Cohen under the term "unbalanced growth") is generally lethal.[125] This lethal effect is related to changes in the ratio protein/DNA rather than the ratio RNA/DNA. Thus, on the one hand, RNA synthesis without concomitant synthesis of protein and DNA does not give rise to the death of the bacteria[133]; on the other hand, *E. coli* during thymine starvation does not die if protein synthesis is blocked by an inhibitor (5-methyltryptophan).[125] It is not known why protein formation when not accompanied by DNA synthesis should be a lethal process.

During unbalanced growth however, the DNA exhibits some instability, since the rate of spontaneous mutation is much higher amongst the survivors than in the population supplemented with thymine.[134, 135] In addition starvation for thymine favors the synthesis of atypical bases.[136]

[125] S. S. Cohen and H. D. Barner, *Federation Proc.* **13**(1), (1954).
[126] S. S. Cohen and H. Barner, *J. Bacteriol.* **69**, 59 (1958).
[127] F. Gros-Doulcet, F. Gros, and S. Spiegelman, *3rd Intern. Congr. Biochem., Brussels, 1955* p. 74 (1956).
[128] A. B. Pardee, *Proc. Natl. Acad. Sci. U. S.* **40**, 263 (1954).
[129] R. M. Herriot, *J. Gen. Physiol.* **34**, 761 (1951).
[130] E. I. Sher and M. F. Mallette, *Arch. Biochem. Biophys.*, in press, quoted by Spiegelman.[146]
[131] A. Kelner, *J. Bacteriol.* **65**, 252 (1953).
[132] L. S. Baron, S. Spiegelman, and H. J. Quastler, *J. Gen. Physiol.* **36**, 631 (1953).
[133] E. Borek, A. Ryan, and J. Rockenbach, *J. Bacteriol.* **71**, 318 (1956).
[134] R. Weinberg, and A. B. Latham, *J. Bacteriol.* **72**, 570 (1956).
[135] C. A. Coughlin and E. A. Adelberg, *Nature* **178**, 531 (1956).
[136] D. B. Dunn and J. D. Smith, *Nature* **175**, 336 (1955).

A direct application of the uncoupling of DNA and RNA synthesis will be discussed later in relationship to experiments based on the decay of P^{32} in bacteria labeled in their nucleic acids.

2. Protein Synthesis in the Absence of RNA Synthesis, or during Formation of an Atypical RNA

As will appear in the general discussion, one of the most commonly accepted hypotheses concerning the relationships between RNA and protein is that protein formation depends on RNA synthesis. In other words, the RNA would serve only once as a template for protein formation instead of acting as a catalyst in the process.

This hypothesis is derived mostly from experiments in which attempts have been made to dissociate protein and enzyme formation either from net RNA synthesis (by using purines or pyrimidine mutants) or from the synthesis of chemically normal RNA (by the use of base analogs).

a. Studies with Mutants

Contrary to what is observed during thymine starvation, specific starvation for uracil in *E. coli* leads to complete cessation of growth and does not permit the induction of β-galactosidase.[137, 138] This is generally interpreted as proof that protein synthesis, and especially synthesis of specific protein, requires the concomitant synthesis of RNA.

This interpretation may have to be modified however in the light of the experiments of Magasanik *et al.*[139] on the control of enzyme synthesis in bacteria. Using a mutant of *Aerobacter aerogenes* which requires guanine, they observed that bacteria deprived of guanine can synthesize large amounts of certain enzymes, the nature of which depends on the carbon source. For instance, when maintained on inositol in the absence of guanine, the bacteria can synthesize inositol dehydrogenase and inosinic dehydrogenase; if instead of inositol, glycerol, histidine, or glucose are added, the bacteria synthesize the inosinic dehydrogenase but not the inositol dehydrogenase.

These results can be explained by the well-known phenomenon of enzyme repression. If one supposes that deprivation of guanine considerably retards growth for a reason which may be independent of the cessation of nucleic acid synthesis, it is clear that since the bacterium metabolizes the carbon source, it will accumulate large pools of endogenous repressors; the synthe-

[137] J. Monod, A. M. Pappenheimer, and G. Cohen-Bazire, *Biochim. et Biophys. Acta* **9**, 648 (1952).

[138] A. B. Pardee, *Proc. Natl. Acad. Sci. U. S.* **40**, 263 (1954).

[139] B. Magasanik, A. K. Magasanik, and F. C. Neidhardt, *in* "The Regulation of Cell Metabolism," A. Ciba Symposium, p. 334. Churchill, London, 1959.

sis of all the enzymes will then become impossible. But the strain is genetically unable to make guanine from any simple carbon source, and no endogenous guanine will be formed. Therefore since guanine represses the enzymes for its own biosynthesis, these enzymes will be synthesized after guanine starvation. This state of affairs is observed for inosinic dehydrogenase, which is synthesized in the absence of guanine whatever the nature of the carbon source is. Inositol dehydrogenase, on the other hand, is generally induced by inositol in the wild strain, which means according to recent hypotheses on enzyme induction,[140] that inositol can antagonize the cytoplasmic repressor of inositol dehydrogenase. Therefore, since the effects of the repressor for inositol dehydrogenase, which is accumulated by *A. aerogenes* during guanine starvation, are neutralized by inositol, the strain not only manufactures inosinic dehydrogenase (for the reasons cited above) but also the inositol dehydrogenase.

Similar results have been obtained by Pardee.[141] Thus pyrimidine mutants of *E. coli*, in the absence of their essential base, produce very large quantities of the enzymes involved in pyrimidine biosynthesis (ureidosuccinate synthetase, dihydroorotate dehydrogenase) (see Chapter 36). The level of these enzymes during pyrimidine starvation can reach 10 to 100 times that observed in the normal strain.

All the above results taken together indicate that in the absence of RNA synthesis, the cell can manufacture enzymes whose repression is not possible under the conditions of the experiment.

Preferential synthesis of enzyme in the absence of RNA synthesis can also be observed during diauxic growth. It is known from the work of Monod,[96] that when *E. coli* is cultivated in the presence of a mixture of glucose and of another carbon source, the utilization of which requires "adaptation," the cell grows first at the expense of glucose which is constitutively metabolized. When glucose is exhausted there is a phase of very slow growth during which the cell adapts itself to the second carbon source, followed by a phase of rapid growth at the expense of this carbon source. During the intermediate phase the cell elaborates no RNA, but increases its protein content by 30%, and synthesizes the enzymes involved in the utilization of the second carbon source.[139]

b. Effect of Base Analogs on the Synthesis of Bacterial Enzymes

The discovery that certain base analogs (see Chapter 39) can be incorporated into the RNA of bacteria, of animal cells, and of plant viruses[142–145]

[140] A. B. Pardee, F. Jacob, and J. Monod, *Compt. rend. acad. sci.* **246**, 3125 (1958).

[141] A. B. Pardee, quoted by B. Magasanik in *Ann. Rev. Microbiol.* **2**, 221 (1953).

[142] J. Monod, "Recherches sur la croissance des cultures bactériennes." Herman et Cie, Paris, 1942.

has led to a study of the effect of these analogs on protein and enzyme formation in bacteria.

5-Hydroxyuridine and 8-azaguanine[146] inhibit the induction of β-galactosidase in *E. coli* without affecting appreciably its growth. Similar results have been obtained with 8-azaguanine and 2,6-diaminopurine in *Staphylococcus aureus*, where the analog inhibits the formation of inducible enzymes (catalase, galactosidase) rather than that of the constitutive enzymes or enzymic systems (glucose oxidase).[147, 148]

These results must be related to the observations of Gale and Folkes[149] that in disrupted staphylococci, induced synthesis of β-galactosidase can be stimulated by a mixture of purines and pyrimidines and that after removal of the RNA from the preparation, constitutive synthesis of catalase can be restored by complete RNA, while induced synthesis of β-galactosidase can only be restored by a mixture of bases.

These observations have led to the conclusion that *de novo* synthesis of a specific protein (enzyme) requires the synthesis of a specific RNA, and that synthesis of constitutive enzymes is not affected by the analog because it is controlled by a preexisting RNA specific for this constitutive enzyme.

Such a conclusion, which is probably the most highly developed form of theory on cosynthesis, is open to a series of objections.

(1) As has been mentioned above, considerable enzyme synthesis is observed in mutants starved of an essential purine or pyrimidine if the carbon source is chosen so as to avoid repression of the enzymes concerned.

(2) Since the early work on inhibition of enzyme synthesis by analogs,[147, 148] many cases of selective inhibition of the formation of constitutive enzymes have been reported: in *E. coli*, 5-hydroxyuridine,[150] and 5-fluorouracil[151] inhibit the constitutive synthesis of β-galactosidase as much as the inducible synthesis. In both cases growth is considerably less affected than is enzyme synthesis. In *Bacillus cereus*, constitutive synthesis of penicillinase is selectively inhibited by 8-azaguanine,[152] to the same extent as is inducible synthesis.

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[143] R. E. F. Matthews, *Nature* **171**, 1061 (1953).

[144] F. Weygand, A. Wacker, and H. Dellweg, *Z. Naturforsch.* **7b**, 19 (1952).

[145] R. Jeener and J. Rosseels, *Biochim. et Biophys. Acta* **11**, 438 (1953).

[146] S. Spiegelman, H. O. Halvorson, and R. Ben Ishaï, *in* "A Symposium on Amino Acid Metabolism" (W. D. McElroy and B. Glass, eds.), p. 1048. Johns Hopkins Press, Baltimore, 1955.

[147] E. H. Creaser, *Nature* **176**, 556 (1955).

[148] E. H. Creaser, *Biochem. J.* **64**, 539 (1956).

[149] E. F. Gale and J. P. Folkes, *Biochem. J.* **59**, 675 (1955).

[150] F. Gros, Françoise Gros, and S. Spiegelman, unpublished results (1954).

[151] A. B. Pardee, personal communication (1958).

[152] H. Chantrenne, *Rec. trav. chim.* **77**, 586 (1958).

These observations show that no discrimination can be made between inducible and constitutive enzymes in respect of their sensitivity to the base analogs and do not favor the interpretation of an obligatory cosynthesis between inducible enzymes and specific RNA.

(3) The main problem, therefore, in the mechanism of action of the analogs, is not to explain why they influence inducible rather than constitutive synthesis of enzymes but, rather, why they inhibit the synthesis of a specific enzyme protein without modifying appreciably the synthesis of total protein (growth).

A possible explanation is that the analog modifies the specificity of the stable RNA templates. These templates could still function in the building of a peptide chain; but this peptide chain would lack biological specificity. This hypothesis would be supported by the fact that the RNA of virus particles which contain thiouracil loses its infectivity.[145]

Such an interpretation cannot be retained however for purely kinetic reasons; in most of the cases studied, the synthesis of enzymes[151, 152] is inhibited selectively, as soon as the analog is added. Since we know that the RNA particle is metabolically stable, the RNA templates existing prior to the addition of the analog should go on catalyzing enzyme formation. Enzyme synthesis should be inhibited only after a lag period corresponding to the time required for the accumulation of the altered templates.

Chantrenne put foward the hypothesis that the inhibitory effect of the analog on enzyme synthesis could be based on a mechanism other than alteration of the RNA templates and suggested that base analogs could be incorporated very rapidly into an RNA fraction capable of very active renewal, such as the sRNA, and this would explain their immediate effect on the synthesis of certain proteins.[153]

Recent results on the metabolic effects of 5-fluorouracil in *E. coli* are in good agreement with Chantrenne's hypothesis.[154] The addition of 5-fluorouracil to *E. coli*, growing exponentially, diminishes within 1 minute the relative rates of incorporation of proline and of tyrosine into the protein, stimulates the rate of incorporation of arginine, and does not influence appreciably the incorporation of fourteen other amino acids (the diminutions observed for proline and tyrosine are, respectively, 27 and 20%). Similar specificity appears to exist at the level of the fixation to the RNA. Amongst seven amino acids tested (valine, methionine, phenylalanine, arginine, isoleucine, proline, and tyrosine) only proline and tyrosine are affected in rate and extent of fixation to the RNA. The saturation level of the RNA proline pool is lowered by about one-third, that of the RNA

[153] H. Chantrenne, *Biochem. Pharmacol.* **1**, 233 (1959).

[154] S. Naono and F. Gros, *Compt. rend. acad. sci.* (1960), in press.

tyrosine pool by one-half, whereas this level is not affected in the case of the other amino acids studied.

Thus, 5-fluorouracil by being incorporated very rapidly into an "acceptor RNA fraction" (probably the sRNA) modifies certain of its properties, such as the capacity to bind proline and tyrosine, as to "adapt" these amino-acids to the proper template. As a possible consequence of this fact, the protein, newly synthesized in the presence of the analog, contains about 20 to 30 % less of these two amino acids than does normal protein. These two amino acids are probably replaced by others (probably arginine) and this explains why certain enzymes are not synthesized (or certain enzyme activities are not increased) after the addition of 5-fluorouracil to the culture.

3. Nucleic Acid Synthesis in the Absence of Protein Synthesis

It has just been shown that the cell machinery involved in the synthesis of protein can function, at least for a limited period of time, in the absence of nucleic acid synthesis: for instance, enzymes can be manufactured in a bacterium no longer able to make either RNA or DNA as a result of base starvation. The reverse is also true: a cell in which protein synthesis is actively prevented can go on making RNA or DNA in quite large amounts. This situation was first described by Gale in his classic observations on bacteria treated by chloramphenicol. This type of uncoupling is of interest mainly for two reasons: (1) it sheds some light on the mechanism of particle formation; (2) it has led to the observation that free amino acids are involved in RNA synthesis.[27, 28]

Bacteria treated with chloramphenicol can double (or triple) their content of nucleic acid; while synthesis of cytoplasmic protein is almost completely inhibited. This observation was first made in *S. aureus*[155] and has been extended to other sensitive strains.[156] Chloramphenicol is not the only agent able to produce this type of uncoupling. Aureomycin[155] and erythromycin[157] have similar effects within a certain range of concentration. This is also true for some amino acid analogs such as 5-methyltryptophan which reduces protein synthesis by about 80 %, and leaves the rate of nucleic acid synthesis unchanged. Finally, in some very exceptional cases a net increase in RNA content occurs without protein formation, e.g., in amino acid auxotrophs lacking their essential amino acid. This is found only in some strains of *E. coli* K12 which specifically require methionine.[158] While cytoplasmic proteins and enzymes are not synthesized in the pres-

[155] E. F. Gale, *2nd Intern. Congr. Biochem., Paris* p. 1 (1952).
[156] C. L. Wisseman, J. E. Smadel, F. E. Hahn, and H. E. Hopps, *J. Bacteriol.* **67**, 662 (1954).
[157] T. D. Brock and M. L. Brock, *Biochim. et Biophys. Acta* **33**, 274 (1955).
[158] E. Borek, A. Ryan, and J. Rockenbach, *J. Bacteriol.* **69**, 460 (1955).

ence of chloramphenicol, the mucopeptides of the cell wall and probably some protein of the cell membrane are still elaborated.[87] This may account for the residual incorporation of radioactive amino acid into the protein fraction after treatment with the inhibitor.

The RNA and the DNA accumulated in the presence of chloramphenicol have been studied both from the chemical and from the biological points of view. It is known from the work of Hershey[159] and of Tomizawa,[160] that the DNA formed after chloramphenicol action has all the characteristics of a normal DNA; thus the chloramphenicol DNA synthesized in T^2-infected bacteria serves as a precursor for T^2 DNA, and behaves, therefore, as a normal free DNA intermediate.

The base composition of the chloramphenicol RNA is very similar to that of normal RNA.[27, 43] Thus the RNA formed in the absence of protein formation seems to keep its chemical specificity. This is probably true also for the biological specificity, at least for some RNA fractions such as the sRNA. This sRNA is normally synthesized in the presence of chloramphenicol, since the ratio of sRNA to total RNA is kept constant as indicated by chemical analysis[43] and by the fact that after doubling of the mass of RNA in the presence of chloramphenicol, the RNA amino acid pool also doubles.[161] It is not known, however, whether the pattern of the amino acids attached to the sRNA is the same in the chloramphenicol sRNA as in the normal sRNA.

Though the chloramphenicol RNA bears some resemblance to the normal RNA, it also exhibits some interesting differences. Neidhardt and Gros[110] observed that after bacteria have accumulated some RNA in the presence of chloramphenicol, more than half of this nucleic acid is destroyed if the bacteria are now transferred to a medium unable to support RNA synthesis (buffer, or minimal medium in the case of an amino acid auxotroph). There is very little destruction if the medium used for resuspending the treated bacteria is able to support resynthesis of RNA.

Since the chloramphenicol RNA can undergo degradation even in the absence of an energy source, it may be considered as the product of a passive mechanism probably due to the effect of cellular ribonuclease. Consequently, the amount of chloramphenicol RNA in the cell results from an equilibrium between passive destruction and *de novo* resynthesis, and chloramphenicol appears to shift the balance of the reactions towards net synthesis when an energy source is available.

[159] A. D. Hershey and N. E. Melechen, *Virology* **3**, 207 (1957).
[160] J. Tomizawa, *Virology* **6**, 55 (1958).
[161] S. Lacks and F. Gros, unpublished results (1959).

These observations have been confirmed by several authors.[111] It is interesting to note that, in *B. cereus*, azaguanine gives rise to an accumulation of RNA without protein synthesis and that the RNA thus formed is metabolically stable.[108] Similarly, the RNA accumulated during methionine starvation in certain mutants of *E. coli* K12, does not breakdown when growth is resumed on addition of methionine.[158]

The metabolic instability of the chloramphenicol RNA might be related to its peculiar physicochemical properties.[42, 43] In fact, treatment of bacteria by chloramphenicol results in a drastic change in the distribution of the cytoplasmic particles. Extracts of treated cells contain very little of the 50 *S* and 30 *S* component but appreciable amounts of a new type of particle, called 15 *S* which contains 75% RNA and 25% protein as compared with 60% and 40% respectively, in the normal particles. Since the 15 *S* component contains 70 to 80% of the RNA built up after chloramphenicol addition, its RNA is probably associated with preexisting protein derived from the normal 30 *S* or 50 *S* particles. Unlike the normal particles, the 15 *S* is very sensitive to ribonuclease probably on account of insufficient protection of the RNA by the protein envelope. This ribonuclease sensitivity seems to be concerned with the metabolic instability of the chloramphenicol RNA reported above.

It may well be that the chloramphenicol RNA, or more precisely the 15 *S* particle, is an artifact resulting from the cessation of protein synthesis, or that it is a natural "free" intermediate of the particle, which would accumulate when its protein counterpart could not be formed. There are good reasons to believe that the 15 *S* particles of Nomura and Watson are identical with the natural intermediate of the ribosomes discovered by Roberts *et al.*,[51] which consists of free, highly polymerized RNA associated with a small amount of protein, and which accumulates after chloramphenicol addition.

4. Necessity of Amino Acids for the Synthesis of RNA

Since RNA synthesis can take place in the presence of chloramphenicol it might be expected also to occur when inhibition of protein formation results from another cause, such as the omission of an essential amino acid. Contrary to expectation, an amino acid auxotroph, when starved of its essential amino acid, does not synthesize any nucleic acid.[27, 28, 162] This observation, which has been confirmed for a large variety of auxotrophs, has been interpreted as a proof that amino acids are necessary for the synthesis of RNA at a stage where they are not yet assembled into a peptide chain. This interpretation is strengthened by the following experiment: if,

[162] M. K. Sands and R. B. Roberts, *J. Bacteriol.* **63**, 505 (1952).

to a suspension of an auxotroph previously starved of its essential amino acid, the missing amino acid is added, normal growth is restored and RNA synthesis is proportional to the amount of the amino acid added. If, to the same suspension, the amino acid is added in the presence of chloramphenicol, RNA synthesis is restored equally well, although this amino acid cannot be incorporated into protein.[27, 28, 163] In addition, restoration of RNA synthesis in the presence of chloramphenicol can be observed on addition of traces of the essential amino acid.[28]

This experiment shows (1) that amino acids play a role as such (in the "free" or "activated" form) during the synthesis of RNA; (2) that amino acids function as catalysts in the synthesis of RNA; calculation shows that for each molecule of essential amino acid added after starvation and in the presence of chloramphenicol, at least ten nucleotides are incorporated into RNA.[28]

These results are considered to indicate that bacteria, and possibly all cells, contain common precursors for protein and RNA. These precursors could consist of complementary associations between amino acids and specific oligonucleotides. In order for the oligonucleotides to be distributed at the surface of a specific template in the right sequence, all the amino acids to which these nucleotides are attached should have to be distributed also according to a complementary sequence. The lack of an amino acid would result in an interruption in the sequence of nucleotides. On the other hand the mechanism for joining the nucleotide subunits would function even if the complementary amino acids were not bound by peptide linkages, providing all are present in the right sequence at the surface of the template.

Other workers have interpreted the necessity of the amino acids for RNA synthesis in a completely different manner, assuming the formation of a specific protein, the synthesis of which would be insensitive to chloramphenicol, but whose presence would be required for RNA synthesis.[164] The difficulties associated with this interpretation have already been discussed elsewhere.[28]

It has also been suggested that free amino acids could be involved in the recycling of the sRNA after it has undergone specific molecular splitting during the adaptation of the amino acids on the template.[165] This interpretation could not account, however, for the mechanism of RNA synthesis during chloramphenicol inhibition, since most of the chloramphenicol RNA —the formation of which depends on the presence of free amino acids— exists in a particulate form (15 *S*).

[163] M. Yčas and G. Brawerman, *Arch. Biochem. Biophys.* **68,** 118 (1957).

[164] A. I. Aronson and S. Spiegelman, *Biochim. et Biophys. Acta* **29,** 214 (1958).

[165] F. H. C. Crick, *Symposia Soc. Exptl. Biol.* **12,** 138 (1958).

V. Protein Synthesis After Selective Destruction or Removal of a Nucleic Acid

The fact that specific protein can be formed in a cell without a corresponding increase in its nucleic acid content, restricts the choice of hypotheses on the nature of the relationship between protein and nucleic acid. This relationship appears therefore to be catalytic rather than stoichiometric, and it suggests that the capacity to synthesize protein depends on the presence or the integrity of the nucleic acid rather than on its net synthesis.

1. Enzyme and Protein Synthesis After Transmutation of the Phosphorus Atoms in Bacterial DNA

The size of the bacteria does not allow enucleation experiments of the type performed with algae or amoebae. In enucleated portions of *Acetabularia mediterranae*, protein synthesis can take place for quite a long time.[166, 167] For example, $C^{14}O_2$ or glycine-C^{14} are incorporated at a normal rate into the protein fraction and a net increase in the protein mass is also observed.[168] RNA synthesis has also been reported in the nucleated fragments.[167] In amoebae, the situation is rather different, since, according to Mazia,[169] removal of the nucleus leads to an immediate diminution (40 to 50%) in the rate of protein synthesis as measured by incorporation of radioactive methionine. The situation in relation to RNA synthesis is still debatable, since, according to Skreb[170] and to Plaut and Rustad,[171] enucleation of amoebae does not suppress incorporation of adenine-C^{14} or orotic acid-C^{14} into the RNA, while according to other workers[172] it suppresses the incorporation of uracil-C^{14}.

Even if conflicting results and the possibility of artifacts have to be taken into consideration, it seems reasonable to conclude that in the absence of its nucleus, the cytoplasm can still incorporate amino acids into peptide linkages. The mechanism by which an enucleated fragment can perform enzyme synthesis has also been studied.

According to Baltus,[173] enucleated halves of *A. mediterranae* can synthesize aldolase at an almost normal rate. It is not known, however, whether such enucleated fragments can form the enzymes which are normally asso-

[166] J. Brachet and H. Chatrenne, *Nature* **168,** 950 (1951).
[167] J. Brachet and H. Chantrenne, *Cold. Spring Harbor Symposia Quant. Biol.* **21,** 329 (1956).
[168] F. Vanderhaeghe, *Biochim. et Biophys. Acta* **15,** 281 (1954).
[169] D. Mazia and D. M. Prescott, *Biochim. et Biophys. Acta* **17,** 23 (1955).
[170] R. Skreb, quoted by Brachet and Chantrenne.[167]
[171] W. Plaut and R. C. Rustad, *Biochim. et Biophys. Acta* **33,** 59 (1959).
[172] D. M. Prescott, *Exptl. Cell Research* **12,** 196 (1957).
[173] E. Baltus, *Biochim. et Biophys. Acta* **33,** 337 (1959).

ciated with the microsomal particles of the cytoplasm. Mammalian reticulocytes also offer an example of naturally occurring enucleated cells which not only can incorporate amino acids into protein,[174, 175] but can also synthesize a specific protein, hemoglobin.[176, 177] These results seem to indicate that in algae or in reticulocytes, specific protein can be formed in the absence of DNA.

Interesting information concerning the role of the nucleus in the synthesis of specific proteins has also been brought forward recently[63, 178] by studying the metabolic consequences of the radioactive decay in bacteria which have incorporated highly labeled P^{32} into the nucleic acids. As will be seen, however, there is a certain discrepancy between the conclusion of such experiments and the data from enucleation experiments reported above. Usually bacteria are grown for a few generations in a medium containing highly labeled phosphorus (specific radioactivity 200 to 500 mc. per milligram). They are then frozen at $-190°$ C. and stored to allow the incorporated atoms of phosphorus to decay. At various intervals, the suspension is thawed and bacteria are assayed for their ability to produce colonies and to synthesize protein, enzymes, and RNA. Since the use of thymine-requiring mutants permits the labeling either of RNA alone, or of both nucleic acids, it is possible to determine whether the modifications observed in the metabolic pattern have to be correlated with alterations in RNA or DNA.

The results of these ingenious experiments can be summarized as follows. Bacteria which have incorporated P^{32} into their nucleic acids lose the ability to produce colonies according to a precise function of the P^{32} decay. The fraction of the surviving bacteria declines according to "multiple hit" kinetics, as if each individual contained a finite number of sensitive units, the preservation of which would permit its survival. Calculation shows that each bacterium contains three such units, that is, as many as nuclei. Accordingly, in uninucleated bacteria, death is a simple exponential function of the fraction of P^{32} atoms decayed. Loss of reproductive capacity in bacteria therefore results from disintegration of the DNA. Moreover, when *E. coli* 15T is labeled in both nucleic acids (by incorporation of P^{32} in the presence of thymine), its rate of inactivation is 4 times faster than when RNA is the only nucleic acid labeled.

Alteration of the DNA by decay of P^{32} also results in the loss of enzyme-forming capacity. Thus, after incorporation of P^{32}, the capacity to form

[174] I. M. London, D. Shemin, and D. Rittenberg, *J. Biol. Chem.* **183,** 749 (1950).
[175] H. Borsook, C. L. Deasy, A. J. Haagen-Smit, G. Keighley, and P. H. Lowy, *J. Biol. Chem.* **196,** 669 (1952).
[176] A. Nizet and S. Lambert, *Bull. soc. chim. biol.* **35,** 771 (1953).
[177] S. B. Koritz and H. Chantrenne, *Biochim. et Biophys. Acta* **13,** 209 (1954).

β-galactosidase, constitutively or adaptively, declines at the same rate as does the ability to produce colonies. This suggests that the DNA (or the nucleus) functions as a unit in the synthesis of specific enzymes, since, for one particular enzyme, destruction of any part of the DNA structure (and not only the relative destruction of the gene) suppresses the synthesis of this enzyme.

While a few phosphorus disintegrations in the DNA may drastically reduce the capacity of a bacterium to synthesize a specific enzyme, they do not reduce the formation of protein or RNA at a comparable rate. These results give the general impression that DNA could exert an indirect control on the capacity to form RNA and polypeptides, while controlling directly the organization of polypeptides into specific proteins.

If this is so, it would be expected that injection of intact DNA molecules into a bacterium which had lost its ability to form enzymes, as the result of P^{32} decay, would restore this ability immediately. Such an experiment has not been performed as yet with bacterial DNA (by transformation or recombination) but it has been observed that after infection of such "P^{32} inactivated" bacteria by a bacteriophage, the infected bacteria synthesize phage protein, that is protein specific for the newly injected DNA in the usual way.[178]

This experiment has to be considered in the light of our knowledge that in bacteria infected by T^2 phage, the chromosomes are destroyed and the host becomes unable to synthesize protein specific for the missing DNA,[179] although it now produces protein structurally related to the infecting DNA. On the other hand, when the integrity of the DNA is preserved, as after an infection by a temperate phage, synthesis of bacterial enzymes can still be performed.[180]

Interestingly, the fact that DNA integrity is required for enzyme synthesis in *E. coli* is in conflict with the data obtained from enucleation experiments in higher organisms.[173, 176] The mechanism for transferring the genetic information to the protein-forming site, although it may be the same in bacteria as in more complex cells, would appear to function in a discontinuous manner in the latter case, and in a continuous manner in the former case.

2. RNA Content and Rate of Protein Synthesis

The problem of the part played by RNA as an intermediate between DNA and the protein-forming machinery has been studied by two distinct methods. The first method is to establish whether there exists a direct rela-

[178] G. S. Stent and C. R. Fuerst, *Advances in Biol. and Med. Phys.* **7**, in press.
[179] J. Monod and E. Wollman *Ann. inst. Pasteur* **73**, 937 (1947).
[180] L. Siminovitch, *Ann. inst. Pasteur* **84**, 265 (1953).

tionship between the RNA content of a cell and the rate at which it synthesizes protein. The second is based on metabolic studies of bacteria whose RNA has been partially or completely destroyed by ribonuclease treatment.

It is known from Brachet's early observations[181] that the cells of an organism which are richest in RNA are the most rapid in growth and division. While this observation had the great merit of drawing attention to the possible role of the RNA in protein synthesis, it did not define, however, the precise relationship between RNA and protein. More precise and quantitative information might be obtained by trying to correlate protein synthesis and RNA content in a given cell, rather than amongst cells of different origin. This type of study has been made with animal cells[181] and on a more extensive scale with bacteria by relating the growth rate to the cellular RNA content.

Earlier experiments involved an examination of the variation in the RNA content in a bacterial culture at different growth phases. It became clear[182] that bacteria contain less RNA during the stationary phase than during the exponential phase of growth. However, the interpretation of the relationship between growth rate and cellular RNA content during the transition from the lag period to the exponential phase is complicated by a series of factors such as size variations in individual bacteria.

A more satisfactory approach is to consider only bacteria in the steady state (exponential phase), but exhibiting different growth rates according to the nature of the carbon source or to the kind of supplements added to the medium. This has been done by several authors.[139, 183, 184] The kind of relationship obtained is illustrated in Fig. 5.

For rapidly growing cells (growth constant K between 0.6 and 1.1) the growth rate is proportional to the RNA content (expressed by the ratio RNA/protein). For lower values of K (between 0.2 and 0.6) the relationship is no longer linear and the RNA content varies very little while the growth rate varies appreciably.

Herbert[185] has recently reported very similar results in studying the effect of growth rate on nucleic acid and protein content, and mean cell mass of *A. aerogenes* grown in a chemostat on a mineral medium with various limiting concentrations of glycerol.

The results are compatible with the assumption[139] that the protein-forming system is composed of a ribonucleoprotein (or at least contains RNA

[181] J. Brachet, "The Nucleic Acids" (E. Chargaff and J. N. Davidson, eds.), Vol. II, p. 475 Academic Press, New York, 1955.

[182] B. Malmgren and C. G. Heden, *Acta Pathol. Microbiol. Scand.* **24**, 437 (1948).

[183] P. C. Caldwell, E. L. Mackor, and C. N. Hinshelwood, *J. Chem. Soc.* p. 3151 (1950).

[184] R. Jeener, *Biochim. et Biophys. Acta* **8**, 125 (1952).

[185] D. Herbert, *7th Intern. Congr. Microbiol., Stockholm* p. 381 (1959).

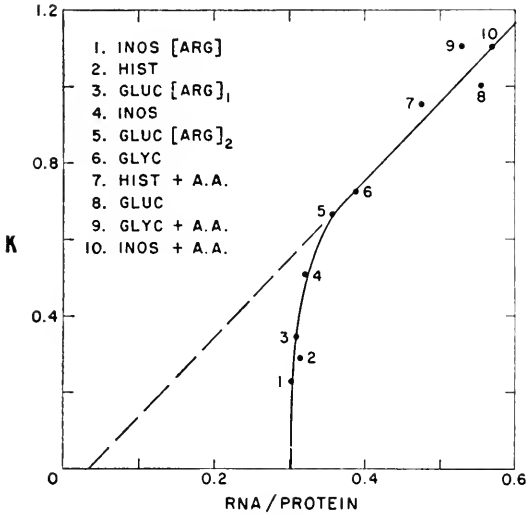

FIG. 5. Relationship between the growth rate and the RNA content of a bacterium grown on different media [B. Magasanik, A. K. Magasanik, and F. C. Neidhardt, *in* "The Regulation of Cell Metabolism," A Ciba Symposium, p. 334. Churchill, London, 1959].

as a fundamental constituent), but with the following restrictions: when the carbon source is not very efficient or, more generally, when the composition of the medium is not optimal, (K between 0.2 and 0.6), it is the cellular content in "building blocks" (amino acids "free" or "activated") and not that of the RNA which limits the growth rate. On the contrary, when the composition of the medium is rich enough to provide protein precursors at a very high rate, the RNA content becomes the limiting factor for the rate of protein synthesis.

If a cell can synthesize protein at a rate proportional to its RNA content, one must expect that RNA destruction leads to almost complete cessation of the protein synthesizing abilities of the cell.

Two different techniques have been used selectively to alter or destroy RNA *in vivo*. The first is based on a selective labeling of the RNA by very highly radioactive P^{32}. The results of such an experiment have already been reported. They show that the capacity to synthesize protein and enzymes declines regularly with the number of phosphorus atoms which have undergone decay in the RNA. This decline is, however, 4 times slower than when both DNA and RNA are labeled.[63, 178]

Complete destruction of the cellular RNA by ribonuclease should lead in principle to more easily interpetable results and this constitutes the second approach which has been followed extensively by Brachet and his school[181]

who have observed that it is possible to destroy RNA *in vivo* by adding ribonuclease to intact cells of various types (onion roots, amoebae, etc.). The effects of ribonuclease on bacterial RNA have been intensively studied by Groth,[186] and by Jeener[187] with *B. megaterium*, by Jerne and Maløe,[188] and by Françoise Gros[189] in *E. coli*.

In amoebae, ribonuclease seems to penetrate according to a mechanism of pinocytosis[190] and the amount of enzyme taken up depends on the external concentration, and can reach 2×10^{11} molecules per cell.

In certain species of bacteria such as *B. megaterium*, growth in a synthetic medium is prevented by ribonuclease; others such as *E. coli* are sensitive only when treated in the absence of salts.[188, 189] It has been found that the action of ribonuclease on *E. coli* takes place in two stages: (1) formation of a complex between ribonuclease and bacteria (and probably between ribonuclease and RNA); and (2) destruction of the RNA.

The first stage is inhibited by salts, whereas the second cannot proceed in their absence. By adding ribonuclease to bacteria in distilled water and by interrupting the first phase by salt addition, followed by incubation for a sufficient period of time to permit the second phase to proceed, one can easily follow the effect of ribonuclease on *E. coli*. Under these conditions, the amount of RNA declines exponentially with the time of contact between enzyme and cells during the first phase.

The metabolic activities of cells whose RNA has been partially or fully destroyed have been extensively studied. In amoebae or in plants, partial RNA destruction by ribonuclease does not affect the energy-yielding mechanisms,[191] since neither the ATP content nor O_2 consumption are sensibly modified. Similar conclusions apply to bacteria[189] in relationship to their capacity to oxidize succinate or glucose. In regard to the ability of synthesizing proteins, it is observed that onion roots or amoebae, which have lost their basophilia after ribonuclease treatment, are unable to incorporate C^{14}-amino acids into protein.[191]

In *E. coli*, ribonuclease influences the ability to synthesize protein by two distinct mechanisms.[189] Even after a very short time of contact with negligible destruction of the RNA, an immediate drop of about 50 per 100 in the ability to synthesize protein (as measured by S^{35} incorporation) is observed. When the incubation proceeds for a longer time a gradual destruc-

[186] D. P. Groth, *Biochem. et Biophys. Acta* **21,** 18 (1956).

[187] R. Jeener, *Biochim. et Biophys. Acta* **32,** 99 (1959).

[188] N. K. Jerne and O. Maaløe, personal communication (1958).

[189] Françoise Gros, unpublished data (1960).

[190] J. Brachet, M. Briers, L. Ledoux, A. Pileri, V. Schumaker, Y. Thomas, and F. Vanderhaeghe, *in* "Radioisotopes in Scientific Research," Vol. 3, p. 285. Pergamon Press, London, 1957.

[191] J. Brachet, "Biochemical Cytology." Academic Press, New York, 1957.

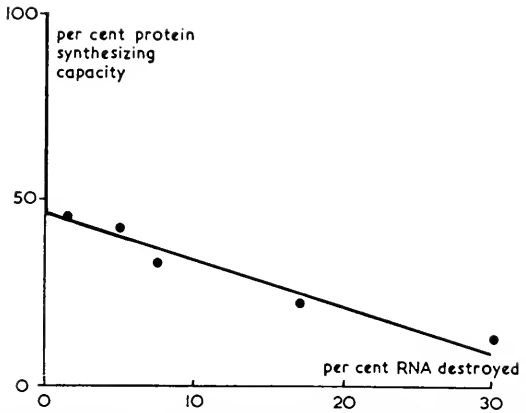

FIG. 6. Protein synthesizing capacity of bacteria as a function of RNA destruction by ribonuclease. To a suspension of *E. coli* (strain K12) in distilled water and containing 150 μg. of bacteria (dry weight) per milliliters, 200 μg. of crystallized ribonuclease per milliliter are added. After various periods of incubation at 15°C., aliquots are sampled and incubated 10 minutes more at 37°C. in the presence of 1 *M* NaCl 0.1. Optical densities at 420 mμ, and RNA content per cell are measured at the end of this second incubation period. Treated cells are then chilled, centrifuged, and resuspended in mineral medium containing per milliliter, 10 μg. of S^{35} in the form of H_2SO_4 (specific activity 20,000 c.p.m. per microgram), and glucose (0.2%). Optical densities are adjusted in each case to that reached after ribonuclease treatment, and cells are incubated at 37°C. with aeration. Initial rates of protein synthesis are determined by measuring the tangents to the various curves of S^{35} incorporation into the protein fraction. Results of Fig. 6 are expressed in relative initial rates of S^{35} incorporation as a function of RNA destruction by ribonuclease.

tion of the RNA occurs, while the rate of protein synthesis goes on diminishing proportionally to the amount of RNA destroyed (see Fig. 6). The ability to synthesize β-galactosidase follows exactly the same kinetics. The first rapid drop observed may be based on a more or less specific interference by the ribonuclease with negatively charged components of the cytoplasm (perhaps the soluble RNA) since it is known that protamine, a basic protein of no enzyme activity, can reduce protein synthesis both in living root tips[192] and in bacteria[189]; the slower decline in protein-synthesizing ability which takes place after a longer period of incubation, is probably due to a gradual destruction of the RNA particles.

It is interesting to note that while alteration in DNA has a more rapid influence on the ability to synthesize β-galactosidase than the capacity to form peptide material, destruction of RNA influences both these properties at the same rate. It appears therefore that the rate of protein formation, rather than the specificity of the protein formed, is under the control of the stable RNA.

[192] B. P. Kaufmann and N. K. Das, *Chromosoma* **7**, 19 (1955).

VI. General Discussion

The general concepts on protein synthesis, which can be deduced from an examination of the experimental results analyzed above, enable some answers to be made to the following questions:

(*a*) What do we know about the intermediate steps in amino acid incorporation into peptide chains?

(*b*) What is the nature of, or what are the main components of, the protein-forming system?

(*c*) How can we visualize the transfer of genetic information to the protein-forming system?

(*d*) How does a cell control its rate of protein synthesis?

1. Intermediate Steps in Protein Formation

It has been suggested from the results of *in vitro* experiments that amino acid activation could be the first biochemical event in the incorporation of amino acids into peptide chains and this hypothesis agrees in certain respects with the results of *in vivo* studies.

The arguments supporting the occurrence of amino acid activation *in vivo* during protein synthesis are as follows:

(1) All the known natural amino acids which compose a protein can be activated.[31]

(2) The steric amino acid analogs, which are known to replace *in vivo* the corresponding natural amino acids in proteins,[193-195] can be activated by those enzymes which are known to activate specifically their natural counterpart. This occurs in the case of the tryptophan analogs (azatryptophan, tryptazan),[196] of some phenylalanine analogs (parafluorophenylalanine),[197] and of selenomethionine.[198] 5-Methyltryptophan which is not incorporated into protein[193, 194] cannot be activated.[196] According to Loftfield *et al.*, however,[199] the absence of incorporation may be due, sometimes, to reasons other than the failure in activation.

In favor of the participation of the sRNA in protein biosynthesis several observations, mostly based on considerations of kinetics can be quoted:

(1) *In vivo*, radioactive amino acids are incorporated earlier into the sRNA than into the protein.[30, 41]

(2) Amino acids bound to the sRNA undergo a very fast renewal during

[193] A. B. Pardee, *Biochim. et Biophys. Acta* **21**, 406 (1956).
[194] R. Munier and G. N. Cohen, *Biochim. et Biophys. Acta* **21**, 592 (1956).
[195] D. B. Cowie and G. N. Cohen, *Biochim. et Biophys. Acta* **26**, 252 (1957).
[196] N. Sharon and F. Lipmann *Federation Proc* **16**, 246 (1957).
[197] A. Ullmann and F. Gros, unpublished data (1958).
[198] B. Nisman and M. L. Hirsch, *Ann. inst. Pasteur* **95**, 615 (1958).
[199] R. B. Loftfield, L. I. Hecht, and E. A. Eigner, *Federation Proc.* **18**, 276 (1959).

the growth of *E. coli*. The rate of this renewal depends to a large extent on the capacity of the cell to synthesize protein. Thus, the bound amino acids are actually utilized for the formation of protein molecules.

(3) Direct transfer of radioactivity can be observed *in vivo*[41] (as well as *in vitro*[30]) from sRNA labeled with radioactive amino acids, to protein fractions.

There is however a possibility that other pathways than amino acid activation and transfer to sRNA exist, either for the formation of peptide linkages in general, or at least for the synthesis of part of the cellular protein. The protein of the membrane or the basic protein of the nucleoprotein particles could belong to this last category. This restriction to the general scheme previously described, is suggested by the results of various experiments with subcellular fractions of bacteria (cf. Chapter 37 by Hoagland on protein synthesis in "*in vitro* systems").

2. Nucleic Acid as Constituents of the Protein-Forming System

The sRNA cannot be considered as a template for the formation of protein owing to its small size (200 nucleotides), nor does it function as a template for the synthesis of peptides, since it is known that *in vivo*, as *in vitro*, only one single molecule of amino acid is attached per molecule of sRNA,[39, 41] and also since the possibility of peptides acting as protein precursors is excluded by several experiments. Therefore the amino acid sequence has to be determined by templates of much higher molecular weight, and the role of the sRNA can be visualized rather as that of a "steric adaptor" of the amino acids to specific loci of this template.[165] Several now well-known hypotheses predict how the base sequence of highly polymerized nucleic acid can "code" the amino acid sequence of the proteins. The critical question is the nature of the nucleic acids which most probably serve as direct template for the synthesis of protein. Put in more precise terms, what are the respective roles of DNA and RNA in protein synthesis?

It is obvious from genetic evidence that the potentiality for a cell to make a specific protein is located in very precise regions of the DNA molecules. Therefore, the simplest hypothesis concerning the mechanism of protein synthesis could be that DNA serves directly as a template for the assembly of free or activated amino acids into a specifically ordered polypeptide chain.[199a]

[199a] The recent observation by Pardee, Jacob, and Monod[140] on enzyme synthesis during genetic recombination in *E. coli* K12 is relevant to this hypothesis. When a strain Hfr injects its lac^+ gene into the cytoplasm of an F^- strain, which is lac^-, β-galactosidase synthesis starts immediately in the zygote and with its maximal rate. Formation of the new protein does not appear to require a macromolecular replicate of the DNA; or at least this replicate would be formed only once by DNA and very rapidly.

However, though no strong argument permits at present to reject such an hypothesis, there is a set of observations showing that the primary site of protein synthesis is RNA rather than DNA.

(1) Kinetic studies show that in animal cells amino acids are incorporated first into "RNA rich" particles (Chapter 37).

(2) The rate of protein synthesis is under certain conditions directly proportional to the RNA content of the cell.[139, 189]

3. Transfer of Information from the DNA

Until now the scheme of protein synthesis which is tentatively proposed can be summarized as follows: activated amino acids are transferred to the sRNA,[30] which adapts them sterically to a template composed of ribonucleoprotein particles. At that stage, the amino acids are incorporated into a peptide chain which is stripped off the template. The free template can then act catalytically.

If it be assumed that RNA is a template for the assembly of the peptide chains, DNA must act at some appropriate stage in controlling the sequence of the amino acids in this chain, since it has been observed that some mutations lead to the formation of altered proteins e.g., hemoglobin,[200] alkaline phosphatase[201] or β-galactosidase.[202] The most logical assumption is that DNA controls protein specificity through the RNA by forming RNA replicates of its own structure. While nothing is known concerning the biochemical relationships between DNA and RNA synthesis, there are many indications, however, suggesting that the nucleus is the RNA forming site[203, 204] or that the presence of DNA is necessary for RNA synthesis.

4. Regulation of the Rate of Protein Synthesis

The rate of protein synthesis depends on at least two types of factors: the concentration of precursors immediately available (amino acids), and the RNA content.

If, as is suggested by certain experiments, amino acids are necessary as such for the synthesis of RNA,[27, 28] it is possible to visualize a mechanism for controlling the rate of protein synthesis. When this rate tends to in-

[200] V. M. Ingram, *Nature* **180**, 326 (1957).

[201] C. Levinthal, *Revs. Modern Phys.* **31**, 249 (1959); Garen, A. *in* "Symposium on Microbial Genetics," London (April 1960)." Cambridge Univ. Press, London and New York, 1960.

[202] J. Monod, *Angew. Chem.* **71**, 685, (1959).

[203] D. Mazia, "Enzymes, Units of Biological Structure and Function" (A. E. Gaebler, ed.), p. 261. Academic Press, New York, 1956.

[204] L. Goldstein and W. Plaut, *Proc. Natl. Acad. Sci. U. S.* **41**, 874 (1955).

crease, the internal concentration of amino acids tends to decrease, and therefore the rate of RNA synthesis also decreases. This should lead to a decrease in the rate of protein formation. This kind of control however, is not very efficient owing to the catalytic nature of the role exercised by amino acids in RNA synthesis[28] (see also the discussion by Magasanik *et al.*[139]).

The rate at which protein precursors are produced offers a much more direct method of control of the rate of protein formation. It has, for example, already been shown that a given metabolite inhibits the activity of, or represses the synthesis of, the enzymes which catalyze its own formation.[205-209]

Although enzyme repression is certainly not as efficient for controlling the immediate supply of protein precursors as is the inhibition of the activity of biosynthetic enzymes, this phenomenon in itself poses very interesting problems concerning the interaction of a metabolite with the protein-forming machinery.

Thus, repression does not usually concern the synthesis of only one enzyme in the metabolic sequence, but of many or of all of the enzymes of the chain.[210] How can a metabolite of simple structure regulate the rate of synthesis of many specific proteins with very different affinities for it? What is the exact nature of the endogenous repressor, and at what stage of protein biosynthesis does it act?

A discussion of these problems would be too far reaching at the present time, but repression is certainly one of the most complex and most interesting problems in relation to the biosynthesis of protein in the intact cell.

VII. Addendum

Since the writing of our article, a great number of new experiments have shed light on some points of main importance in the problem of protein synthesis.

For instance, Roberts and his group[211] have finally succeeded in showing that very short contact (about 10 seconds) of logarithmically growing cells of *E. coli* with S^{35}-labeled sulfate leads to early appearance of radioactivity in the ribosome fraction, and that such labeling precedes the one of the

[205] H. J. Vogel, "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 276. Johns Hopkins Press, Baltimore, 1957.

[206] A. B. Pardee, *in* "The Regulation of Cell Metabolism," A Ciba Symposium, p. 295. Churchill, London 1959.

[207] J. Monod and G. Cohen-Bazire, *Compt. rend. acad. sci.* **236,** 746 (1953).

[208] M. Cohn, G. N. Cohen, and J. Monod, *Compt. rend. acad. sci.* **236,** 746 (1953).

[209] W. Maas and L. Gorini, *Federation Proc.* **16,** 215 (1957).

[210] G. Cohen and Jacob, *Compt. rend. acad. sci.* **248,** 3490 (1959).

[211] E. T. Bolton, R. J. Britten, D. B. Cowie, B. J. McCarthy, K. McQuillen, and R. B. Roberts, Carnegie Institution Year Book 58, p. 259 (1958–1959).

soluble protein. Radioactivity can also be displaced from ribosomes to soluble protein by incubating cells labeled for a very short time in the absence of the tracer.

A very elegant demonstration of the early formation of protein in the microsomal particles and of the RNA in the nucleus has been given by Zalockar[212] in radioautographing cells of *Neurospora*, after incorporation of tritiated precursors of these macromolecules. Similar results have also been obtained by Sirlin[213] in studying the sites of protein and nucleic acid synthesis in salivary glands of *Surittia*.

[212] M. Zalokar, *Exptl. Cell Research* **19,** 114 (1960).
[213] J. L. Sirlin, *Exptl. Cell Research*, **19,** 177 (1960).

Chapter 39

Agents Which Influence Nucleic Acid Metabolism

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I. Introduction

The elucidation of the chemical structure of most of the components of both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) and the pathways of their biosynthesis made possible the design and testing of a variety of compounds which may alter these pathways or modify the composition of the resultant macromolecules. Appropriate alterations in biosynthetic pathways have several practical consequences. First, they allow a more detailed study of individual reactions and pathways, as well as investigations of factors which are important in their control. Second, they

afford a means of influencing rates of cellular reproduction. The implications of such external control techniques to the therapy of bacterial, viral, and neoplastic diseases are obvious. Third, they enable certain analogs of the purines and pyrimidines to be introduced into the nucleic acids, a feature which provides an opportunity for studying the functional significance of various portions of these macromolecules with particular reference to the role of certain types of RNA in the activation of amino acids for protein synthesis, and the role of specific varieties of DNA in the transmission of genetic information in cellular reproduction. In this chapter are discussed a selection of some of the more interesting compounds shown (*a*) to have a relatively specific action on the biosynthesis and incorporation of purine- or pyrimidine-containing compounds, (*b*) to be incorporated themselves into the nucleic acids, or (*c*) to resemble a component of the nucleic acids and to possess striking biological activity. Compounds will be discussed individually in groups chosen either for site of action or general structure (see Table of Contents).

Of the agents to be discussed, several have been designed and synthesized with the specific objective of attaining a blockade of a known reaction or sequence of reactions in nucleotide synthesis. Indeed, certain predictions now are possible concerning some of the structural alterations which it is reasonable to consider, for several compounds have been prepared on this basis which are sufficiently similar to normal metabolites that they either function in or inhibit specific metabolic reactions. Typical of these replacements has been the substitution of halogens for a hydrogen atom or a methyl group in position 5 of the pyrimidines (e.g., fluoro, chloro, bromo, and iodo derivatives of uracil), replacement of oxygen functions by sulfur or amino groups (e.g., mercaptopurine, thioguanine, aminopterin), or alteration in basic ring structures by replacement of carbon by nitrogen (e.g., azaguanine, azauracil, azathymine). The biological activities of some of these and of other compounds were discovered through screening procedures designed to disclose inhibitory activity for the growth of microorganisms or neoplastic tissue, and only later were specific actions on nucleic acid metabolism uncovered (e.g., with azaserine and 6-diazo-5-oxo-L-norleucine). Many experimental techniques for screening potential purine and pyrimidine antimetabolites have been described and these will continue to be of great value in further studies in this area.[1-5] At present these systems are primarily capable of identifying compounds which alter the biosynthesis of nucleotides. It is to be hoped, however, that with the aid of recently developed

[1] G. H. Hitchings and G. B. Elion, *Cancer Research Suppl.* **3,** 66 (1955).
[2] J. D. Davidson and B. B. Freeman, *Cancer Research Suppl.* **3,** 97 (1955).
[3] G. A. LePage and J. L. Greenlees, *Cancer Research Suppl.* **3,** 102 (1955).
[4] C. Heidelberger and R. A. Keller, *Cancer Research Suppl.* **3,** 106 (1955).
[5] J. E. Stone and V. R. Potter, *Cancer Research* **17,** 794 (1957).

knowledge of reactions involved in the formation of macromolecules and their roles in cellular function, a broader outlook may be employed and that new concepts in the design of antimetabolites eventually will evolve.

It has been interesting as well as disquieting to observe the facility with which bacterial and neoplastic populations of cells become immune to the action of most of the compounds to be discussed. Through investigation of the mechanisms of resistance to nucleotide antimetabolites, shifts in the balance between the utilization of preformed purine or pyrimidine derivatives and their synthesis *de novo* have been detected. The many factors which control such balances are at present largely unknown and increased research endeavor will be needed to disclose them and to explain their functional interrelationships.

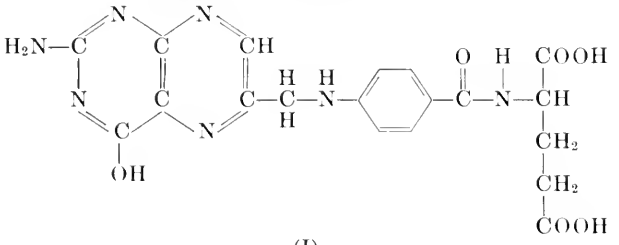
As will be apparent in the discussion of individual agents, it is impossible to consider the sites of actions of antimetabolites without giving proper attention to their physiological disposition, since the primary motivation for the development of these compounds is the treatment of disease states and the more complete elaboration of the biochemical foundations of such states. Thus, the failure of some of the extremely active compounds to be transported into some areas of the body (e.g., the brain), into which neoplastic cells may penetrate very early following the development of a malignancy, has limited very severely their potential effectiveness. In addition, metabolic alteration plays a major role in the activation of a number of the agents to be discussed; this generally proceeds through the enzymic conversion of the analog to a fraudulent mononucleotide, oligonucleotide or even nucleic acid, in order to form the presumed biologically active inhibitor. Catabolism of certain analogs, though frequently neglected, may account for the limitation of the action of these agents in intact animals, as does the velocity of renal excretion in certain cases. Finally, variations in tissue distribution and in the metabolic activities of various cell types may give rise to a selectivity of action which could not be predicted from studies with isolated enzymes.

Several excellent reviews have appeared on the various aspects of the topics to be discussed and should be consulted for detailed information concerning these agents.[6-10] The present chapter is not intended to offer a complete coverage of the literature; rather, it attempts to present some of the highlights of the research on compounds of either chemotherapeutic or more fundamental interest which influence some aspect of nucleic acid metabolism.

[6] J. A. Montgomery, *Cancer Research* **19**, 447 (1959).
[7] R. E. F. Matthews, *Pharmacol. Revs.* **10**, 359 (1958).
[8] H. G. Mandel, *Pharmacol. Revs.* **11**, 743 (1959).
[9] H. E. Skipper and L. L. Bennett, Jr., *Ann. Rev. Biochem.* **27**, 137 (1958).
[10] W. Shive and C. G. Skinner, *Ann. Rev. Biochem.* **27**, 643 (1958).

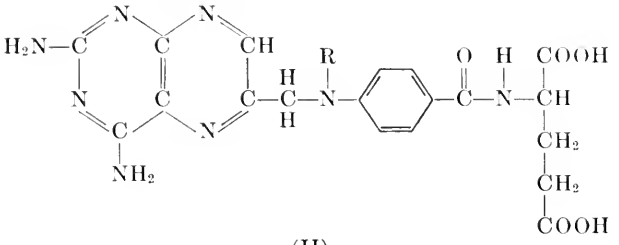
II. Compounds Which Interfere with the Formation of Folic Acid-Derived Coenzymes

1. Aminopterin (4-amino-4-deoxypteroylglutamic acid) and Amethopterin (4-amino-4-deoxy-10-methylpteroylglutamic acid; Methotrexate)



(I)

Pteroylglutamic (folic) acid[11-18]



(II)

Aminopterin (R=H) and amethopterin (R=CH_3)

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[11] The term "folic acid" has often been used generically to include various compounds with related biological activites; however, in this chapter, for reasons of brevity, the term is used synonymously with pteroylglutamic acid. Also, when appropriate, the simplified abbreviations of Huennekens *et al.*[12] are employed: folic acid, F; dihydrofolic acid, FH_2; tetrahydrofolic acid, FH_4; the 5-formyl derivative of FH_4, f^5FH_4, is usually referred to as folinic acid, rather than citrovorum factor (CF), since the organism used in the determination of the factor is now designated *Pediococcus cerevisiae* (ATCC 8081) rather than *Leuconostoc citrovorum*. The term *leucovorin* is retained for the synthetic form of f^5FH_4, of which only one of the two enantiomorphs exhibits the biological activity of folinic acid. Some of the earlier and also the more recent reviews in this field are listed below.[12-18]

[12] F. M. Huennekens, M. J. Osborn, and H. R. Whitely, *Science* **128,** 120 (1958).

[13] A. D. Welch and R. W. Heinle, *Pharmacol. Revs.* **3,** 345 (1951).

[14] A. D. Welch and C. A. Nichol, *Ann. Rev. Biochem.* **21,** 633 (1952).

[15] A. D. Welch, *in* "Enzymes: Units of Biological Structure and Function" (O. H. Gaebler, ed.), p. 547. Academic Press, New York, 1956.

[16] G. R. Greenberg and L. Jaenicke, *in* "Chemistry and Biology of Purines" (G. E. W. Wolstenholme and C. M. O'Connor, eds.), p. 204. Little, Brown, Boston, Mass., 1957.

[17] G. N. Timmis, *J. Pharm. and Pharmacol.* **9,** 81 (1957).

Early studies[19] of the effect of the administration of folic acid to adult individuals with chronic myelocytic leukemia had indicated that such treatment caused the clinical condition to deteriorate rapidly and the peripheral "blast cell" count to increase markedly. Remissions apparently developed in this disease when patients were given a diet low in folic acid, together with a poorly absorbed sulfonamide (to inhibit the biosynthesis of folic acid-derivatives by intestinal flora) and a weak competitive antagonist of folic acid (so-called x-methylfolic acid). Also, in mice this antagonist was used successfully to create a deficiency of folic acid[20]; this state, in animals with a transplanted acute lymphoblastic leukemia, significantly prolonged the survival of the animals.[21] However, the first clinical findings of major importance in the chemotherapy of leukemia were obtained with aminopterin; these showed that remarkable temporary remissions in the course of some cases of acute leukemia in children could be induced by treatment with this agent.[22] Even a decade later, no derivatives of folic acid have been found which offer any significant advantages over aminopterin, except perhaps amethopterin, and, with the exception of recent important findings in choriocarcinoma in the human female,[23, 24] consistent inductions of temporary remissions have not been reported with these agents in other types of neoplastic disease of man. On the other hand, the striking results obtained with amethopterin in a variety of transplantable neoplasms of animals, particularly acute leukemias in mice, have led to much investigation of this and related compounds.

Recent advances in our knowledge of the compounds formed metabolically from pteroylglutamic acid and of their catalytic roles in a variety of enzymic transformations indicate that the 4-amino derivatives of folic acid are particularly effective inhibitors of the enzymic reduction of folic acid (F)[11] to the tetrahydro form (FH_4),[11] i.e., 5,6,7,8-tetrahydropteroylglutamic acid. Although these analogs of folic acid may interfere to some extent with other stages in the metabolic alteration of the vitamin, such inhibitions apparently are obtained only with higher concentrations. For example, the growth of *Pediococcus cerevisiae*, an organism which uti-

[18] T. H. Jukes and H. P. Broquist, *in* "Metabolic Pathways" (D. M. Greenberg, ed.), Vol. 2. Academic Press, New York, in press.

[19] R. W. Heinle and A. D. Welch, *J. Clin. Invest.* **27**, 539 (1948).

[20] D. R. Weir, R. W. Heinle, and A. D. Welch, *Proc. Soc. Exptl. Biol. Med.* **69**, 211 (1948).

[21] D. R. Weir, A. D. Welch, and R. W. Heinle, *Proc. Soc. Exptl. Biol. Med.* **71**, 107 (1949).

[22] S. Farber, L. K. Diamond, R. D. Mercer, R. F. Sylvester, Jr., and J. A. Wolfe, *New Engl. J. Med.* **238**, 787 (1948).

[23] M. C. Li, R. Hertz, and D. B. Spencer, *Proc. Soc. Exptl. Biol. Med.* **93**, 361 (1956).

[24] M. C. Li, R. Hertz, and D. M. Bergenstal, *New Engl. J. Med.* **259**, 66 (1958).

lizes F extremely poorly as compared to a derivative of FH_4, folinic acid (f^5FH_4),[25] can be inhibited by relatively high concentrations of amethopterin; however, when substrains of the organism were selected for their capacity to grow on relatively minute amounts of folic acid, the sensitivity to amethopterin was markedly increased (over sixtyfold); this situation was observed even when the new strain was grown on folinic acid.[26]

Initially, rat liver slices were shown to convert folic acid enzymically to compounds which, in the presence of ascorbic acid (or certain other reducing agents), could be altered (nonenzymically) to folinic acid[27]; the enzymic phase of these alterations of folic acid was markedly inhibited by aminopterin.[28] It has been concluded[12] from studies with avian liver systems that the principal site of action of the 4-amino analogs of F is a TPNH-dependent, dihydrofolic (FH_2) (7,8-dihydropteroylglutamic) acid reductase which catalyzes the reaction: $FH_2 + TPNH + H^+ \rightleftharpoons FH_4 + TPN^+$ (an equilibrium which at neutral reaction lies far to the right); in addition, the conversion of F to FH_2 was reported to be TPNH-dependent. However, very recent studies[29] have indicated that although the conversion of folic acid to FH_2 is TPNH-dependent, that of FH_2 to FH_4 (with extracts of sheep liver at pH 5) requires DPNH. In this system the reduction of F to FH_2 was exceedingly sensitive to amethopterin; in fact, the enzyme could be titrated with the inhibitor, 1.2×10^{-6} μmole being equivalent to that amount of enzyme which, under prescribed conditions, reduced 1×10^{-3} μmole of folic acid in 30 minutes. Under these conditions, the affinity of the enzyme for aminopterin was almost 1,000 times that for folic acid. Earlier evidence also showed that the enzymic reduction of F to FH_2 (by chicken liver) is inhibited by the 4-amino antagonists[30]; and that the overall system of chicken liver (F to FH_4) is dependent on TPNH and is exceedingly sensitive to aminopterin[31] or amethopterin.[32] In the former study,[31] the capacity of DPNH to substitute for TPNH in the reduction of FH_2 was demonstrated. Recent studies[33] have demonstrated firm binding of aminopterin or amethopterin in the supernatant fraction of a sucrose homogenate of rat liver prepared 16 hours after the injection of a large dose

[25] H. E. Sauberlich and C. A. Baumann, *J. Biol. Chem.* **176,** 165 (1948); **181,** 871 (1949).
[26] C. A. Nichol, *Nature* **183,** 550 (1959).
[27] C. A. Nichol and A. D. Welch, *Proc. Soc. Exptl. Biol. Med.* **74,** 52 (1950).
[28] C. A. Nichol and A. D. Welch, *Proc. Soc. Exptl. Biol. Med.* **74,** 403 (1950).
[29] J. M. Peters and D. M. Greenberg, *Nature* **181,** 1669 (1958); *Biochim. et Biophys. Acta* **32,** 273 (1959).
[30] S. Futterman, *J. Biol. Chem.* **228,** 1031 (1957).
[31] S. Futterman and M. Silverman, *J. Biol. Chem.* **224,** 31 (1957).
[32] S. F. Zakrzewski and C. A. Nichol, *Biochim. et Biophys. Acta* **27,** 427 (1958).
[33] W. C. Werkheiser, *Proc. Am. Assoc. Cancer Research* **3,** 72 (1959).

of the antagonist. Extensive dialysis failed to remove the analog unless folic acid was present in a high concentration. A direct correlation was demonstrated between the amount of antagonist required to inhibit the folic acid-reductase activity in the supernatant fraction of normal liver and the amount of enzyme present. The affinity of the reductases for the 4-amino analogs is so great that a value for "K_i" cannot easily be estimated by classic methods; however, the procedure[34] of titrating the inhibitor with the enzyme may be used, as was done in this case.

Clearly, the 4-amino analogs do not inactivate the susceptible enzymes, as had been proposed[35]; rather, the affinity of the enzymes for the antagonists is so great as to make infeasible the physiological attainment of concentrations of folic acid sufficient to displace the inhibitor from the enzymes. Of considerable interest will be the relative affinities of the folic acid reductases of various animal cells and tissues for the 4-amino analogs, since these might account in part for the considerable variations in the responses of various normal tissues, and those of various types of neoplasms. Great differences between the reductases of different animal species are almost certain to be encountered, since in some species, e.g., the guinea pig, aminopterin and amethopterin are toxic only in relatively enormous doses. These antagonists do not appear to be inactivated significantly or excreted more rapidly by the guinea pig, and in this species, folic acid is enzymically reduced by the liver to compounds determinable as folinic acid.[36] Finally, the remarkably greater toxicity of amethopterin in female mice, as compared to males, might be related to the relative amounts of the reductases, or their affinities for the antagonist, in the two sexes.[37]

As a result of the profound inhibition of the F and FH_2 reductases by the 4-amino derivatives of F, the formation and regeneration of the F-derived coenzyme is blocked, and many biochemical transformations are inhibited or prevented. Although FH_4 may be regarded as the coenzyme (or an essential portion of it) in each of these reactions, the exact structure of the derivatives of FH_4 involved in the transformations has not yet been established to the satisfaction of all concerned.[38] In any case, at least five derivatives of FH_4 are involved in reactions of great biological importance; these are[11]: (1) a formyl (*f*) derivative, either $f^{10}FH_4$ or $f^{5\text{-}10}FH_4^+$ (i.e., a compound with a =CH— group linking nitrogens 5 and 10), in which the substituent is at the formic acid level of oxidation; (2) a so-called hydroxy-

[34] W. W. Ackermann and V. R. Potter, *Proc. Soc. Exptl. Biol. Med.* **72**, 1 (1949).
[35] E. M. Greenspan, A. Goldin, and E. B. Schoenbach, *Cancer* **4**, 619 (1951).
[36] D. Wood and C. A. Nichol, unpublished research (1957).
[37] A. Goldin, E. M. Greenspan, B. Goldberg, and E. B. Schoenbach, *Cancer* **3**, 849 (1950).
[38] R. L. Blakley, *Biochem. J.* **65**, 331 (1957).

methyl (*h*) derivative, either $h^{10}FH_4$ or $h^{5\text{-}10}FH_4$ (i.e., a compound with a $-CH_2-$ group linking nitrogens 5 and 10), in which the substituent is at the formaldehyde level of oxidation; (3) a formimino (*fi*) derivative (fi^5FH_4), in which the substituent at position 5 has the structure —CH=NH, (4) folinic acid (CF), the relatively stable form which may be designated as f^5FH_4; and (5) a phosphorylated derivative, pFH_4, which is believed to be N^{10}-phosphoryl FH_4.

Recent articles[12, 16, 18] have summarized much of our present knowledge of the enzymic interconversions of the derivatives of FH_4 and their function in the biosynthesis and degradation of various metabolites. Perhaps the first indications of the involvement of folic acid in the metabolism of formate were obtained (*a*) in studies of the utilization by F-deficient and repleted rats of formate-C^{14} for amino acid synthesis,[39] (*b*) in the initial demonstration of the biosynthesis from formate-C^{14} of labile methyl groups by rat liver slices,[40] and (*c*) in studies of the incorporation of formate-C^{14} into the total nucleoprotein and nucleic acid purines of mice treated with aminopterin and amethopterin.[41] However, studies of purine metabolism made definitive the participation of folic acid in the activation of formate. As discussed in Chapter 35, recent developments in our knowledge of the biosynthesis of purines have shown that the conversion of glycinamide ribonucleotide to the formyl derivative is dependent upon FH_4. The introduction of this formate-carbon into what is to become carbon 8 of the hypoxanthine moiety of inosine-5′-phosphate (i.e., inosinic acid), is to be compared to the subsequent addition, by an apparently analogous reaction which also involves fFH_4, of formate-carbon to the amino group of 4-amino-5-imidazolecarboxamide ribonucleotide, which after a TPNH-dependent ring closure becomes inosinic acid. Possibly the most susceptible to inhibition by amethopterin, of the FH_4-dependent reactions concerned with the formation of nucleotides, is the conversion of deoxyuridine-5′-phosphate and formaldehyde to thymidylic acid and, therefore, the synthesis of DNA. Early studies with formate-C^{14} and deoxyuridine,[42-46] which served as the

[39] G. W.E. Plaut, J. J. Betheil, and H. A. Lardy, *Abstr. 116th Meeting Am. Chem. Soc., Atlantic City, New Jersey*, p. 65C (1949); *J. Biol. Chem.* **184**, 795 (1950).

[40] A. D. Welch and W. Sakami, *Federation Proc.* **9**, 245 (1950); W. Sakami and A. D. Welch, *J. Biol. Chem.* **187**, 379 (1950).

[41] H. E. Skipper, J. H. Mitchell, Jr., and L. L. Bennett, Jr., *Cancer Research* **10**, 510 (1950).

[42] J. R. Totter, E. Volkin, and C. E. Carter, *J. Am. Chem. Soc.* **73**, 1521 (1951); J. R. Totter, *ibid.* **76**, 2196 (1954).

[43] P. Reichard, *Acta Chem. Scand.* **9**, 1275 (1955).

[44] M. Friedkin and D. Roberts, *J. Biol. Chem.* **220**, 653 (1956); M. Friedkin and A. Kornberg, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 609. Johns Hopkins Press, Baltimore, 1957.

[45] R. L. Blakley, *Biochim. et Biophys. Acta* **24**, 224 (1957); *Nature* **182**, 1719 (1958).

[46] E. A. Phear and D. M. Greenberg, *J. Am. Chem. Soc.* **79**, 3737 (1957).

basis of these findings, have now been made much more definitive (see Chapter 36). Recently, a transfer of H^3 from substrate amounts of $FH_4{}^3$ to thymidine-5′-phosphate has been shown to occur during its formation from deoxyuridine-5′-phosphate and formaldehyde.[47] It was proposed that "hydroxymethyl-FH_4" (presumably as $h^{5\text{-}10}FH_4$) and deoxyuridylic acid are linked, each through their respective position 5, by the "active methylene" group derived from formaldehyde. Cleavage of the complex to form thymidylic acid is thought to involve the transfer of one atom of H^3 (from position 6 of the $FH_4{}^3$-derivative) to yield the 5-methyl group, with the separation of 7,8-$FH_2{}^3$. Such a mechanism would require the continued action of dihydrofolic acid reductase for regeneration of FH_4 and this situation would result in the process being exceedingly sensitive to inhibition by the 4-amino analogs of folic acid.

The importance to nucleic acid biosynthesis in mammalian cells of interference with the formation of FH_4 was convincingly demonstrated when the inhibition of reproduction of sarcoma-180 cells in culture by amethopterin was shown to be prevented completely (if glycine and other amino acids were present) by the simultaneous addition of a utilizable purine and thymidine.[48]

Both severe depletion of folic acid by dietary means and the administration of 4-amino analogs of the vitamin cause remarkably variable effects in different organs and tissues, and considerable variations also are seen among various animal species.[14] The rates of formation and degradation of FH_4 and its derivatives probably are quite variable in various tissues and under varying conditions. Also, marked differences exist with respect to the operation in various tissues, under varying circumstances, of the many metabolic reactions which are dependent on coenzymes derived from folic acid.[14] In almost all cases, however, the most profound effects of administration of aminopterin and amethopterin are observed in those tissues in which proliferation is essential and continuous (e.g., the bone marrow and the intestinal mucosa). In the liver, in which there exists not only an extremely active system for the enzymic reduction of folic acid, but also many of the metabolic reactions which are dependent upon folic acid-derived coenzymes, the uptake of formate-C^{14} into purine-containing nucleotides is usually sharply depressed by the administration of the antagonist. In addition, liver homogenates inactivate irreversibly a folic acid-derived, normal constituent of that tissue, the enzymic formation of which is blocked by amethopterin.[30] The enzymic inactivation is diphosphopyridine nucleotide (DPN)-dependent[30] and is inhibited by xanthopterin,[14, 30, 49-51] as well as by arsenite and tetraethylthiuram disulfide.[49]

[47] M. Friedkin, *Federation Proc.* **18**, 230 (1959).
[48] M. T. Hakala, *Science* **126**, 255 (1957).
[49] M. Silverman and J. C. Keresztesy, *Federation Proc.* **12**, 268 (1953).

It was shown several years ago that the incorporation of formate-carbon into positions 2 and 8 of nucleic acid purines occurs to an equal extent *in vivo*, but, as has been noted,[14] it is not clear why this is so when a very active inosinic acid transformylase system, dependent upon FH_4, operates effectively to exchange with formate the carbon is position 2, at least in a cell-free system obtained from pigeon liver. Similarly, the accumulation, in many biological systems inhibited by folic acid-antagonists, of aminoimidazolecarboxamide (or derivatives of it), suggests that the earlier formylation of the ribonucleotide of glycinamide is far from completely inhibited, under conditions which lead to a profound interference with the formylation of the imidazole derivative. This difference in the apparent inhibitory effect of amethopterin may be a reflection of different affinities of the two transformylases for FH_4 (the coenzyme in both reactions).

In the enzymic degradation of purines, at least by *Clostridium cylindrosporum*, an additional FH_4-dependent reaction occurs: the breakdown of formiminoglycine (derived by opening of the ring of 4-imidazolone arising from xanthine). This sequence of reactions, utilized by this organism as its only source of energy, also has been exploited for the determination of adenosine triphosphate (ATP), formate, and FH_4, and is very useful in the detection of a state of folic acid-deficiency.[52] The key reactions are:

$$\text{Forminoglycine} + FH_4 \rightleftharpoons \text{glycine} + fi^5FH_4 \text{ (formimino transferase)} \quad (1)$$

$$fi^5FH_4 \rightleftharpoons f^{5-10}FH_4 + NH_3 \text{ (cyclodeamimase)} \quad (2)$$

$$f^{5-10}FH_4 + ADP + P_i \rightleftharpoons \text{formate} + FH_4 + ATP \text{ (tetrahydrofolic formylase)} \quad (3)$$

Although only indirectly concerned with nucleic acid metabolism, mention should be made of recent advances in our knowledge of the catabolism of L-histidine. This is because of the involvement of a FH_4-dependent transfer of the formimino group from the intermediate formiminoglutamic acid (in a manner similar to that discussed above), and the important analytical applications of these reactions in the detection of a state of folic acid-deficiency, in which formiminoglutamic acid appears in the urine, particularly following the administration of L-histidine. A large volume of literature concerning this field exists to which only a few key references can be given here.[53-56]

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[50] L. D. Wright and A. D. Welch, *Science* **98**, 179 (1943).

[51] A. D. Welch, E. M. Nelson, and M. F. Wilson, *Federation Proc.* **8**, 346 (1949).

[52] J. C. Rabinowitz and W. E. Pricer, Jr., *J. Am. Chem. Soc.* **78**, 5702 (1956); J. C. Rabinowitz, *Abstr., Gordon Research Conf., New London, New Hampshire*, p. 83 (1957).

[53] H. Bakerman, M. Silverman, and F. S. Daft, *J. Biol. Chem.* **188**, 177 (1951).

[54] B. Borek and H. Waelsch, *J. Biol. Chem.* **205**, 459 (1953).

[55] J. C. Rabinowitz and H. Tabor, *J. Biol. Chem.* **233**, 252 (1958).

[56] H. H. Hiatt, M. Goldstein, and H. Tabor, *J. Clin. Invest.* **37**, 829 (1958).

Folic acid antagonists have found their greatest field of usefulness in the treatment of acute leukemia in children, although remissions have been observed occasionally in other types of neoplasia. In many cases of acute leukemia in children, the response to amethopterin is not sufficient for significant remissions to develop. Although fair remissions are seen in perhaps one-half to two-thirds of the cases, the number of "complete remissions," in which the leukemic state appears (temporarily) to have been abolished, is small. As a rule, the diagnosis is rarely made until the leukemic state is well advanced, and infiltration of most of the tissues of the body with leukemic cells may be presumed to have occurred. In human neoplasias, a cure appears never to have been attained following therapy with amethopterin, or, for that matter, with other chemotherapeutic agents, used singly or in combination. On the other hand, under some conditions, a high proportion of mice inoculated with some types of leukemia cells can be cured with amethopterin; however, this result usually is attained only with early treatment following the introduction of a relatively small number of cells. Usually, antagonists of folic acid cause only a prolongation of the survival time. Clearly, however, the action of amethopterin on many types of lymphoblastic cells in mice is sufficiently selective to permit very favorable results to be obtained despite the influence of this extremely toxic agent on some cells of the mammalian host.

Mouse leukemia cells (lymphoblasts L-5178-Y and mast cells P-815) reproducing in culture have a very high requirement for folic acid, as compared to other types of neoplastic and "normal" cells. However, the needs of these leukemic cells can be met by minute amounts of folinic acid, a finding which implies that the cells are deficient in the enzyme responsible for the formation of FH_4 from F_2. This circumstance may account for the very high sensitivity to amethopterin of such cells, *in vivo* as well as in culture.[57, 58]

The preferential incorporation of formate-C^{14} into leukemic blood cells of mice (*in vivo*) has been demonstrated by autoradiographic techniques.[59, 60] These studies indicated that the leukemic cells have a much more active formate metabolism than the various normal cells of the blood. Studies with similar mouse leukemic cells incubated *in vitro* demonstrated the incorporation of formate-C^{14} into proteins and into the purine components of nucleic acids; this incorporation was inhibited (though far from completely) by amethopterin.[61]

[57] G. A. Fischer and A. D. Welch, *Science* **126**, 1018 (1957).

[58] R. Schindler, M. Day, and G. A. Fischer, *Cancer Research* **19**, 47 (1959).

[59] H. E. Skipper, J. B. Chapman, G. A. Boyd, W. H. Riser, Jr., and M. Bell, *Proc. Soc. Exptl. Biol. Med.* **77**, 849 (1951).

[60] H. E. Skipper, J. B. Chapman, and M. Bell, *Proc. Soc. Exptl. Biol. Med.* **78**, 787 (1951).

[61] A. D. Williams, G. G. Slater, and R. J. Winzler, *Cancer Research* **15**, 532 (1955).

Studies *in vitro* with leucocytes obtained from human subjects have demonstrated remarkable differences between the cells characteristic of acute leukemia, chronic granulocytic leukemia, and chronic lymphocytic leukemia.[62] Thus, the two former types of cells utilized formate-C^{14} rapidly and steadily for at least 6 hours *in vitro*, incorporations which were stimulated by folinic, but not by folic acid, and markedly inhibited by high concentrations of amethopterin (an inhibition prevented by folinic acid). On the other hand, cells from normal individuals or from patients with chronic lymphocytic leukemia took up but little formate and were essentially unaffected by amethopterin. Since amethopterin, even at very high levels, as with mouse leukemia cells, did not suppress completely the incorporation of formate by cells of patients with chronic granulocytic leukemia, the existence of an amethopterin-resistant biochemical pathway for formate utilization was considered. However, it should be emphasized here that the effect of amethopterin appears to be exerted primarily on the formation of FH_4 and probably affects but little, if at all, the activity of preformed coenzyme stored within the leukemic leucocytes. That the coenzyme content of such cells may not be optimal, however, is indicated by the finding that the addition of folinic acid *in vitro* stimulated the incorporation of formate, while the finding that folic acid was without such activity may indicate that the folic acid reductase activity is extremely low in these cells, or that DPNH or TPNH may be limiting. It would be of much interest to examine FH_2-reductase activity of the cells, since, as previously discussed, the synthesis of thymidylic acid (and presumably of other compounds, e.g., methionine-methyl) appears to involve the formation from $f^{5\text{-}10}FH_4$ of FH_2 and its amethopterin-sensitive reconversion to FH_4.

In view of these results (and those described in the first paragraph of this section), it will perhaps appear surprising that amethopterin is so rarely used in the treatment of chronic granulocytic leukemia. However, the host-toxicity observed with amethopterin is usually much greater in adults than in children and other agents are employed if possible.

Recent clinical studies with halogenated derivatives of amethopterin in which the *p*-aminobenzoic acid portion of the molecule is substituted with a single (3′-) or two (3′,5′-) chlorine atoms, or with bromine (3′-) and chlorine (5′-) atoms, although based on very encouraging findings in the treatment of mouse leukemia,[63] and markedly lower toxicity in rats and dogs

[62] R. J. Winzler, A. D. Williams, and W. R. Best, *Cancer Research* **17,** 108 (1957); R. J. Winzler, *in* "The Leukemias: Etiology, Pathophysiology, and Treatment" (J. W. Rebuck, F. H. Bethell, and R. W. Monto, eds.), p. 567. Academic Press, New York, 1957.

[63] A. Goldin, *J. Natl. Cancer Inst.* **22,** 811 (1959); A. Goldin and R. Humphreys, *Proc. Am. Assoc. Cancer Research* **3,** 22 (1959).

than with amethopterin,[64] have not indicated that the derivatives offer any clinical advantages over the parent compound.[65]

Although much research effort has been expended in search of a definitive explanation of the mechanisms of amethopterin-resistance, this goal has not been attained. However, many studies with both bacteria and neoplastic cells in culture, as well as in mice with transplanted neoplasms, indicate that spontaneously appearing, genetically stable, drug-resistant mutant cells are selected by amethopterin, and that the development of a high degree of resistance in a cell population is a stepwise process of selection of the products of successive mutational events.[66, 67] Therapy with subcurative doses of amethopterin in successive mice, inoculated with cells obtained progressively from the drug-treated animals, leads to the gradual selection of amethopterin-resistant strains of leukemia cells.[66, 68, 69]

Studies with *Streptococcus faecalis* have indicated that cells which are profoundly resistant to amethopterin (e.g., inhibited only by a 1,000-fold to 3,000,000-fold higher concentration than that required for the parent sensitive strain[70]) convert folic acid to functional forms (measured as folinic acid) more efficiently (about 100-fold) than do the drug-sensitive cells.[71-73] However, this phenomenon per se does not appear to be sufficient to account for the tremendous changes in drug-sensitivity. Indeed, results obtained with several strains of mouse leukemic cells have not indicated that greater efficiency in the utilization of folic acid, or in its conversion to derivatives measured as folinic acid, is a concomitant event in the development of amethopterin-resistance.[57, 72, 74, 75] In *S. faecalis* the enzyme system which is

[64] D. P. Rall, A. J. Pallotta, and J. R. Elsea, *Proc. Am. Assoc. Cancer Research* **3,** 54 (1959).

[65] E. J. Freireich, M. Lane, and R. K. Shaw, *Proc. Am. Assoc. Cancer Research* **3,** 20 (1959).

[66] L. W. Law, *Cancer Research* **16,** 698 (1956).

[67] A. D. Welch, *Cancer Research* **19,** 359 (1959).

[68] J. H. Burchenal, E. Robinson, S. F. Johnston, and M. N. Kushida, *Science* **111,** 116 (1950).

[69] L. W. Law and P. J. Boyle, *Proc. Soc. Exptl. Biol. Med.* **74,** 599 (1950).

[70] J. H. Burchenal, G. B. Waring, and D. J. Hutchison, *Proc. Soc. Exptl. Biol. Med.* **78,** 311 (1951).

[71] H. P. Broquist, A. R. Kohler, D. J. Hutchison, and J. H. Burchenal, *J. Biol. Chem.* **202,** 59 (1953).

[72] C. A. Nichol and A. D. Welch, *in* "Antimetabolites and Cancer" (C. P. Rhoads, ed.), p. 63. Am. Assoc. Advancement Sci., Washington, D. C., 1955.

[73] A. Anton and C. A. Nichol, *Proc. Am. Assoc. Cancer Research* **2,** 91 (1956); *Biochem. Pharmacol.* **3,** 1 (1959).

[74] C. A. Nichol, *in* "The Leukemias: Etiology, Pathophysiology, and Treatment" (J. W. Rebuck, F. H. Bethell, and R. W. Monto, eds.), p. 583. Academic Press, New York, 1957.

[75] G. A. Fischer, *Ann. N. Y. Acad. Sci.* **76,** 673 (1958); *Cancer Research* **19,** 372 (1959).

concerned with the conversion of folic acid to materials measured as folinic acid, and which is highly susceptible to inhibition by amethopterin in the sensitive but not in the resistant bacterial cells, nevertheless, can be extracted from the latter in an active form which is only slightly less susceptible to inhibition by the drug than the enzyme system extracted from drug-sensitive cells.[72-74] Unfortunately, however, cellfree extracts of leukemic cells have not yet been described which have exhibited suitable activity in the formation of FH_4 from folic acid[72, 74]; since aqueous extracts of acetone powders and high-speed supernatant fractions of sucrose homogenates of liver have exhibited high folic acid-reductase activity, similar results with neoplastic cells may soon be forthcoming.

The successful progressive growth in culture of L-5178-Y lymphoblasts of mice and the progressive selection from them of amethopterin-resistant stable mutant strains, the growth of which is resistant to inhibition by up to 100,000 times the concentration of drug which inhibits the parent strain,[75] afford some degree of optimism concerning comparative studies of the possible mechanisms of drug-resistance in mammalian cells using both intact cells and cell-free extracts.

The data now available are compatible with the concept that amethopterin-resistant bacterial (and probably mammalian) cells (*1*) are dependent on a source of folic acid-derived coenzymes, and (*2*) do not metabolize either folic acid or the antagonist in a manner very significantly different from that of drug-sensitive cells; rather, in some manner not yet clear, the drug-sensitive enzyme system of drug-resistant cells appears to be relatively less accessible to the antagonist, while remaining available to folic acid in the extracellular environment. The nature and locus of the "barrier" between the enzyme and the drug is the subject of much investigation.

Mention should be made of studies of a condition referred to as amethopterin-dependence which occurred in a strain of L-1210 leukemia in mice.[66, 76] Animals inoculated with these cells died of leukemia in a significantly shorter period of time when given folic acid-antagonists in doses of the usual magnitude, an effect partially prevented by folinic acid. The incorporation of formate-C^{14} into the ribonucleotide of aminoimidazolecarboxamide, purines, nucleic acids, and other components of tumors which was observed in these cells was unequivocally increased by treatment of the mice with amethopterin. However, formate incorporation was sharply diminished in the analogous components of the normal tissues of the host and in tumors derived from the parent, drug-sensitive L-1210 cells.[77] In studies by another group of a similarly dependent line of L-1210, the pres-

[76] L. W. Law, *Proc. Soc. Exptl. Biol. Med.* **77**, 340 (1951); *J. Natl. Cancer Inst.* **11**, 849 (1951).

[77] H. E. Skipper, L. L. Bennett, Jr., and L. W. Law, *Cancer Research* **12**, 677 (1952).

ence of an amethopterin-sensitive infectious agent was evident.[78] The explanation was offered that the infectious agent (presumably lymphocytic choriomeningitis virus) retards the rate of tumor growth, while amethopterin by inhibiting the effect of the virus on the amethopterin-resistant tumor cells, permits the more rapid proliferation of the neoplastic cells and the earlier death of the mice. It had been shown earlier that a strain of L-1210 lymphoma indeed can carry a filtrable, self-propagating contaminant (presumably lymphocytic choriomeningitis virus).[79] Treatment with amethopterin (or azaguanine) was found to protect mice from the otherwise fatal effects of infection with the virus of lymphocytic choriomeningitis, even though the infection was not eradicated[80]; a similar result was obtained in mice given a folic acid-deficient diet.[81] Under either of these conditions the proliferation of the virus was not interfered with, but its damaging effects on susceptible cells were reduced or prevented.[82] Even more remarkable findings have recently been obtained in mice given an amethopterin-resistant line of the tetraploid lymphocytic neoplasm, P-288, intentionally infected with lymphocytic choriomeningitis.[83] Although passage of the infected cells through mice which had been immunized against the virus caused the latter to disappear, the administration of amethopterin prevented this disappearance of virus These findings, and others,[84] have suggested that this antimetabolite can interfere markedly with either the formation of or the response to antibodies, or both. A most remarkable example of this type of interference is the finding that a drug-resistant subline of P-288 (free of lymphocytic choriomeningitis) could grow progressively and kill mice of genetically foreign strains (in which mice the neoplastic cells ordinarily will not proliferate continuously), when the animals were treated with amethopterin.[83]

Whether a phenomenon of this type contributed to the early observations concerning partial dependence on amethopterin is not known. Certainly, the evidence has not indicated that lymphocytic choriomeningitis or any other demonstrable infectious agent was present in the tumor cells, and manifestations of illness were not seen in the host-animals.[85] Thus, it is not yet possible to offer a biochemical explanation of the phenomenon of

[78] S. R. Humphreys, J. M. Venditti, M. Mantel, and A. Goldin, *J. Natl. Cancer Inst.*, **17**, 447 (1956).

[79] L. W. Law and T. B. Dunn, *J. Natl. Cancer Inst.* **11**, 1037 (1951).

[80] V. H. Haas and S. E. Stewart, *Virology* **2**, 511 (1956).

[81] V. H. Haas, G. M. Briggs, and S. E. Stewart, *Science* **126**, 405 (1957); V. H. Haas, S. E. Stewart, and G. M. Briggs, *Virology* **3**, 15 (1957).

[82] E. M. Lerner, II, and V. H. Haas, *Proc. Soc. Exptl. Biol. Med.* **98**, 395 (1958).

[83] M. Potter and V. H. Haas, *J. Natl. Cancer Inst.* **22**, 801 (1959).

[84] D. E. Uphoff, *Proc. Soc. Exptl. Biol. Med.* **99**, 651 (1958).

[85] L. W. Law, personal communication (1959).

partial dependence of leukemia cells upon amethopterin. However, in view of the profound effect of amethopterin on the folic acid reductases, the failure of the analog to be reduced by these enzymes,[29, 86] and the remarkable resistance of the agent to metabolic alteration (see below), it appears most unlikely that amethopterin could function in a manner somewhat resembling folic acid.[69, 77] For these reasons, therefore, the concept of interference by amethopterin with the function of an unknown infectious agent harbored by certain strains of amethopterin-resistant tumor cells, or with some deleterious host reaction upon such cells, appears worthy of further consideration. It must be concluded that the phenomenon of amethopterin-dependence has yet to be explained, and that the presumed role of FH_4 in the formation or activity of certain antibodies is deserving of intensive investigation.

Some aspects of the pharmacology of amethopterin should be mentioned briefly, since recent studies in man,[87] using fluorimetric procedures, indicate that the drug not only is rapidly and completely absorbed from the alimentary tract, but also is excreted in the urine without very significant metabolic alteration. However, earlier studies,[88] using a biological assay, indicated partial inactivation of the analog, as have findings with certain microorganisms.[89, 90] In the tissues, amethopterin appears to be distributed largely in the total extracellular water,[87] despite the fact that its toxic effects are exerted primarily on the folic acid reductases of the cytoplasm (at least of liver cells). Since, at body pH levels, the compound is more than 99% dissociated as an acid, it is now predictable[90, 91] that only minute amounts of amethopterin should be transported from the blood to the cerebrospinal fluid and the tissues of the brain.[92] Only by intrathecal administration has it been possible to obtain high concentrations of amethopterin in the cerebrospinal fluid.[92] The grave chemotherapeutic importance of this failure of amethopterin to pass from the blood to the cerebral extracellular fluid and thus to reach those neoplastic cells which enter the central nervous system early in various initially amethopterin-sensitive malignant diseases, has been indicated by important recent investigations in patients with acute leukemia[92] or choriocarcinoma.[23, 24] The possibility of altering the structure

[86] S. F. Zakrzewski, *Federation Proc.* **18,** 357 (1959).

[87] M. V. Freeman, *J. Pharmacol. Exptl. Therap.* **122,** 154 (1958).

[88] J. H. Burchenal, G. B. Waring, R. R. Ellison, and H. C. Reilly, *Proc. Soc. Exptl. Biol. Med.* **78,** 603 (1951).

[89] M. Webb, *Biochim. et Biophys. Acta* **17,** 212 (1955); *Biochem. J.* **70,** 472 (1958).

[90] B. B. Brodie and C. A. M. Hogben, *J. Pharm. and Pharmacol.* **9,** 345 (1957).

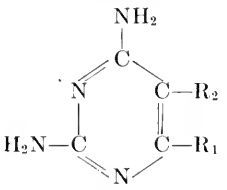
[91] D. P. Rall, J. R. Stabenau, and C. G. Zubrod, *J. Pharmacol. Exptl. Therap.* **125,** 185 (1959).

[92] J. W. Whiteside, F. S. Philips, H. W. Dargeon, and J. H. Burchenal, *A.M.A. Arch. Internal Med.* **101,** 279 (1958).

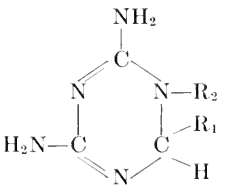
of amethopterin in such a way as to permit rapid crossing of the "blood-brain barrier," without too great a diminution of the high intrinsic activity of the analog, is a reasonable expectation for the future.

2. Diaminopyrimidines and Diaminodihydrotriazines

A sizable group of compounds of the following type structures [(III) and (IV)] has been the subject of much investigation during the past decade.



(III)

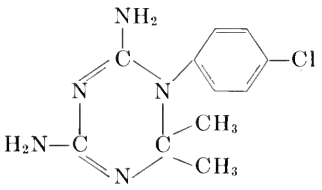


(IV)

Although it is not possible entirely to exclude effects of these compounds on other systems, several members of both series which have been investigated in considerable detail have been shown to interfere with the formation of coenzymes derived from folic acid. A similar statement can be made concerning the aliphatic antimalarial compound, chloroguanide (Paludrine),

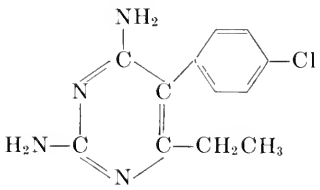
$$(p)\text{-Cl}—C_6H_4—NH—C(=NH)—NH—C(=NH)—NH—CH(CH_3)_2$$

since it appears that this drug is conveted metabolically to a diaminodihydrotriazine (V).[93, 94]



(V)

Chloroguanide metabolite



(VI)

Pyrimethamine (Daraprim)

The close structural resemblance of the metabolite to the more potent and generally more useful antimalarial, pyrimethamine (VI), a 2,4-diaminopyrimidine, is readily apparent. The evidence strongly indicates that each of these antimalarial agents acts primarily by interfering with the formation of FH_4 or a closely related compound by certain plasmodia.[95-99] The capacity of pyrimethamine and related compounds

[93] F. Hawking and W. L. M. Perry, *Brit. J. Pharmacol.* **3**, 320 (1948).

[94] H. C. Carrington, A. F. Crowther, D. G. Davey, A. A. Levi, and F. L. Rose, *Nature* **168**, 1080 (1951).

[95] G. H. Hitchings, *Trans. Roy. Soc. Trop. Med. Hyg.* **46**, 467 (1952).

[96] E. J. Modest, G. E. Foley, M. M. Pechet, and S. Farber, *J. Am. Chem. Soc.* **74**, 855 (1952).

[e.g., 2,4-diamino-5(3′,4′-dichlorophenyl)-6-methyl-pyrimidine] and the analogous triazines to inhibit hematopoiesis and, particularly, to cause leucopenia has been investigated in several animal species and in patients with leukemia.[98-103] These activities and the toxicity of the compounds appear to be exerted in a manner resembling that of amethopterin; analogous conclusions may be drawn from studies of the diaminodihydrotriazines. However, direct studies of the effect of these agents on the folic acid reductases, analogous to those described with amethopterin, have not been reported.

An interesting situation is found in the RH strain of *Toxoplasma*, for this pathogenic organism probably obtains its supply of coenzymes related to FH_4 exclusively from small molecular precursors, since sulfadiazine inhibits significantly the progress of infections with this organism (in mice), an effect prevented by *p*-aminobenzoic acid, but only very ineffectively by folic acid or even folinic acid.[104] The inefficiency of action of the latter compounds may be attributed to the absence of a system in the parasite for the utilization of these substances; in fact, traces of activity in these compounds probably reflect the presence, or the catabolic formation in the tissues of the mouse, of utilizable derivatives of *p*-aminobenzoic acid. This suggestion is made more reasonable by the fact that the course of infections in mice with this *Toxoplasma* also is not affected by aminopterin.[104, 105] However, the progress of the disease is inhibited by sulfonamides to about the same extent as by pyrimethamine, and it has been suggested that the diamino-pyrimidine affects a stage in the intracellular synthesis *de novo* of FH_4 (or its functional derivatives) by the parasite. Studies of combinations of a sulfonamide and pyrimethamine have demonstrated marked synergistic activity, since cures could be obtained in mice with the combinations, whereas only a delay in the progress of the lethal infection was obtained with either drug alone. It is of considerable interest that the therapeutic efficiency of the combination was not impaired, either in the mouse or in man, by the simultaneous administration of folinic acid and yeast, while the occurrence of thrombocytopenia and leucopenia was prevented.

These important observations offer much encouragement to those interested in the logical development of agents which can attack certain types of cells selectively or the undesirable effects of which on normal or host cells can be nullified or minimized selectively.

A striking synergism also has been observed between the dihydrotriazines and sulfadiazine in the therapy of experimental infections in mice with *Diplococcus pneu-*

[97] G. E. Foley, *Proc. Soc. Exptl. Biol. Med.* **83,** 740 (1953).

[98] A. D. Welch, *in* "Cellular Metabolism and Infections" (E. Racker, ed.), Part I, p. 61. Academic Press, New York, 1954.

[99] M. N. Swaffield, G. E. Foley, E. J. Modest, and C. L. Maddock, *Proc. Am. Assoc. Cancer Research* **3,** 68 (1959).

[100] L. H. Schmidt, H. B. Hughes, and I. G. Schmidt, *J. Pharmacol. Exptl. Therap.* **107,** 92 (1953).

[101] R. J. Dern, E. Beutler, J. Arnold, A. Lorinez, M. Block, and A. S. Alving, *Am. J. Trop. Med. Hyg.* **4,** 217 (1955).

[102] M. L. Murphy, R. R. Ellison, D. Karnofsky, and J. H. Burchenal, *J. Clin. Invest.* **33,** 1338 (1954).

[103] S. Farber, R. Toch, E. M. Sears, and D. Pinkel, *Advances in Cancer Research* **4,** 1 (1956).

[104] J. K. Frenkel and G. H. Hitchings, *Antibiotics & Chemotherapy* **7,** 630 (1957).

[105] L. G. Goodwin and I. M. Rollo, *in* "Biochemistry and Physiology of Protozoa" (S. H. Hutner and A. Lwoff, eds.), Vol. 2, p. 225. Academic Press, New York, 1955.

moniae, Type II.[106] In this disease, unlike toxoplasmosis, a synergism can be demonstrated between aminopterin and either sulfadiazine or the dihydrotriazines. Similar studies of a wide variety of pathogenic microorganisms has indicated that certain streptococci are particularly susceptible to a diaminodihydrotriazine and that the action of the latter is potentiated markedly by a sulfonamide in experimental infections.[107]

A consideration of resistance to compounds of the diaminodihydrotriazine and diaminopyrimidine types is of considerable theoretical and practical importance, since cross-resistance between these agents is usually quite marked. Not only does this finding support the concept of a common mechanism of action, but also it has practical significance in that the relatively easy development of resistance of malarial plasmodia to the relatively weak agent, chloroguanide, predisposes them to much more rapid development of resistance to the more potent agent, pyrimethamine.[98]

Of considerable interest to the problem of resistance to the diaminopyrimidines is the finding, in a strain of *S. faecalis* selected for its ability to grow in a 1,000-fold greater concentration of pyrimethamine than the parent strain, that following exposure to folic acid, materials related to that substance could not be extracted from the cells. However, following exposure of the sensitive cells to the same concentrations of folic acid, the folic acid-like compounds were recovered quantitatively. The resistant strain may be one which possesses the ability to bind assimilated folic acid within the cells.[108]

The conclusion that the diaminopyrimidines and the diaminodihydrotriazines act primarily by interfering with the formation of a coenzyme related to tetrahydrofolic acid is supported by recent findings in mice with P-1534 leukemia. In these experiments, the prolongation of survival and the toxicity caused by a diaminodihydrotriazine were nullified by the coadministration of folinic acid, but not by therapy with folic acid.[99] On the other hand, as the result of recent studies with *Lactobacillus arabinosus*, it was concluded that diaminodihydrotriazines compete with DPN, not with pteroylglutamic acid (PGA), in a DPN-mediated reduction of a folic acid-like compound.[106] An even greater complication has been introduced by the finding, with *S. pyogenes* (C-203) grown in a chemically defined medium, that the inhibitory activity of diaminodihydrotriazines is not prevented by either folic or folinic acids, although a combination of acetate and DNA (or of acetate, deoxyguanylic acid, and thymidine, but not of acetate and RNA) is effective.[109]

Although the mechanism of action of the diaminodihydrotriazines may not be precisely the same in all forms of life, it may be concluded that the actions of these compounds are directed primarily against the formation of coenzymes concerned with the transfer of one-carbon intermediates and therefore with the synthesis of nucleic acids.

III. Compounds Which Interfere with Amination Reactions in Purine and Pyrimidine Synthesis

The potential importance of compounds which interfere with amination reactions in the synthesis of nucleic acid precursors will be indicated by a consideration of the role of glutamine in the synthesis of purine and pyrim-

[106] G. E. Foley, E. J. Modest, J. R. Cataldo, and H. D. Riley, *Biochem. Pharmacol.* **3**, 18 (1959).
[107] M. W. Fisher and L. Doub, *Biochem. Pharmacol.* **3**, 10 (1959).
[108] R. C. Wood and G. H. Hitchings, *Federation Proc.* **17**, 339 (1958).
[109] V. M. McGlohon and O. D. Bird, *Biochem. Pharmacol.* **2**, 299 (1959).

idine nucleotides. As described in detail in Chapters 35 and 36, each of several steps in the biosynthesis of purine- and pyrimidine-containing compounds requires glutamine as the amino-donor. These steps are: (*a*) the amination of 1-pyrophosphoryl-ribose-5-phosphate (PRPP) to form 1-amino-ribose-5-phosphate (ribosylamine-5-phosphate)[110, 111] (*b*) the conversion of formylglycinamide ribonucleotide to the corresponding formamidino derivative,[112] (*c*) the conversion of xanthine ribonucleotide to guanylic acid,[113, 114] and (*d*) the formation of a ribonucleotide of cytosine from the corresponding derivative of uracil.[115-117] The introduction of these ring-nitrogen atoms and amino groups into the purines and pyrimidines is to be distinguished from the incorporation of nitrogen into position 1 of the pyrimidine rings, which is mediated by carbamylphosphate, or into position 1 of the purine ring and position 3 of the pyrimidines, for which aspartic acid is the donor.

Although it might be the fond conviction of the theoretical chemist and biologist that the rational design of structures capable of interfering with nucleic acid metabolism will obviate the need for random screening of compounds, that time has not yet arrived. Thus, it was through routine testing of fermentation "beers" for antimicrobial and carcinostatic activity that the antibiotics azaserine [*O*-diazoacetyl-L-serine; (VII)][118-123] and DON [6-diazo-5-oxo-L-norleucine; (VIII)][124-126] were discovered (see also Chapter 35).

[110] D. A. Goldthwait, G. R. Greenberg, and R. A. Peabody, *Biochim. et Biophys. Acta* **18,** 148 (1955).

[111] S. C. Hartman, B. Levenberg, and J. M. Buchanan, *J. Biol. Chem.* **221,** 1057 (1956).

[112] D. A. Goldthwait, R. A. Peabody, and G. R. Greenberg, *J. Am. Chem. Soc.* **76,** 5258 (1954).

[113] M. Bentley and R. Abrams, *Federation Proc.* **15,** 218 (1956).

[114] R. Abrams and M. Bentley, *Arch. Biochem. Biophys.* **79,** 91 (1958).

[115] M. L. Eidinoff, J. E. Knoll, B. Marano, and L. Cheong, *Cancer Research* **18,** 105 (1958).

[116] N. P. Salzman, H. Eagle, and E. D. Sebring, *J. Biol. Chem.* **230,** 1001 (1958).

[117] H. O. Kammen and R. B. Hurlbert, *Biochim. et Biophys. Acta* **30,** 195 (1958).

[118] S. A. Fusari, R. P. Frohardt, A. Ryder, T. H. Haskell, D. W. Johannessen, C. C. Elder, and Q. R. Bartz, *J. Am. Chem. Soc.* **76,** 2878 (1954).

[119] C. C. Stock, H. C. Reilly, S. M. Buckley, D. A. Clarke, and C. P. Rhoads, *Nature,* **173,** 71 (1954).

[120] J. Ehrlich, L. E. Anderson, G. L. Coffey, A. B. Hillegas, M. P. Knudsen, H. J. Koepsell, D. L. Kohberger, and J. E. Ozaas, *Nature* **173,** 72 (1954).

[121] S. A. Fusari, T. H. Haskell, R. P. Frohardt, and Q. R. Bartz, *J. Am. Chem. Soc.* **76,** 2881 (1954).

[122] J. A. Moore, J. R. Dice, E. D. Nicolaides, R. D. Westland, and E. L. Wittle, *J. Am. Chem. Soc.* **76,** 2884 (1954).

[123] E. D. Nicolaides, R. D. Westland, and E. L. Wittle, *J. Am. Chem. Soc.* **76,** 2887 (1954).

[124] H. W. Dion, S. A. Fusari, Z. L. Jakubowski, J. G. Zora, and Q. R. Bartz, *J. Am. Chem. Soc.* **78,** 3075 (1956).

$$\bar{N}{=}\overset{+}{N}{=}CH{-}CO{-}O{-}CH_2{-}CH(NH_2){-}COOH$$

(VII)

$$\bar{N}{=}\overset{+}{N}{=}CH{-}CO{-}CH_2{-}CH_2{-}CH(NH_2){-}COOH$$

(VIII)

These closely related compounds, each produced by a strain of *Streptomyces*, exhibit considerable chemical reactivity as a result of the diazo function. Synthesis of each of these compounds has been devised and only the L-isomers are active as biological inhibitors. Both azaserine and DON are unstable, and below pH 2 evolve nitrogen at room temperature with complete loss of biological activity. Alkaline solutions of azaserine also slowly lose inhibitory activity as a result of migration of the diazo-acetyl group from the hydroxyl to the amino group of the serine moiety. Despite their names, which imply a structural similarity to serine or norleucine, azaserine and DON are both structural analogs of L-glutamine

$$H_2N{-}CO{-}CH_2{-}CH_2{-}CH(NH_2){-}COOH$$

as will be evidenced by an examination of their molecular configurations.

The original biological studies with azaserine attempted to relate its action to inhibition of the synthesis of amino acids or proteins, because the antimicrobial activity could be prevented by a variety of amino acids; in particular, leucine, phenylalanine, tyrosine, and tryptophan were effective.[127-129] In contrast to these results with azaserine, inhibition of the growth of *E. coli* by DON could not be prevented by an excess of any of the amino acids.[130]

In the first report of an effect of these compounds on nucleic acid metabolism, a profound inhibition by azaserine of the incorporation of formate-C^{14} into the nucleic acids of sarcoma-180, adenocarcinoma E_0771, liver, and intestine, was noted.[131] These findings were quickly followed by studies which indicated that the primary site of action was probably the conversion of formylglycinamide to formylglycinamidine (each as the ribonucleotide)[132] (see Chapter 35). Using soluble enzyme preparations from pigeon liver, the

[125] R. D. Westland, S. A. Fusari, and H. M. Crooks, *Abstr. 129th Meeting Am. Chem. Soc., Dallas, Texas* p. 14M (1956).

[126] H. A. DeWald and A. M. Moore, *Abstr., 129th Meeting Am. Chem. Soc., Dallas, Texas* p. 13M (1956).

[127] L. Kaplan and C. C. Stock, *Federation Proc.* **13,** 239 (1954).

[128] H. C. Reilley, *Proc. Assoc. Cancer Research* **1,** 40 (1954).

[129] H. Halvorson, *Antibiotics & Chemotherapy* **4,** 948 (1954).

[130] R. E Maxwell and V. S. Nickel, *Antibiotics & Chemotherapy* **7,** 81 (1957).

[131] H. E. Skipper, L. L. Bennett, Jr., and F. M. Schabel, Jr., *Federation Proc.* **13,** 298 (1954).

[132] S. C. Hartman, B. Levenberg, and J. M. Buchanan, *J. Am. Chem. Soc.* **77,** 501 (1954).

inhibition caused by azaserine not only was partially nullified by the presence of excess glutamine, but also led to the accumulation of sufficient glycinamide ribonucleotide and its *N*-formyl derivative for rigorous characterization studies.[133] Confirmation of these findings was rapid [134] and further work with *E. coli* indicated that preformed purines or aminoimidazolecarboxamide, as well as the amino acids mentioned above, could prevent the inhibitory action of azaserine.[135] The fact that these antagonists did not inhibit the incorporation of formate into the thymine of DNA, and of preformed adenine into liver,[136] intestine, and sarcoma-180 in mice (indeed, stimulation of the utilization of preformed purines has been observed) is indicative of the specificity of its action.[137] In fact, profound blockade of the synthesis of purines *de novo* by *E. coli* was obtained with doses which were only one-tenth those needed to disturb amino acid metabolism.[138] It has been reported that azaserine caused a marked increase in the size of the glutamine pool and a decrease in other free amino acids in *Scenedesmus* and *Chlorella* cells.[139] Presumably, this represents a blockade in many of the transamination reactions upon which amino acid formation depends; however, the concentrations of azaserine required were far greater than those which inhibited the synthesis *de novo* of purines. Accumulation of glutamine in the acid-soluble fraction of *E. coli* inhibited by azaserine also has been reported.[140]

More detailed studies[141] of the nature of the interaction between azaserine and the enzyme involved in the transfer of amino groups from glutamine to formylglycinamide ribonucleotide have revealed competitive kinetics over short periods when azaserine was added simultaneously. Under these same conditions DON had an affinity for this enzyme which was approximately forty times greater than that of azaserine. If, however, the enzyme was preincubated with azaserine or DON, an irreversible inactivation occurred. Similarly, in the presence of excess formylglycinamide ribonucleotide, the reaction kinetics would change from competitive to noncompeti-

[133] S. C. Hartman, B. Levenberg, and J. M. Buchanan, *J. Biol. Chem.* **221,** 1057 (1956).
[134] A. J. Tomisek, H. J. Kelly, and H. E. Skipper, *Arch. Biochem. Biophys.* **64,** 437 (1956).
[135] L. L. Bennett, Jr., F. M. Schabel, Jr., and H. E. Skipper, *Arch. Biochem. Biophys.* **64,** 423 (1956).
[136] R. K. Barclay, E. Garfinkel, and M. Phillips, *Proc. Am. Assoc. Cancer Research* **2,** 93 (1956).
[137] J. F. Fernandes, G. A. LePage, and A. Lindner, *Cancer Research* **16,** 154 (1956).
[138] A. J. Tomisek, H. J. Kelly, and H. E. Skipper, *Abstr. 128th Meeting Am. Chem. Soc., Minneapolis, Minn.* p. 5C (1955).
[139] S. A. Barker, J. A. Bassham, M. Calvin, and U. C. Quarck, *J. Am. Chem. Soc.* **78,** 4632 (1956).
[140] A. J. Tomisek, M. R. Reid, and H. E. Skipper, *Cancer Research* **19,** 489 (1959).
[141] B. Levenberg, I. Melnick, and J. M. Buchanan, *J. Biol. Chem.* **225,** 163 (1957).

tive during the increased time required for complete reaction. Preliminary evidence for a direct interaction between azaserine and the enzyme had been reported earlier[142, 143]; thus, washing of Ehrlich ascites carcinoma cells previously exposed *in vivo* to azaserine did not restore their normal capacity for the synthesis of purines. Experiments *in vitro* with these cells also demonstrated that a finite time was required before inhibition was established. These diazo compounds appear to react with the enzyme to form a stable complex which cannot be dissociated by glutamine. The correctness of this view has been shown by the isolation of two peptides containing azaserine residues, after reaction of the highly purified enzyme with *O*-(diazoacetyl-2-C^{14})-L-serine, and subsequent digestion of the product with trypsin[144]; analogous covalent bonding did not result when crystalline bovine serum albumin was treated with azaserine-C^{14}. Identification of the radioactive peptides should provide valuable information concerning the structure of the active center of this enzyme.

In general, the most rapidly proliferating tissues, such as tumor and intestine, experience the most profound inhibition of purine synthesis by azaserine or DON, while liver is relatively immune to their action.[145] This may be attributable in part to deamination of the azaserine, known to occur in rat liver.[146, 147] A number of sublines of transplantable tumors of mice have been selected for resistance to azaserine or DON. One of these, a resistant strain of plasma cell neoplasm 70429, appeared abruptly during chronic treatment with azaserine of mice bearing this tumor.[148] The situation resembled the appearance of full resistance to streptomycin, which may occur in one step in certain bacterial strains. In chemotherapeutic studies in children with acute leukemia,[149] and also in studies with *E. coli*,[150] refractoriness to these agents has developed so promptly as again to suggest a one-step mechanism; however, an example of what might be considered stepwise increases in the degree of resistance, similar to those noted with penicillin, also has been recorded.[151] In a cell-free preparation from an azaserine-resistant strain of the plasma cell neoplasm (70429), the incorporation of glycine into acid-soluble purines was inhibited by azaserine to the

[142] G. A. LePage, J. Greenlees, and J. F. Fernandes, *Ann. N. Y. Acad. Sci.* **63,** 999 (1956).

[143] J. Greenlees and G. A. LePage, *Cancer Research* **16,** 808 (1956).

[144] R. L. Herrmann, R. A. Day, and J. M. Buchanan, *Abstr. 135th Meeting Am. Chem. Soc., Boston, Mass.*, p. 45C (1959).

[145] E. C. Moore and G. A. LePage, *Cancer Research* **17,** 804 (1957).

[146] H. C. Reilly, *Proc. Am. Assoc. Cancer Research* **2,** 41 (1955).

[147] G. A. LePage and A. C. Sartorelli, *Texas Repts. Biol. and Med.* **15,** 169 (1957).

[148] M. Potter and L. W. Law, *J. Natl. Cancer Inst.* **18,** 413 (1957).

[149] G. S. Tarnowski and C. C. Stock, *Cancer Research* **17,** 1033 (1957).

[150] R. E. Maxwell and V. S. Nickel, *Antibiotics & Chemotherapy* **7,** 81 (1957).

[151] A. C. Sartorelli and G. A. LePage, *Cancer Research* **18,** 457 (1958).

same extent as with extracts obtained from the parent line, while intact resistant cells *in vitro* were unaffected by the same concentrations of the antagonist.[152] Similar results have been obtained with a subline of TA3 carcinoma[151]; however, in this case, the resistant cells which were exposed to azaserine *in vivo* recovered over 50% of their normal ability to synthesize purines after 24 hours, at a time when these reactions in the cells of the sensitive line were still inhibited by about 85%.

Although the primary site of action of azaserine and DON appears to be the enzyme concerned with the donation of the amino group of glutamine to formylglycinamide ribonucleotide, several other sites of action have been demonstrated. Inhibition of the initial step in the synthesis of purine ribonucleotides, i.e., the formation of ribosylamine-5-phosphate from glutamine and pyrophosphorylribose-5-phosphate, requires approximately fifty times the level of azaserine which is necessary to inhibit the enzyme discussed above.[141] In this initial reaction, the simultaneous addition of glutamine in excess will nullify most of the inhibition caused by azaserine, and DON is a much more effective inhibitor than azaserine.[153] Similar results have been obtained by others,[145, 154] and, at even higher levels of azaserine, inhibition of the conversion of xanthylic acid to guanylic acid has been observed.[113, 114]

Interference in pyrimidine metabolism by DON and azaserine has been detected only in the conversion of uracil nucleotides to cytidine nucleotides. Thus, DON inhibited the incorporation of carbamyl-L-aspartic and orotic acids into cytosine, as compared to uracil or thymine, in the nucleic acids of tumors and normal tissues of rats *in vivo*.[115] Similar results were obtained with tissue slices, but prevention of the effects of DON by glutamine was not very effective. In whole cell suspensions of the Novikoff hepatoma, DON profoundly inhibited the conversion of orotic acid to cytidine ribonucleotides, and these findings were confirmed in studies with a cytoplasmic fraction.[155]

Although the sites of enzymic inhibition established by either azaserine or DON have been clearly indicated as those which utilize glutamine, the mechanisms by which apparently unrelated amino acids, such as phenylalanine and tryptophan, prevent inhibition of growth by azaserine in microbial systems, have yet to be explained.[140,156] It may be that these compounds nullify the action of azaserine by preventing access to receptor sites

[152] E. P. Anderson, B. Levenberg, and L. W. Law, *Federation Proc.* **16,** 145 (1957).

[153] W. Barg, E. Boggliano, N. Sloane, and E. C. DeRenzo, *Federation Proc.* **16,** 150 (1957).

[154] J. S. Gots and E. G. Gollub, *J. Bacteriol.* **72,** 858 (1956).

[155] H. O. Kammen and R. B. Hurlbert, *Cancer Research* **19,** 654 (1959).

[156] H. E. Skipper and J. R. Thomson, *in* "Ciba Foundation Symposium on Amino Acids and Peptides with Antimetabolite and Cytotoxic Action," (G. E. W. Wolstonholme and C. M. O'Connor, eds.), p. 38. Churchill, London, 1958.

for glutamine; however, if this is the case, these amino acids should be competitive inhibitors of glutamine in the isolated enzyme systems. In spite of the fact that azaserine and DON cause profound inhibition of the growth of a variety of experimental neoplasms, the use of these agents in the treatment of human cancer has been disappointing. Remission of acute leukemia in children has been observed, but it is usually of very short duration; however, combinations of azaserine and mercaptopurine appear to delay the evolution of populations of cells which are resistant to these agents in both experimental neoplasms and acute leukemia in children.

Two other analogs of glutamine, γ-glutamylhydrazine

$$H_2N—NH—CO—CH_2—CH_2—CH(NH_2)—COOH$$

and *O*-carbamylserine

$$H_2N—CO—O—CH_2—CH(NH_2)—COOH$$

have been demonstrated to inhibit the amination of formylglycinamide ribonucleotide, but they are much weaker antagonists than azaserine or DON.[141] More recently *O*-carbazylserine

$$H_2N—NH—CO—O—CH_2—CH(NH_2)—COOH$$

has been synthesized and reported to have a similar inhibitory action on the amination reactions required in the synthesis of purines and pyrimidines.[157] However, this is an interpretation of microbial growth studies with *Streptococcus lactis*, and the corresponding enzymic results are not available.

IV. Structural Analogs of Purines and Their Metabolic Activity

Although the presence of purines in the nucleic acids had been well established by the turn of the century, it has only been during the last fifteen years that purposeful attempts have been made to design purine analogs with the specific aim of interfering with nucleic acid synthesis or function. A considerable portion of the progress in this field can be attributed to the synthetic efforts of workers at the Wellcome Research Laboratories (U.S.A.) where a program of synthesis of purine (and pyrimidine) analogs was begun in 1942.[158, 159] Since that time a large number of compounds have been prepared and examined for biological activity in microbial[160] or experimental tumor systems.[161] Although much information has been obtained concern-

[157] T. J. McCord, J. M. Ravel, C. G. Skinner, and W. Shive, *J. Am. Chem. Soc.* **80**, 3762 (1958).

[158] G. H. Hitchings and G. B. Elion, *Ann. N. Y. Acad. Sci.* **60**, 195 (1954).

[159] G. H. Hitchings and G. B. Elion, *Proc. 3rd Intern. Congr. Biochem., Brussels, 1955* p. 55 (1956).

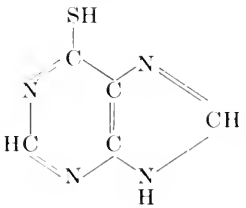
[160] G. B. Elion, G. H. Hitchings, and H. Vander Werff, *J. Biol. Chem.* **192**, 505 (1951).

[161] D. A. Clarke, G. B. Elion, G. H. Hitchings, and C. C. Stock, *Cancer Research* **18**, 445 (1958).

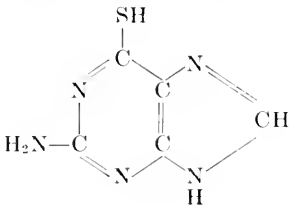
ing the relationship of structure to action,[6] the more interesting derivatives generally closely resemble the naturally occurring purines. As will be apparent in the discussion to follow, the primary sites of action of many of these derivatives cannot be strictly defined at the present time, but their biological activities are clearly related to nucleic acid metabolism and in some cases have been responsible for the attainment of desirable therapeutic effects in human disease.

1. 6-Mercaptopurine

Among the very large number of purine analogs[159, 161] which have been prepared, 6-mercaptopurine (IX) is still regarded as the most useful therapeutic agent of this type in cancer chemotherapy. Although definitive statements concerning its mechanism of action as an inhibitor of growth cannot be made, several areas in which it functions as an antimetabolite have been demonstrated. This compound was prepared[162] as one of a large series of 2- and 6-substituted purine derivatives containing mercapto or substi-



(IX)
6-Mercaptopurine



(X)
Thioguanine

tuted mercapto groups. Mercaptopurine may be considered an analog of either hypoxanthine or adenine and indeed it is in the formation or function of these purine derivatives that the most prominent inhibitory activity of this antimetabolite is expressed.

Results from the preliminary screening of mercaptopurine as an inhibitor of the growth of *Lactobacillus casei*[160] and experimental tumors[163] indicated the biological activity of the compound. Although initial tests on sarcoma-180 in mice disclosed only borderline activity, transplants from the tumors following mercaptopurine treatment frequently were noted to be nonviable. Subsequent studies by many workers, using this and other transplantable neoplasms, have confirmed the antitumor activity and led to the widespread use of the compound in the treatment of certain types of leukemia.[164, 165]

[162] G. B. Elion, E. Burgi, and G. H. Hitchings, *J. Am. Chem. Soc.* **74,** 411 (1952).

[163] D. A. Clarke, F. S. Philips, S. S. Sternberg, C. C. Stock, G. B. Elion, and G. H. Hitchings, *Cancer Research* **13,** 593 (1953).

[164] H. E. Skipper, J. R. Thomson, G. B. Elion, and G. H. Hitchings, *Cancer Research* **14,** 294 (1954).

[165] Summarized in *Ann. N. Y. Acad. Sci.* **60,** 365–499 (1954).

The early microbial experiments with *L. casei* and *S. faecalis*, microorganisms which have absolute purine requirements in the absence of folic acid, defined the specific anti-purine action of this compound. The inhibition of growth caused by mercaptopurine could be reversed in both organisms by the four natural purines, hypoxanthine, xanthine, adenine, and guanine.[166-168] In a medium containing pteroylglutamic acid, xanthine was the most effective agent in overcoming mercaptopurine inhibition in *S. faecalis* and a strain selected for resistance to this analog was unable to utilize adenine, guanine, or hypoxanthine for growth in the absence of pteroylglutamic acid, while xanthine remained active as a purine source and as a reversing agent for mercaptopurine toxicity.[168] Such data led to the view[169] that one of the primary sites of action of mercaptopurine may lie in the interconversion of guanine and adenine nucleotides through a hypoxanthine derivative, as was recently demonstrated.[114] This concept is supported by the accumulation of a large amount of inosine and hypoxanthine following growth of a mercaptopurine-resistant strain (MPR) of *L. casei* in a medium containing adenine-8-C^{14}.[170] More recently, purine metabolism has been studied in growing cultures of strains of *S. faecalis* which were either sensitive or resistant to the inhibitory action of the purine analogs, mercaptopurine and azaguanine. The resistant strains varied greatly in their ability to utilize guanine or hypoxanthine for the synthesis of the purines of nucleic acids, but could incorporate adenine into the adenine of nucleic acid, and xanthine into both of the purines of nucleic acids.[171, 172] Similar studies with a subline of L-1210 leukemia which was selected for resistance to mercaptopurine and azaguanine revealed marked depression in the metabolism of guanine or hypoxanthine, but little change in the utilization of adenine.[173] However, it has not been established unequivocally that the nutritional and, in fact, even the enzymic differences between drug-sensitive strains and resistant sublines are related to the mechanism of action of an antimetabolite, although it is probable that this is the case. Certainly, such studies have great value in achieving an understanding of the mechanism of resistance and thereby in suggesting means of circumventing this ubiquitous problem in chemotherapy.

[166] G. B. Elion, S. Singer, G. H. Hitchings, M. E. Balis, and G. B. Brown, *J. Biol. Chem.* **202**, 647 (1953).

[167] G. B. Elion, S. Singer, and G. H. Hitchings, *Ann. N. Y. Acad. Sci.* **60**, 200 (1954).

[168] D. J. Hutchison, *Ann. N. Y. Acad. Sci.* **60**, 212 (1954).

[169] G. B. Elion, S. Singer, and G. H. Hitchings, *J. Biol. Chem.* **204**, 35 (1953).

[170] G. B. Elion and G. H. Hitchings, *Federation Proc.* **13**, 203 (1954).

[171] D. J. Hutchison, *Cancer Research* **18**, 214 (1958).

[172] M. E. Balis, V. Hylin, M. K. Coultas, and D. J. Hutchison, *Cancer Research* **18** 220 (1958).

[173] R. W. Brockman, C. Sparks, M. S. Simpson, and H. E. Skipper, *Biochem. Pharmacol.* **2**, 77 (1959).

Before discussing in further detail the proposed sites or mechanisms of action of mercaptopurine, the metabolic disposition of this drug must be considered. Most of the conversions are the counterparts of enzymic reactions for which the natural purines are substrates. Oxidation of mercaptopurine by xanthine oxidase preparations forms thiouric acid (2,8-dihydroxy-6-mercaptopurine),[174, 175] which is a major catabolite found both in mammalian[174] and bacterial systems.[176] From resting cell suspensions of *Bacillus cereus*, the intermediate oxidation product thioxanthine (2-hydroxy-6-mercaptopurine) has also been isolated.[176] Following administration of mercaptopurine to mice, rats, and leukemic patients, small amounts of 6-methylmercaptopurine[177] and an unidentified metabolite have been isolated from the urine.[178] Another route of catabolism is desulfurization of mercaptopurine,[174, 179, 179a] presumably to yield hypoxanthine, a reaction which although limited in some species has considerable significance, since hypoxanthine effectively overcomes many of the inhibitory effects of the analog. This reaction accounts in part for the incorporation of the radioactivity into the purines of the nucleic acids of animals[174] and particularly of microorganisms[180] treated with mercaptopurine-8-C^{14}. Of greater interest, however, are the reactions which result in the formation of anabolic derivatives of this antimetabolite.

The formation *in vivo* of the ribonucleotides of mercaptopurine has been demonstrated in mammalian and bacterial systems.[173, 179, 179a, 181, 182] In almost all cases, populations of cells selected for resistance to mercaptopurine are inefficient in this conversion as well as in the utilization of the analog as a source of natural purines via desulfurization. Such evidence prompted the chemical synthesis of mercaptopurine ribonucleoside[183] in an attempt to overcome the inefficient conversion in resistant lines of cells, as well as to reduce the steps required in the formation of the ribonucleotide derivatives

[174] G. B. Elion, S. Bieber, and G. H. Hitchings, *Ann. N. Y. Acad. Sci.* **60**, 297 (1954).

[175] T. L. Loo, *J. Am. Chem. Soc.* **81**, 3039 (1959).

[176] N. H. Carey and H. G. Mandel, *Abstr. Meeting Am. Soc. Pharmacol. Exptl. Therap., Ann Arbor*, p. 8 (1958).

[177] E. J. Sarcione and L. Stützman, *Proc. Am. Assoc. Cancer Research* **2**, 342 (1958).

[178] G. B. Elion and G. H. Hitchings, *Federation Proc.* **16**, 177 (1957).

[179] R. W. Brockman, C. Sparks, D. J. Hutchison, and H. E. Skipper, *Cancer Research* **19**, 177 (1959).

[179a] R. W. Brockman, L. L. Bennett, Jr., M. S. Simpson, A. R. Wilson, J. R. Thomson, and H. E. Skipper, *Cancer Research* **19**, 856 (1959).

[180] M. E. Balis, V. Hylin, M. K. Coultas, and D. J. Hutchison, *Cancer Research* **18**, 440 (1958).

[181] R. W. Brockman, C. Sparks, and M. S. Simpson, *Biochim. et Biophys. Acta* **26**, 671 (1957).

[182] A. R. P. Paterson, *Proc. Am. Assoc. Cancer Research* **3**, 50 (1959).

[183] J. A. Johnson and H. J. Thomas, *J. Am. Chem. Soc.* **78**, 3863 (1956).

which have been postulated to be the active inhibitors of growth.[181, 184, 185] Unfortunately, however, there is little if any difference between the biological activity of the ribonucleoside and that of mercaptopurine as inhibitors of sensitive or resistant populations of cells[184], a result which suggested either rapid cleavage of the ribonucleoside by nucleosidases to the free analog or its further metabolism to the ribonucleotide at a rate comparable to the formation of this derivative from mercaptopurine. A number of workers have studied formation of the unnatural nucleotides in cell-free systems. Mercaptopurine ribonucleotide has been prepared[186] by the condensation of the analog with pyrophosphorylribose-5-phosphate catalyzed by inosinic pyrophosphorylase from beef liver, an enzyme which also forms the ribonucleotides of hypoxanthine and guanine. Recently a purified pyrophosphorylase has been prepared from *E. coli* and found to exhibit approximately equal affinities for mercaptopurine and hypoxanthine, but a very much lower affinity for guanine.[187] Crude extracts of mouse leukemic cells form the ribonucleotide of mercaptopurine[173]; the ribonucleotide also is formed by a pyrophosphorylase from either yeast or liver[188] which appears to be different from the enzyme responsible for the formation of the ribonucleotides of guanine or hypoxanthine. Further phosphorylation of the ribonucleotide of mercaptopurine to the di- and triphosphates has been accomplished with ATP and an enzyme preparation from hog kidney.[189] Mercaptopurine deoxyribonucleoside has been formed by reaction of mercaptopurine with deoxyribose-1-phosphate catalyzed by nucleoside phosphorylase obtained from liver.[190]

Although convincing evidence is at hand concerning the conversion of mercaptopurine to ribonucleotides, the incorporation of this analog into nucleic acids has not been completely documented. Experiments with mercaptopurine-S^{35} and -C^{14} in mice indicate significant labeling of the nucleic acid fraction of rapidly proliferating tissues; upon hydrolysis to the free purines, several radioactive derivatives were found, one of which had the properties of mercaptopurine.[174] The other radioactive derivatives may be thioguanine or acid-degradation products of mercaptopurine nucleotides; incorporation into RNA appeared to be greater than that into DNA.

[184] H. E. Skipper, J. R. Thomson, D. J. Hutchison, F. M. Schabel, Jr., and J. J. Johnson, Jr., *Proc. Soc. Exptl. Biol. Med.* **95,** 135 (1957).

[185] M. E. Balis, D. H. Levin, G. B. Brown, G. B. Elion, H. C. Nathan, and G. H. Hitchings, *Arch. Biochem. Biophys.* **71,** 358 (1957).

[186] L. N. Lukens and K. A. Herrington, *Biochim. et Biophys. Acta* **24,** 432 (1957).

[187] C. E. Carter, *Biochem. Pharmacol.* **2,** 105 (1959).

[188] J. L. Way and R. E. Parks, Jr., *J. Biol. Chem.* **231,** 1467 (1958).

[189] J. L. Way, J. L. Dahl, and R. E. Parks, Jr., *Federation Proc.* **17,** 418 (1958); *J. Biol. Chem.* **234,** 1241 (1959).

[190] M. Friedkin, *Biochim. et Biophys. Acta* **18,** 447 (1955).

The nutritional and enzymic studies described above, coupled with information concerning the effect of mercaptopurine on biosynthetic reactions *in vivo* and *in vitro*, indicate that this antimetabolite has several sites of action. What cannot be said with certainty is that these actions are necessarily those which lead to inhibition of growth, or that the most critical site of action has been disclosed. Formation of ribonucleotides of the natural purines, particularly those of guanine and hypoxanthine, is competitively inhibited by mercaptopurine and, thus, at sufficiently high levels of the analog, utilization of preformed purines for the biosynthesis of nucleic acids and coenzymes is restricted.[180, 185, 187, 191] Generally, incorporation of adenine into nucleic acid or acid-soluble adenine derivatives is not affected appreciably by mercaptopurine.

Of much interest have been repeated observations indicating that mercaptopurine blocks the synthesis *de novo* of purines; this was evidenced by some of the earlier microbial growth experiments in defined media in the absence of exogenous purines.[187, 168] The incorporation of formate[4, 191-193] and of glycine[3, 137, 143] into both purines of the acid-soluble fraction and the nucleic acids of mammalian as well as microbial systems is depressed by mercaptopurine. These results, however, do not conform with the observation that neither mercaptopurine nor its ribonucleotide inhibits the synthesis of inosinic acid from glycine in soluble enzyme preparations from pigeon liver.[186] The resolution of this apparent impasse may be found in a preliminary report which shows that mercaptopurine ribonucleotide inhibits the conversion of inosinic acid to adenylosuccinate (the intermediate in the formation of adenylic acid) in a cell-free extract of *S. faecalis*.[194] Less marked inhibition was also seen in the oxidation by an unidentified system of inosinic acid to xanthylic acid, the presumed precursor of guanine ribonucleotides. The same report confirmed earlier studies[172, 179] showing that a resistant strain of this organism exhibited an impaired capacity for the formation of the ribonucleotides of mercaptopurine, guanine, and hypoxanthine; however, it was suggested that a sufficient amount of the analog ribonucleotide was formed to block the conversion of all the natural purines, except xanthine, into ribonucleotides and their subsequent incorporation into nucleic acids.[181]

These findings would imply that the apparent inhibition of the synthesis *de novo* of purines by mercaptopurine may be related to interference with the conversion of inosinic acid to the appropriate adenine and guanine

[191] R. W. Brockman, L. L. Bennett, Jr., and H. E. Skipper, *Proc. Am. Assoc. Cancer Research* **2**, 191 (1957).

[192] H. E. Skipper, *Ann. N. Y. Acad. Sci.* **60**, 315 (1954).

[193] H. G. Mandel, J. K. Inscoe, H. M. Maling, and P. K. Smith, *J. Pharmacol. Exptl. Therap.* **120**, 195 (1957).

[194] J. S. Salser and M. E. Balis, *Federation Proc.* **18**, 314 (1959).

derivatives. This assumes that a major part of purine synthesis *de novo* passes through this common intermediate. An additional site of action which has been proposed for microbial systems is in the formation of a purine precursor prior to the ribonucleotide of aminoimidazolecarboxamide. The accumulation of aminoimidazolecarboxamide by suspensions of a purine-requiring mutant of *E. coli* was suppressed not only by the presence of natural purines in the medium, but also by mercaptopurine[195]; this result was interpreted as a possible negative feedback control of the pathways of purine synthesis by the analog.

Numerous other effects of mercaptopurine in systems *in vivo* have been reported, but these may be referable to reduced production of purine ribonucleotides and coenzymes. Thus, not only the increase in the level of DPN in the liver caused by the injection of nicotinamide, but also the rate of the subsequent diminution of the DPN-level, were reduced by mercaptopurine.[196] The adaptive formation of β-galactosidase by *Mycobacterium tuberculosis* was inhibited by this analog.[197] Mercaptopurine also appears to reduce or eliminate the formation of antibodies in rabbits[198] and to inhibit the acetylation of sulfanilamide.[199]

The possible relationship of this latter finding to reversal by coenzyme A[200, 201] of the inhibition of mitosis of sarcoma-180 and of lipogenesis in mouse fibroblasts in cell culture by mercaptopurine will be apparent. In a detailed study of the effects of this antimetabolite on a number of biosynthetic systems in growing cultures of *E. coli* B,[202] temporary inhibition of growth was seen at levels of the analog which had demonstrated no effect on other aspects of cellular chemistry. Other parameters of cell function were affected in the following order of decreasing sensitivity: acetate utilization for lipid and protein synthesis, formate incorporation, and nucleic acid synthesis. Effects of mercaptopurine on glycolysis, presumably indirect, have been noted at high levels of the agent.[203-206] However, as pointed out by many of the workers quoted above, the subtle effects of purine nucleotide or coenzyme deprivation on cell growth by the mechanisms discussed may be difficult to detect when growth phenomena are studied in the living cell.

Of the many derivatives of mercaptopurine, certain S-glycosyl compounds[207-209]

[195] J. S. Gots and E. G. Gollub, *Proc. Am. Assoc. Cancer Research* **2**, 207 (1957).

[196] N. O. Kaplan, A. Goldin, S. R. Humphreys, M. M. Ciotti, and F. E. Stolzenbach, *J. Biol. Chem.* **219**, 287 (1956).

[197] L. Ottey, *J. Pharmacol. Exptl. Therap.* **115**, 339 (1955).

[198] R. Schwartz, A. Eisner, and W. Dameshek, *Clin. Research* **7**, 39 (1959).

[199] S. Garattini and R. Paoletti, *Giorn. ital. chemioterap.* **3**, 55 (1956); *Chem. Abstr.* **51**, 4452 (1957).

[200] J. J. Biesele, *Ann. N. Y. Acad. Sci.* **71**, 1054 (1958).

[201] J. J. Biesele, *Ann. N. Y. Acad. Sci.* **60**, 228 (1954).

[202] E. T. Bolton and H. G. Mandel, *J. Biol. Chem.* **227**, 833 (1957).

[203] P. Hochstein, *Proc. Am. Assoc. Cancer Research* **2**, 214 (1957).

[204] D. Burk, J. Laszlo, J. Stengle, and K. Wight, *Federation Proc.* **16**, 160 (1957).

[205] J. Laszlo, J. Stengle, K. Wight, and D. Burk, *Proc. Am. Assoc. Cancer Research* **2**, 224 (1957).

[206] D. Burk, *Klin. Wochschr.* **35**, 1102 (1957).

[207] I. Goodman, G. B. Elion, and G. H. Hitchings, *Federation Proc.* **14**, 219 (1955).

[208] I. Goodman, J. R. Fouts, and G. H. Hitchings, *Federation Proc.* **17**, 232 (1958).

are of interest because they are slowly cleaved to mercaptopurine in the intestine following oral administration.[208, 210, 211] Similarly, 6-(1′-methyl-4′-nitro-5′-imidazolyl)-thiopurine and the analogous derivative of thioguanine apparently serve as sources of the corresponding thiopurines *in vivo*[212, 213]; these compounds have shown sufficient promise to justify clinical trials which are now in progress.[214] Substitution of selenium for sulfur results in a compound, 6-selenopurine,[215] with increased biological activity against certain microorganisms.[216] Studies with experimental tumors in mice indicate that, in general, selenopurine is less active than mercaptopurine,[217] but it profoundly inhibits the incorporation of formate into nucleic acid purines of Ehrlich ascites tumor cells *in vitro*.[216]

2. Thioguanine

The guanine analog, thioguanine (2-amino-6-mercaptopurine) (X), was first prepared in 1948. Although the method of synthesis was similar to that described for 6-mercaptopurine, the yields were variable and a satisfactory synthesis involving thiation of guanine by phosphorus pentasulfide in pyridine did not appear until 1955.[218] However, sufficient material was available in the intervening years for demonstration of the marked inhibition of the growth of *L. casei* by the analog,[160] an effect which could be prevented by adenine, guanine, hypoxanthine, or xanthine at one-tenth the level of thioguanine.[166] Studies with *S. faecalis* indicated similar inhibition except that xanthine was ineffective as a reversing agent[168]; strains of these organisms selected for resistance to thioguanine were cross-resistant to 6-mercaptopurine.[167, 168] Thioguanine was also shown to inhibit the growth of experimental neoplasms in mice at one-twentieth the dosage levels required for mercaptopurine[161, 219]; however, toxicity to the host, manifested as extreme depression of the bone marrow and resultant agranulocytosis and thrombocytopenia, was observed. Nevertheless, these preliminary results led to a clinical trial of thioguanine in human leukemia[107]; although the results were encouraging, the compound appeared to offer no advantage over mercaptopurine and dosage levels similar to those of mercaptopurine were required, in contrast to the results obtained in mice.

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209 I. Goodman, *Federation Proc.* **18,** 236 (1959).

210 D. A. Clarke and G. H. Hitchings, *Proc. Am. Assoc. Cancer Research* **2,** 287 (1958).

211 G. H. Hitchings, J. R. Fouts, F. S. Philips, and S. S. Sternberg, *Proc. Am. Assoc. Cancer Research* **2,** 307 (1958).

212 G. B. Elion and G. H. Hitchings, *Federation Proc.* **18,** 221 (1959).

213 G. H. Hitchings and G. B. Elion, *Proc. Am. Assoc. Cancer Research* **3,** 27 (1959).

214 G. B. Elion, G. H. Hitchings, and R. W. Rundles, *Proc. Am. Assoc. Cancer Research* **3,** 18 (1959).

215 H. G. Mautner, *J. Am. Chem. Soc.* **78,** 5292 (1956).

216 H. G. Mautner, *Biochem. Pharmacol.* **1,** 169 (1958).

217 H. G. Mautner and J. J. Jaffe, *Cancer Research* **18,** 294 (1958).

218 G. B. Elion and G. H. Hitchings, *J. Am. Chem. Soc.* **77,** 1676 (1955).

219 D. A. Clarke, F. S. Philips, S. S. Sternberg, and C. C. Stock, *Ann. N. Y. Acad. Sci.* **60,** 235 (1954).

The ribonucleoside, thioguanosine, has been synthesized[220] and found in most respects to resemble thioguanine as an inhibitor of the growth of sarcoma-180 and other mammalian cells in tissue culture. However, preliminary clinical reports[221] have suggested that the ribonucleoside, at dose levels considerably lower than those required for thioguanine, has some value in the treatment of leukemia, a circumstance which implies that the derivative either has greater potency or is more favorably absorbed, distributed, and excreted than is the free base.

Recently, more detailed studies of the mechanism of action of thioguanine in mammalian systems have been described.[222] These studies were facilitated by the use of thioguanine-C^{14} and by the fact that this analog and its various derivatives have absorption maxima in the range of 320 to 360 mμ. Following intraperitoneal injection of the analog into mice bearing Ehrlich ascites tumor cells, and sacrifice of the animals after 45 minutes, the majority of the compound in the tumor tissue could be recovered as thioguanosine-5′-phosphate. The ribonucleotide was also prepared by condensation of thioguanine with pyrophosphorylribose-5-phosphate; this reaction was catalyzed by a dialyzed extract (obtained from an acetone powder prepared from Ehrlich ascites cells) in the presence of Tris buffer, Mg^{++}, and inorganic pyrophosphatase. Small amounts of what appeared to be di- and triphosphates of thioguanosine also were present in acid-soluble extracts of tumors, but their identity has not been established unequivocally. Other metabolites isolated from tumor cells exposed to thioguanine *in vivo* included small amounts of thioguanine ribonucleoside and thiouric acid (6-mercapto-2,8-dihydroxypurine). When thioguanine or thioxanthine (2-hydroxy-6-mercaptopurine) were incubated with a partially purified preparation of xanthine oxidase, thioxanthine was readily converted to thiouric acid; however, thioguanine was not affected by the enzyme, a finding which indicates the necessity for deamination (of position 2) before oxidation in position 8 will occur. In contrast to tumor cells, extracts obtained at the same time from the intestine contained considerably less thioguanylic acid and more thiouric acid. It is of interest that pretreatment of the animals with azaserine, which has been shown to deplete the pools of acid-soluble purine ribonucleotides,[151] resulted in an enhanced conversion of thioguanine to the ribonucleotides in tumor cells; however, in the intestine, in which azaserine at this particular dosage level had little effect on the pools of purine-containing metabolites, ribose-containing derivatives of thioguanine did not accumulate. This finding resembles the compensatory

[220] J. J. Fox, I. Wempen, A. Hampton, and I. L. Doerr, *J. Am. Chem. Soc.* **80**, 1669 (1958).

[221] I. H. Krakoff, R. R. Ellison, and C. T. Tan, *Proc. Am. Assoc. Cancer Research* **3**, 34 (1959).

[222] E. C. Moore and G. A. LePage, *Cancer Research* **18**, 1075 (1958).

stimulation of the uptake of adenine seen in animals treated with azaserine; however, with thioguanine the result is additional injury to the cell and the potentiation of its antitumor activity by azaserine.[223, 224]

Studies on the metabolism of thioguanine by the bone marrow of guinea pigs were conducted in an attempt to reveal the biochemical reason for the primary sensitivity of this organ[222]; however, a pattern not unlike that seen with intestine was obtained. It was proposed, however, that bone marrow, which is remarkably dependent upon preformed purines,[225, 226] would have a higher sensitivity to an antagonist of preformed purines than would tissues (such as intestine) which are capable of extensive synthesis *de novo*. Other studies,[224] in which a thioguanine-resistant subline of the Ehrlich ascites carcinoma was used, appear to substantiate the suggestion that formation of the ribonucleoside or its phosphorylated derivatives is essential for tumor inhibition. Extracts of sensitive and resistant tumors obtained at various time periods after injection of thioguanine revealed a lower uptake and a more rapid clearance of thioguanine from the resistant cells. Furthermore, the anabolic conversion to thioguanylic acid was more extensive in the sensitive cells, while the resistant cell line appeared to possess a more active degradative system, giving rise to thioxanthine and thiouric acid; however, the more extensive degradation by the resistant cells might be a reflection of the inefficient conversion to thioguanylic acid. Another manifestation of the difference between sensitive and resistant cells was found in the incorporation of thioguanine into the combined nucleic acids. Although less than 1% of the nucleic acid purines of the sensitive cells was replaced by the analog, little if any intact analog appeared in the nucleic acids of the resistant cells.[227] As with mercaptopurine, a small amount of thioguanine was converted in the mouse to acid-soluble and nucleic acid adenine and guanine, a conversion which also was reduced in the resistant-cell line.

Information concerning the primary site or sites of action of thioguanine is not yet complete, but certain areas are indicated at this time. Preliminary microbial reversal studies implied a blockade in the utilization of preformed purines. However, in mammalian systems there may be several mechanisms of action.[228] In mice bearing Ehrlich ascites carcinoma cells, the intraperitoneal injection of thioguanine, followed by injections of guanine-8-C^{14} at various time intervals, resulted in an inhibition of incorporation of the guanine into RNA and DNA of the tumor cells to the extent of from 60 to 80% of that occurring in untreated animals. However, the inhibition

[223] A. C. Sartorelli and G. A. LePage, *Cancer Research* **18,** 938 (1958).
[224] A. C. Sartorelli, G. A. LePage, and E. C. Moore, *Cancer Research* **18,** 1232 (1958).
[225] R. A. Abrams and M. Bentley, *Arch. Biochem. Biophys.* **58,** 109 (1955).
[226] L. G. Lajtha and J. R. Vane, *Nature* **182,** 191 (1958).
[227] G. A. LePage, *Proc. Am. Assoc. Cancer Research* **3,** 36 (1959).
[228] A. C. Sartorelli and G. A. LePage, *Cancer Research* **18,** 1329 (1958).

was not sustained, and little or no effect on the appearance of the C^{14} of guanine in the adenine of the nucleic acids was seen, even at early time periods. Similarly, thioguanine did not exert a primary effect on the incorporation of adenine per se into the purines of nucleic acids. When the incorporation of glycine into the purines of nucleic acids was challenged by pretreatment with thioguanine, incorporation into adenine was suppressed by 75% and that into guanine by 60%. This effect was achieved under conditions in which no blockade in the incorporation of glycine into proteins was observed. Similar studies have been carried out on the effect of thioguanine on the incorporation of formate into purines and of methionine into proteins of L-1210 leukemia *in vitro*.[229] In experiments with glycine-C^{14} the accumulation of formylglycinamide ribonucleotide, which normally results from azaserine treatment, was sharply reduced by the subsequent administration of thioguanine.[228] It was suggested that this apparent interference at an early stage of the synthesis of purines *de novo* may be attributable to a competition between thioguanine and normal intermediates for pyrophosphorylribose-5-phosphate. A third possible site of action, revealed in part by the data concerning the incorporation of guanine and glycine, is suggested by experiments *in vitro* with Ehrlich ascites carcinoma cells. In these, a marked depression of the incorporation of inosinic acid into adenine of the nucleic acids occurred at levels of thioguanine which had a less striking or no effect on the incorporation of adenine, guanine, or even glycine.[228]

In a preliminary study with *Bacillus cereus*,[230] thioguanine caused inhibition of growth at levels which were one-fifth those required for effects on nucleic acid content of the cell. Under these conditions, interference with the formation or function of purine-containing cofactors may be the primary cause of inhibition of growth; however, proportional restriction of growth and synthesis of nucleic acids may occur at these low levels of inhibitor.

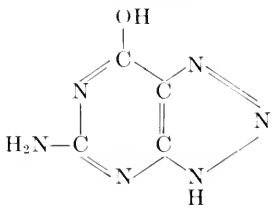
Thus, it can be seen that in many respects thioguanine resembles mercaptopurine in the types of inhibition established, even with respect to effects involving specific metabolites. This has led to the speculation that mercaptopurine may exert a portion of its effects following conversion to thioguanine nucleotide. However, precise definition of the mechanisms of action of thioguanine must await enzymic studies with isolated portions of the anabolic pathways involving purine-containing compounds.

3. Azaguanine

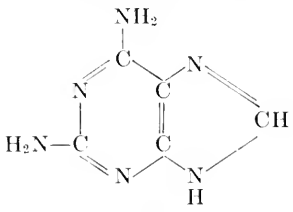
By ring closure of diaminopyrimidines with nitrous acid a group of 8-azapurines (*v*-triazolo-[*d*]-pyrimidines) has been prepared; analogs of the sev-

[229] F. U. Brown and H. G. Mandel, *Proc. Assoc. Cancer Research* **2**, 284 (1958).
[230] H. G. Mandel and R. G. Latimer, *Proc. Am. Assoc. Cancer Research* **3**, 40 (1959).

eral physiologically occurring purines were synthesized in 1945 and their biological properties tested.[231] The initial microbial assays with *E. coli* in-



(XI)
8-Azaguanine



(XII)
2,6-Diaminopurine

dicated that 8-azaguanine (XI) is an antagonist of guanine and that growth could be restored with either guanine or xanthine, but not with hypoxanthine or adenine. Conversely, inhibition of growth by 8-azaadenine was nullified by supplementing the medium with adenine or hypoxanthine. Subsequently, azaguanine was shown to be a potent inhibitor of the growth of *Tetrahymena pyriformis* (formerly designated *T. gelii*), an organism which requires preformed guanine.[232, 233] In this system up to 50 moles of guanine were required to overcome the inhibition caused by 1 mole of azaguanine. These early results with azaguanine have been extended to a number of viral,[234-238] bacterial,[239, 240] protozoal,[241] and avian[242] systems. Azaguanine also has been reported to increase the mutation rate of *E. coli* B/It to T5 phage resistance,[243] an effect which is prevented by guanosine.

Considerable stimulation of interest in azaguanine resulted from the demonstration of inhibition of the growth of certain tumors in experimental animals treated with the analog.[244-250] In microbial and mammalian systems

[231] R. O. Roblin, Jr., J. O. Lampen, J. P. English, Q. P. Cole, and J. R. Vaughan, Jr., *J. Am. Chem. Soc.* **67,** 290 (1945).
[232] G. W. Kidder and V. C. Dewey, *J. Biol. Chem.* **179,** 181 (1949).
[233] M. Flavin and M. Engleman, *J. Biol. Chem.* **200,** 59 (1953).
[234] R. E. F. Matthews, *Nature* **167,** 892 (1951).
[235] R. E. F. Matthews, *Nature* **169,** 500 (1952).
[236] R. E. F. Matthews, *J. Gen. Microbiol.* **8,** 277 (1953).
[237] R. E. F. Matthews, *J. Gen. Microbiol.* **10,** 521 (1954).
[238] H. R. Morgan, *J. Exptl. Med.* **95,** 277 (1952).
[239] G. H. Hitchings, G. B. Elion, E. A. Falco, P. B. Russell, and H. Vander Werff, *Ann. N. Y. Acad. Sci.* **52,** 1318 (1950).
[240] R. E. F. Matthews and J. D. Smith, *Nature* **177,** 271 (1956).
[241] D. M. Lilly, F. S. Sterbenz, and V. Tarantola, *Proc. Soc. Exptl. Biol. Med.* **83,** 434 (1953).
[242] J. S. Younger, E. N. Ward, and J. E. Salk, *Proc. Soc. Exptl. Biol. Med.* **75,** 151 (1950).
[243] A. Novick and L. Szilard, *Nature* **170,** 926 (1952).
[244] G. W. Kidder, V. C. Dewey, R. E. Parks, Jr., and G. L. Woodside, *Science* **109,** 511 (1949).

azaguanine appeared to be a competitive inhibitor of the incorporation of guanine; this was indicated by experiments in which guanine was effective in overcoming the inhibition produced by this analog. However, subsequent studies have shown that its effects are more complicated and probably are related to the action of the nucleotide derivatives of azaguanine. The noninhibitory nature of 9-ethyl-8-azaguanine, a compound substituted at the normal point of ribose attachment, would tend to support this concept.[179] The formation of nucleotides was implied by the discovery of this analog in the nucleic acids of mice treated with azaguanine,[251, 252] a finding which has been extended to viruses,[253-255] plants,[255] microorganisms,[255-258] and animal tumors.[252, 257, 259] The extent of incorporation into DNA appears to be very small,[251, 252] while that into RNA is usually less than 5% of the total guanine (an exception is found in *Bacillus cereus*, in which up to 40% of the guanine of the RNA may be replaced by azaguanine under appropriate conditions[255]). That incorporation into RNA was not the result of purine exchange was established by the isolation of azaguanine ribonucleoside from the medium, after additional growth of a culture of *B. cereus* (previously treated with the analog) in the presence of the reversing agent, guanosine.[260]

The form of the incorporated analog has been clearly verified by the isolation and characterization of the mixed 2′- and 3′-phosphates of azaguanosine following alkaline digestion of the RNA.[253, 257, 261] Data obtained by analysis of RNA from *B. cereus* fractionated by ethanol, dialysis, or en-

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[245] G. W. Kidder, V. C. Dewey, R. E. Parks, Jr., and G. L. Woodside, *Cancer Research* **11**, 204 (1951).

[246] K. Sugiura, G. H. Hitchings, L. F. Cavalieri, and C. C. Stock, *Cancer Research* **10**, 178 (1950).

[247] L. W. Law, *Cancer Research* **10**, 186 (1950).

[248] M. Finkelstein and P. A. Thomas, *Cancer Research* **11**, 801 (1951).

[249] M. R. Murray, E. R. Peterson, E. Hirschberg, and J. L. Pool, *Ann. N. Y. Acad. Sci.* **58**, 1147 (1954).

[250] A. Gellhorn, E. Hirschberg, and A. Kells, *J. Natl. Cancer Inst.* **14**, 935 (1954).

[251] J. H. Mitchell, Jr., H. E. Skipper, and L. L. Bennett, Jr., *Cancer Research* **10**, 647 (1950).

[252] H. G. Mandel, P. E. Carlo, and P. K. Smith, *J. Biol. Chem.* **206**, 181 (1954).

[253] R. E. F. Matthews, *Nature* **171**, 1065 (1953).

[254] R. E. F. Matthews, *Virology* **1**, 165 (1955).

[255] J. D. Smith and R. E. F. Matthews, *Biochem. J.* **66**, 323 (1957).

[256] M. R. Heinrich, V. C. Dewey, R. E. Parks, Jr., and G. W. Kidder, *J. Biol. Chem.* **197**, 199 (1952).

[257] I. Lasnitzki, R. E. F. Matthews, and J. D. Smith, *Nature* **173**, 346 (1954).

[258] H. G. Mandel, *J. Biol. Chem.* **225**, 137 (1957).

[259] L. L. Bennett, Jr., H. E. Skipper, and L. W. Law, *Federation Proc.* **12**, 300 (1953).

[260] H. G. Mandel and R. Markham, *Biochem. J.* **69**, 297 (1958).

[261] H. G. Mandel, G. I. Sugarman, and R. A. Apter, *J. Biol. Chem.* **225**, 151 (1957).

zymic digestion, indicate marked incorporation of the analog into the terminal positions of shorter polynucleotide chains, a circumstance which suggests that the presence of azaguanine in some cases may terminate further additions to the polymer.[255, 260] The form of the analog in the DNA of *B. cereus* has been established by hydrolysis to the corresponding deoxyribonucleosides and isolation of deoxyazaguanosine.[240, 255] In neither the RNA nor the DNA has an azaadenine derivative been detected, even when the growth of a bacterial culture was inhibited by azaadenine; however, azaadenine was converted to derivatives of azaguanine, with subsequent incorporation of these into nucleic acid.[255] Azaguanosine and the corresponding deoxyribonucleoside have been prepared enzymically by condensation of azaguanine with ribose-1-phosphate or deoxyribose-1-phosphate, a reaction catalyzed by nucleoside phosphorylases from horse liver.[262] Azaguanosine-5′-phosphate (as well as azaguanosine[240]) has been isolated from *B. cereus* exposed to azaguanine[255, 260]; and the enzymic synthesis of the ribonucleotide has been reported by direct reaction of azaguanine with pyrophosphorylribose-5-phosphate.[188] Correlations have been noted in both mouse tumors and *S. faecalis* between resistance to azaguanine and the capacity to form azaguanine nucleotides *in vivo*[179a, 181, 183, 259]; a reflection of this is seen in the levels of the nucleotide pyrophosphorylase activity *in vitro*.[173] Differences in cellular permeability in the various strains of mouse leukemias has been eliminated as a possible mechanism of resistance.[263] These results support the concept, derived from earlier suggestions, that the analog must be converted to a ribonucleotide before it becomes an active inhibitor.[232, 264, 166] However, preliminary trials with azadeoxyguanosine as an inhibitor of the growth of *Tetrahymena*[262] or with the mixed 2′-3′-monophosphates of azaguanosine as an inhibitor of tumor growth[261] did not indicate enhanced activity as compared to that of azaguanosine. The partial identification of the di- and triphosphates of azaguanine, which must certainly be formed prior to incorporation into nucleic acids, has been reported for extracts of *S. faecalis*.[179] Also, these ribonucleotides have been prepared by chemical and enzymic methods; in the latter a preparation from hog kidney was employed.[189]

A considerable limitation of the action of azaguanine in mammalian systems results from its rapid deamination by guanase *in vivo*[265] or *in vitro*[266-268] to azaxanthine, biologically a relatively inert compound.[236, 269]

[262] M. Friedkin, *J. Biol. Chem.* **209,** 295 (1954).
[263] J. D. Davidson, *Proc. Am. Assoc. Cancer Research* **2,** 290 (1958).
[264] G. B. Elion, S. Singer, and G. H. Hitchings, *Federation Proc.* **15,** (1956).
[265] H. G. Mandel, E. L. Alpen, W. D. Winters, and P. K. Smith, *J. Biol. Chem.* **193,** 63 (1951).
[266] A. Roush and E. R. Norris, *Arch. Biochem.* **29,** 124 (1950).
[267] J. Kream and E. Chargaff, *J. Am. Chem. Soc.* **74,** 4274 (1952).

Indeed, it was felt for some time that the selective action of azaguanine on certain experimental tumors and its lack of toxic effect on naturally resistant tumors, and on host organs such as liver and intestine, could be attributed to the low levels of guanase in these latter tissues.[269, 270] However, all of the data are not in agreement with this general statement, and other factors must be concerned with the susceptibility of various tissues to this analog.[269, 271] That the action of azaguanine is limited by guanase, however, became apparent when a marked increase in azaguanine toxicity[268, 272] and carcinostatic activity[273] was noted following the administration of aminoimidazolecarboxamide, a compound which acts not only as a purine precursor but also as an excellent inhibitor of guanase. As predicted,[268] this resulted in greater incorporation of guanine into nucleic acids when aminoimidazolecarboxamide was administered with guanine.[274, 274a]

Although it is not a substrate for xanthine oxidase of milk,[275] or for adenosine deaminase from rabbit intestine and adenocarcinoma 755,[276] azaguanine is a potent inhibitor of these enzymes. With the deaminase it is thought to combine in noncompetitive manner with the enzyme-substrate complex. Such inhibition is well established by even subtherapeutic levels of azaguanine administered to inact animals,[277] but the suggested connection between these findings and growth inhibition requires further substantiation.

As indicated above, the sites of action of azaguanine in nucleic acid metabolism, or indeed in the complex phenomena of growth, are by no means established. Despite its metabolic conversion to nucleotides, its incorporation into nucleic acids, and changes in the pattern of utilization of purines by cell lines resistant to this analog, the effects of azaguanine on the incorporation of precursors into the purines of nucleic acids have not yet afforded a satisfactory formulation of its mechanism of action. The activities observed with azaguanine were the inhibition of the incorporation

[268] P. E. Carlo and H. G. Mandel, *Cancer Research* **14,** 459 (1954).
[269] E. Hirschberg, J. Kream, and A. Gellhorn, *Cancer Research* **12,** 524 (1952).
[270] E. Hirschberg, M. R. Murray, E. R. Peterson, J. Kream, R. Schafranek, and J. L. Pool, *Cancer Research* **13,** 153 (1953).
[271] B. Shacter and L. W. Law, *J. Natl. Cancer Inst.* **18,** 77 (1957).
[272] P. E. Carlo and H. G. Mandel, *J. Biol. Chem.* **201,** 343 (1953).
[273] H. G. Mandel and L. W. Law, *Cancer Research* **14,** 808 (1954).
[274] L. L. Bennett, Jr. and H. E. Skipper, *Cancer Research* **17,** 370 (1957).
[274a] H. G. Mandel, J. L. Way, and P. K. Smith, *Biochim. et Biophys. Acta* **23,** 402 (1957).
[275] P. Feigelson and J. D. Davidson, *Cancer Research* **16,** 352 (1956).
[276] P. Feigelson and J. D. Davidson, *J. Biol. Chem.* **223,** 65 (1956).
[277] J. E. Ultman and P. Feigelson, *Cancer Research* **18,** 1319 (1958).

of formate[4, 278] and glycine[3, 279] into guanine (but not adenine) of the nucleic acids of tumors or internal organs of mice. Similarly, incorporation of phosphate-P^{32} into the DNA of adenocarcinoma 755 of mice treated with azaguanine was slightly reduced,[2] but uptake into Flexner-Jobling tumors in rats treated with this compound on a different dosage schedule, was stimulated.[4] The incorporation of adenine[233, 279, 280] or guanine[272] into nucleic acids, and their interconversion[233] in several systems, were essentially unaffected by azaguanine, a surprising result when compared to the growth experiments in which guanine proved to be a good reversing agent. Perhaps one of the most striking actions of this analog is the reproducible increase in the content of ribonucleic acid per cell which has been noted with *B. cereus*,[260] since it appears that this "extra" RNA is abnormal material in which a high percentage of the guanine is replaced by azaguanine. Confirmatory increases in the uptake of guanine and hypoxanthine,[179] glycine,[3] uracil,[281] and radioactive phosphate[4] have been reported in this and other systems. These results, coupled with increases in the nucleotide pools of tumor and liver,[282] and of *S. faecalis*,[179] have suggested that the primary effect of azaguanine is probably not on the early stages of purine nucleotide synthesis but on the organization and function of nucleic acids and purine-containing coenzymes.

One likely result of the suggested effect of azaguanine would be disruption of protein synthesis. Supporting this idea was the greater inhibition of the uptake of methionine or cystine[283] in *B. cereus* than of growth. Incorporation of other amino acids, such as lysine, valine, and histidine, also was strongly inhibited, while the uptake of aspartic acid and leucine was essentially unchanged, and that of serine and alanine was greatly stimulated.[284] Such results suggest a considerable disorganization of normal protein synthesis and perhaps amino acid exchange following exposure to azaguanine. Similar studies by another group would appear to indicate that intracellular protein synthesis is interrupted by the analog, but that the formation of cell wall material continues essentially unaltered, as does the production of DNA.[281, 285] As noted above, the production of RNA increases, but this probably represents an abnormal form of RNA in that it is rapidly degraded, with rejection of the analog from the RNA, when the cells are removed to

[278] H. E. Skipper, J. H. Mitchell, Jr., L. L. Bennett, Jr., M. A. Newton, L. Simpson, and M. Eidson, *Cancer Research* **11**, 145 (1951).
[279] R. Abrams, *Arch. Biochem. Biophys.* **33**, 436 (1951).
[280] J. L. Way, H. G. Mandel, and P. K. Smith, *Cancer Research* **14**, 812 (1954).
[281] H. Chantrenne and S. Devreux, *Nature* **181**, 1737 (1958).
[282] P. A. Zahl and H. G. Albaum, *Proc. Soc. Exptl. Biol. Med.* **88**, 263 (1955).
[283] H. G. Mandel, *Arch. Biochem. Biophys.* **76**, 230 (1958).
[284] D. B. Roodyn and H. G. Mandel, *Federation Proc.* **18**, 439 (1959).
[285] H. Chantrenne, *Biochem. Pharmacol.* **1**, 233 (1959).

fresh medium.[258, 260] Such a circumstance is similar to that seen with chloramphenicol, in which abnormal RNA is formed and protein synthesis is interrupted. Azaguanine also blocks the adaptive formation of amylase in *B. subtilis*,[286] as well as that of β-galactosidase and catalase in a strain of *S. aureus*.[287, 288] The production of antibodies by mice[289] *in vivo* and by rabbit spleen *in vitro*[290] also has been shown to be blocked by azaguanine; however, these observations must be examined at the enzyme level before the analog or its derivatives can be assigned a direct causal relationship to the phenomena.

4. Diaminopurine

Consideration of 2,6-diaminopurine (XII) as an agent which can interfere with nucleic acid metabolism is difficult, since the agent also serves as a precursor of purines, particularly guanine, in most mammalian[291-297] and some bacterial systems.[298-301] Nevertheless, this amino derivative of guanine is a good inhibitor of the growth of certain bacteria,[302-304] viruses,[305, 306] and animal tissues,[307-312] possibly through an interference with some aspect of

[286] J. Fukumoto, T. Yamamoto, and D. Tsuru, *Nature* **180**, 438 (1957).
[287] E. H. Creaser, *Nature* **176**, 556 (1955).
[288] E. H. Creaser, *Biochem. J.* **64**, 539 (1956).
[289] R. A. Malmgren, B. E. Bennison, and T. W. McKinely, *J. Natl. Cancer Inst.* **12**, 807 (1952).
[290] R. W. Dutton, A. H. Dutton, and M. George, *Nature* **182**, 1377 (1958).
[291] A. Bendich and G. B. Brown, *J. Biol. Chem.* **176**, 1471 (1948).
[292] A. Bendich, S. S. Furst, and G. B. Brown, *J. Biol. Chem.* **185**, 423 (1950).
[293] L. D. Hamilton, *Nature* **172**, 457 (1953).
[294] E. Goldwasser, *J. Biol. Chem.* **202**, 751 (1953).
[295] L. L. Bennett, Jr., and H. E. Skipper, *Arch. Biochem. Biophys.* **54**, 566 (1955).
[296] L. L. Bennett, Jr., H. E. Skipper, H. W. Toolan, and C. P. Rhoads, *Cancer Research* **16**, 262 (1956).
[297] M. E. Balis, D. V. Praag, and F. Aezen, *Cancer Research* **16**, 628 (1956).
[298] M. E. Balis, D. H. Levin, G. B. Brown, G. B. Elion, H. Vander Werff, and G. H. Hitchings, *J. Biol. Chem.* **196**, 729 (1952).
[299] S. E. Kerr and F. Chernigoy, *J. Biol. Chem.* **200**, 887 (1953).
[300] L. Hamilton, *Ann. N. Y. Acad. Sci.* **56**, 961 (1953).
[301] M. E. Balis, M. S. Brooke, G. B. Brown, and B. Magasanik, *J. Biol. Chem.* **219**, 917 (1956).
[302] G. H. Hitchings, G. B. Elion, H. Vander Werff, and E. A. Falco, *J. Biol. Chem.* **174**, 765 (1948).
[303] G. B. Elion and G. H. Hitchings, *J. Biol. Chem.* **187**, 511 (1950).
[304] G. H. Hitchings, G. B. Elion, E. A. Falco, P. B. Russell, M. B. Sherwood, and H. Vander Werff, *J. Biol. Chem.* **183**, 1 (1950).
[305] R. L. Thompsen, M. L. Price, S. A. Minton, Jr., G. B. Elion, and G. H. Hitchings, *J. Immunol.* **65**, 529 (1950).
[306] C. Friend, *Proc. Soc. Exptl. Biol. Med.* **78**, 150 (1951).
[307] J. H. Burchenal, A. Bendich, G. B. Brown, G. B. Elion, G. H. Hitchings, C. P. Rhoads, and C. C. Stock, *Cancer* **2**, 119 (1949).

the metabolism of nucleic acids or nucleotide-containing coenzymes.[298] Attempts to reverse the inhibitory effects of this compound on the growth of *L. casei* have indicated that it is a weak antagonist of folic acid, probably by virtue of its diaminopyrimidine nucleus.[302] More specific and complete prevention of the effects of diaminopurine have been noted with purines, particularly adenine, e.g., with *L. casei*,[302] *E. coli*,[313] *Aerobacter aerogenes*,[301] vaccinia virus growing in chick embryo tissue,[305] and sarcoma-180 cells in culture.[312]

Metabolic studies with N^{15}- or C^{14}-labeled diaminopurine indicated clearly that conversion of this purine derivative into nucleic acid guanine was a prominent aspect of its metabolism.[291, 292] Minor incorporation in the form of nucleic acid adenine was also noted,[295, 296] but no unchanged diaminopurine was detected. A partially purified nucleoside phosphorylase from beef liver formed guanosine from diaminopurine and ribose-1-phosphate[313]; presumably deamination occurred after condensation with ribose, since diaminopurine ribonucleoside,[314] but not the free purine, is a substrate for the deaminase contaminating this preparation. The 8-hydroxy derivative of diaminopurine is formed during its incubation with xanthine oxidase from milk.[315] Reaction of diaminopurine ribonucleoside with a purified phosphokinase from yeast (which is apparently specific for adenosine and diaminopurine ribonucleoside) gave the monophosphate; in turn, this reacted with ATP to form the corresponding di- and triphosphates.[316] These ribonucleotides also have been detected in the tissues of mice after the administration of diaminopurine.[317] Following incubation of diaminopurine with resting cell suspensions of *E. coli*, diaminopurine ribonucleotides and 2-methylaminoadenine ribonucleotides, as well as 2-methylamino-6-hydroxypurine and xanthine, have been isolated from the acid-soluble components.[318] The latter compounds, but not the ribonucleotides, were formed from diaminopurine by suspensions of a resistant strain of this organism.

[308] R. Hertz and W. W. Tullner, *Science* **109,** 539 (1949).

[309] G. E. Cartwright, J. G. Palmer, G. H. Hitchings, G. B. Elion, F. D. Gunn, and M. M. Wintrobe, *J. Lab. Clin. Med.* **35,** 518 (1950).

[310] J. J. Biesele, R. E. Berger, A. Y. Wilson, G. H. Hitchings, and G. B. Elion, *Cancer* **4,** 186 (1951).

[311] J. H. Burchenal, D. A. Karnofsky, E. M. Kingsley-Pillers, C. M. Southam, W. P. L. Myers, G. C. Escher, L. F. Craver, H. W. Dargeon, and C. P. Rhoads, *Cancer* **4,** 549 (1951).

[312] J. J. Biesele, R. E. Berger, and M. Clarke, *Cancer Research* **12,** 465 (1952).

[313] E. D. Korn and J. M. Buchanan, *J. Biol. Chem.* **217,** 183 (1955).

[314] J. Davoll and B. A. Lowy, *J. Am. Chem. Soc.* **73,** 1650 (1951).

[315] J. B. Wyngaarden, *J. Biol. Chem.* **224,** 453 (1957).

[316] A. Kornberg and W. E. Pricer, Jr., *J. Biol. Chem.* **193,** 481 (1951).

[317] G. P. Wheeler and H. E. Skipper, *J. Biol. Chem.* **205,** 749 (1953).

[318] C. N. Remy and M. S. Smith, *J. Biol. Chem.* **228,** 325 (1957).

The situation is unclear concerning the mechanism of action of diaminopurine as an inhibitor of growth. Although the reversal studies and the specificity of formation of the ribonucleotide suggest that this compound is an adenine antagonist, studies with a mutant of *A. aerogenes*,[301] in which diaminopurine can substitute for guanine and appear as guanine in the nucleic acids, as well as its deamination to guanosine derivatives,[313] would assign it a role in guanine metabolism. However, resistance to diaminopurine generally is associated with resistance to other antimetabolites of adenine, such as 8-azaadenine,[319, 320] and, in one case at least, such resistance results in a reduced capacity to form ribonucleotides of this analog.[318] Hence, several reports[298, 318, 319] have suggested that it may exert its antimetabolite function either by competing with adenine or by forming a 2-aminoadenine ribonucleotide derivative which blocks the formation or function of one of the critical adenine-containing coenzymes. Although diaminopurine inhibits the incorporation of formate,[4, 278, 299] glycine,[3] and aminoimidazolecarboxamide,[321] the utilization of this analog in the synthesis of purine nucleotides makes it difficult to distinguish between true inhibition and dilution of the purine pools by diaminopurine. Furthermore, in at least one instance in which formate and phosphate uptake was measured, even phospholipid metabolism was strongly inhibited,[322, 323] a circumstance which suggests a general depression of a number if not most parameters of cellular function. The prevention by diaminopurine or its riboside of inhibition of *E. coli* by urethane or *N*-methylformamide, and the nullification of these effects by the simultaneous addition of adenine, confuse the issue even more.[324, 325] In summary, then, although its metabolism has been considerably explored, the mechanism of action of diaminopurine is still undefined.

5. Miscellaneous Purines

A great variety of naturally occurring or chemically prepared compounds which resemble the purines or their derivatives possess biological activity. Since their precise relationship to nucleic acid metabolism is impossible to define at this point, space limitations permit only brief mention of their properties. Isomers of the natural purines in which the nitrogen and carbon in position 7 and 8 have been interchanged to

[319] G. B. Elion, H. Vander Werff, G. H. Hitchings, M. E. Balis, D. H. Levin, and G. B. Brown, *J. Biol. Chem.* **200**, 7 (1953).

[320] G. B. Elion, S. Singer, G. H. Hitchings, M. E. Balis, and G. B. Brown, *J. Biol. Chem.* **202**, 647 (1953).

[321] Z. Miller and L. Warren, *J. Biol. Chem.* **205**, 331 (1953).

[322] W. C. Werkheiser, R. J. Winzler, and D. W. Visser, *Cancer Research* **15**, 641 (1955).

[323] W. C. Werkheiser and D. W. Visser, *Cancer Research* **15**, 644 (1955).

[324] H. E. Skipper and F. M. Schabel, Jr., *Arch. Biochem. Biophys.* **40**, 476 (1952).

[325] H. E. Skipper, F. M. Schabel, Jr., V. Binns, J. R. Thomson, and G. P. Wheeler, *Cancer Research* **15**, 143 (1955).

give pyrazalo[3,4-*d*]pyrimidines have been extensively investigated.[326] Of a large series, only the adenine analogs, the 4-amino or 4-alkylamino derivatives, inhibit the growth of certain microorganisms or experimental tumors. Although the inhibitory activity of these compounds is reversed by adenine, so also is it by certain other pyrazalopyrimidines, a finding which suggests that the action of this type of compound may not be specifically related to nucleotide metabolism. Further evidence concerning this point is to be found in the inhibitory activity of the 1-alkyl-4-amino derivatives, since the normal point of attachment of ribose in these compounds is blocked. However, these substances are sufficiently like the natural purines to be substrates for xanthine oxidase and to inhibit the action of this enzyme on normal purines, an effect which may be related to their biological properties. In this reaction, pyrazaloadenine is converted into pyrazoloisoguanine, an even more effective inhibitor of xanthine oxidase.[327, 328] Furthermore, in the presence of a pyrophosphorylase from liver or yeast the ribonucleotides of both the adenine and guanine analogs were formed.[329]

Among the antibiotics which contain a purine nucleoside structure, puromycin is particularly interesting. It is an active trypanocidal agent[330, 331] and inhibitor of the growth of other protozoa and multicellular organisms[332]; in addition, the growth of certain experimental neoplasms is suppressed by puromycin.[333] Each of these activities is exhibited more markedly by the aminonucleoside fragment of puromycin, derived from 6-dimethylaminopurine, than by the parent compound.[334] The elegant chemical studies on the structure and synthesis of this compound and related purine-3′-amino-3′-deoxyribofuranosides,[335] as well as their biological properties,[332] have been reviewed. Unfortunately, definitive statements concerning their effects on the metabolism of nucleic acids cannot be made, except to note that certain unnatural as well as natural purines will antagonize some but not all of the activities of this type of compound.[336, 337] Since 3′-amino-3′-deoxyadenosine also exhibited considerable inhibitory activity, the corresponding pyrimidine derivatives were prepared, but these were inactive as inhibitors of the growth of trypanosomes or experimental tumors.[338]

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[326] H. E. Skipper, R. K. Robins, and J. R. Thomson, *Proc. Soc. Exptl. Biol. Med.* **89,** 594 (1955); *Cancer Research* **17,** 579 (1957).

[327] P. Feigelson, J. D. Davidson, and R. K. Robins, *J. Biol. Chem.* **226,** 993 (1957).

[328] P. Feigelson and J. D. Davidson, *Cancer Research* **18,** 226 (1958).

[329] J. L. Way and R. E. Parks, Jr., *J. Biol. Chem.* **231,** 467 (1958).

[330] R. I. Hewitt, W. S. Wallace, A. R. Gumble, E. R. Gill, and J. H. Williams, *Am. J. Trop. Med. Hyg.* **2,** 254 (1953).

[331] M. Agosin and T. von Brand, *Antibiotics & Chemotherapy* **4,** 624 (1954).

[332] B. L. Hutchings, *in* "The Chemistry and Biology of Purines" (G. E. W. Wolstenholme and C. M. O'Connor, eds.), p. 177. Little, Brown, Boston, Mass., 1957.

[333] W. Troy, S. Smith, G. Personeus, L. Moser, E. James, S. J. Sparks, M. Stevens, S. Halliday, D. McKenzie, and J. J. Oleson, *in* "Antibiotics Annual" (H. Welch and F. Marti-Ibanez, eds.), p. 186. Medical Encyclopedia, New York, 1954.

[334] E. J. Tobie and B. Highman, *Am. J. Trop. Med. Hyg.* **5,** 504 (1956).

[335] B. R. Baker, *in* "The Chemistry and Biology of Purines" (G. E. Wolstenholme and C. M. O'Connor, eds.), p. 120. Little, Brown, Boston, Mass., 1957.

[336] R. I. Hewitt, A. R. Gumble, W. S. Wallace, and J. H. Williams, *Antibiotics & Chemotherapy* **4,** 1222 (1954).

[337] L. Bortle and J. J. Oleson, *in* "Antibiotics Annual" (H. Welch, ed.), p. 770. Medical Encyclopedia, New York, 1955.

[338] H. M. Kissman and M. J. Weiss, *J. Am. Chem. Soc.* **80,** 2575 (1958).

Unsubstituted purine ribonucleoside (nebularine) has been isolated from a species of mushroom[339] and the structure confirmed by synthesis.[340] This ribonucleoside is much more toxic (about 25-fold) than is free purine.[340] The formation of the mono-, di-, and triphosphates of this ribonucleoside, *in vivo* and *in vitro*,[341, 342] the partial reversal of its cytotoxic action by adenine,[343] and the fact that a portion of the purine ribonucleoside administered to rats is found in the adenine and guanine of the nucleic acids,[341] would suggest an action related to ribonucleotide metabolism. The inability of mammalian nucleoside phosphorylases to cleave purine ribonucleoside[342] and the equally ineffective reverse reaction (i.e., synthesis of the ribonucleoside from free purine),[344] may account for the striking difference in the toxicities of these two compounds. Mention also should be made of the inability of the polynucleotide phosphorylase of *Azotobacter vinelandii* to bring about phosphorolysis of purine ribonucleoside diphosphate; thus, if the mechanisms of synthesis of RNA in the rat involve a similar enzyme, the failure of purine ribonucleoside to appear in the nucleic acids of the rat may be explained.[342]

Three other purine-containing antibiotics have been isolated: (*a*) nucleocidin, a glycoside of adenine in which there is a sulfamic ester of the carbohydrate residue[345]; (*b*) cordycepin, a 9-glycosyl derivative of adenine in which the sugar is a branched-chain deoxy-hexose[346]; and (*c*) angustmycin-C, a 9-D-psicofuranosyl glycoside of adenine.[347, 348] Several other synthetic purines or purine derivatives, such as 6-chloropurine,[349, 350] 6-methylpurine, and their ribonucleosides,[351] and 2-fluoroadenosine,[352] also are extremely toxic to cells in culture, as well as to whole animals,[353] but little evidence concerning the mechanism of action of these compounds is available.

The methylated purines, caffeine, theobromine, and theophylline, which are mildly mutagenic,[243] have been reported to inhibit both ribo- and deoxyribonucleoside phosphorylases at high concentrations, although they themselves are apparently not substrates[354]; accordingly, their incorporation into the nucleic acids of *E. coli* was negligible.[355]

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[339] N. Löfgren, B. Lüning, and H. Hedström, *Acta Chem. Scand.* **8**, 670 (1954).
[340] G. B. Brown and V. S. Weliky, *J. Biol. Chem.* **204**, 1019 (1953).
[341] M. P. Gordon and G. B. Brown, *J. Biol. Chem.* **220**, 927 (1956).
[342] G. R. Barker, J. B. Lloyd, M. D. Montague, and N. F. Wood, *Biochem. J.* **72**, 9P (1959).
[343] D. A. Clarke, F. S. Philips, S. S. Sternberg, and C. C. Stock, *Proc. Am. Assoc. Cancer Research* **2**, 10 (1955).
[344] M. P. Gordon, O. M. Intrieri, and G. B. Brown, *J. Biol. Chem.* **229**, 641 (1957).
[345] C. W. Waller, J. B. Patrick, W. Fulmor, and W. E. Meyer, *J. Am. Chem. Soc.* **79**, 1011 (1957).
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[347] H. Yunsten, *J. Antibiotics (Japan)* **A11**, 244 (1958), quoted from Schroeder and Hoeksema.[348]
[348] W. Schroeder and H. Hoeksema, *J. Am. Chem. Soc.* **81**, 1767 (1959).
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[354] A. L. Koch and W. A. Lamont, *J. Biol. Chem.* **219**, 189 (1956).
[355] A. L. Koch, *J. Biol. Chem.* **219**, 181 (1956).

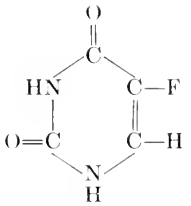
V. Structural Analogs of Pyrimidines and Their Effects on Nucleic Acid Metabolism

During the last decade, knowledge of the metabolism of pyrimidines has increased greatly and agents have been sought which might influence the synthesis or utilization of pyrimidine-containing compounds. The extensive background of information on the pyrimidine requirements of certain strains of microorganisms has provided a valuable tool for screening potentially active compounds.[356, 357] Further interest was generated by findings[358-361] which indicated that marked differences in the capacity to utilize preformed uracil are to be found in various mammalian tissues. More recently, the important phenomenon of the "thymineless death" of bacteria[362] also has stimulated interest in the potential usefulness of pyrimidine antimetabolites as therapeutic agents. In some tumors there is a greatly diminished activity of several enzymes involved in the catabolism of pyrimidines,[363, 364] which are present in many tissues, but predominantly in liver. Despite the newness of many of the antimetabolites to be discussed, much can be said about their specific mechanisms of action.

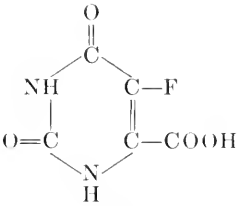
1. 5-Fluoropyrimidines

The similarities in the physical and chemical properties of the 5-halogenouracils and thymine have prompted extensive study of the actions of such compounds in the synthesis and utilization of thymine derivatives for DNA formation. The first member of the fluorine-substituted pyrimidines, 5-fluorouracil (XIII) was not synthesized until 1957.[365] This synthesis, unlike that of the previously known halogenated pyrimidines, was not accomplished by direct replacement of hydrogen in the pyrimidine ring. In the preparation, the diethyl ester of 2-fluoro-3-oxosuccinic acid was condensed with *S*-ethylisothiouronium bromide; hydrolysis of the product gave 5-fluoroorotic acid (XIV), a compound of much interest per se, which also could be decarboxylated to form 5-fluorouracil. This synthetic route was particularly appropriate for the preparation[366] from thiourea-

[356] G. H. Hitchings and G. B. Elion, *Cancer Research* Suppl. **3**, 66 (1955).
[357] J. L. Stokes, *J. Bacteriol.* **48**, 201 (1944).
[358] R. J. Rutman, A. Cantarow, and K. E. Paschkis, *Cancer Research* **14**, 119 (1954).
[359] C. Heidelberger, K. C. Liebman, and E. Harbers, *Cancer Research* **17**, 399 (1957).
[360] V. Lagerkvist and P. Reichard, *Acta Chem. Scand.* **8**, 361 (1954).
[361] P. Reichard and O. Sköld, *Biochim. et Biophys. Acta* **28**, 376 (1958).
[362] S. S. Cohen, *J. Bacteriol.* **71**, 588 (1956).
[363] E. S. Canellakis, *J. Biol. Chem.* **227**, 701 (1957).
[364] V. R. Potter, A. F. Brumm, and F. J. Bollum, *Proc. Am. Assoc. Cancer Research* **2**, 336 (1958).
[365] R. Duschinsky, E. Pleven, and C. Heidelberger, *J. Am. Chem. Soc.* **79**, 4559 (1957).
[366] N. K. Chaudhuri, B. J. Montag, and C. Heidelberger, *Cancer Research* **18**, 318 (1958).



(XIII)
5-Fluorouracil



(XIV)
5-Fluoroorotic acid

C^{14} of 5-fluoroorotic acid-2-C^{14} and 5-fluorouracil-2-C^{14}, compounds of great value in metabolic studies with these antimetabolites. Alternatively, 5-fluorouracil could be obtained by condensing the potassium enolate of the ethyl ester of 2-fluoro-3-oxopropionic acid with S-ethylisothiouronium bromide, followed by hydrolysis of the ethylthiol grouping.

Several important concepts were involved in the development of the 5-fluoropyrimidine derivatives.[367] First, the fluorine atom bears close similarity to the H-atom in its physical and certain chemical properties, since it has an atomic radius of 1.35 Å., as compared to that of 1.20 Å. for H, and its substitution for hydrogen in certain biologically important compounds has given rise to antagonistic activities. Second, a substitution in position 5 of the appropriate uracil derivative would block the addition of a "1-C" unit via a folic acid-derived coenzyme and perhaps would provide thereby an inhibitor of the synthesis of thymine. Finally, from the standpoint of potential usefulness as a therapeutic agent in the treatment of cancer, observations relative to the differences in the ability of certain normal and neoplastic tissues to utilize uracil, as discussed earlier, served as a major motivation for the investigation of this compound.

Preliminary screening of 5-fluoropyrimidines against a number of microorganisms revealed a wide spectrum of inhibitory activities for fluorouracil, while other fluoro derivatives were considerably less active.[367, 368] The inhibitory activity of fluorouracil could be reversed best by thymidine in a noncompetitive manner or less well by uracil or cytosine. Both fluorouracil and 5-fluoroorotic caused marked inhibition in the rate of growth of a variety of experimental tumors in mice; however, 5-fluorocytosine, 2-methylthio-5-fluorouracil and 2-thio-5-fluorouracil were without such activity.[367, 369] That the inhibition produced by fluorouracil is the result of a specific effect on nucleic acid metabolism was demonstrated by experiments in which a profound depression of the incorporation of formate-C^{14}

[367] C. Heidelberger, N. K. Chaudhuri, P. Danneberg, D. Mooren, L. Griesbach, R. Duschinsky, R. J. Schnitzer, E. Pleven, and J. Scheiner, *Nature* **179**, 663 (1957).

[368] J. Scheiner, E. Kostelak, and R. Duschinsky, *Federation Proc.* **16**, 242 (1957).

[369] C. Heidelberger, L. Griesbach, B. J. Montag, P. Mooren, O. Cruz, R. J. Schnitzer, and E. Grunberg, *Cancer Research* **18**, 305 (1958).

into DNA thymine of Ehrlich ascites tumor cells *in vivo* resulted from the administration of fluorouracil at a dosage level which did not interfere with incorporation of formate into nucleic acid purines or proteins.[367, 370] Similarly, the incorporation of orotic acid and uracil into DNA thymine of Ehrlich ascites cells was depressed to a degree resembling that of formate, while the incorporation of thymidine per se was markedly increased, a finding which suggests localization of the primary metabolic inhibition at the conversion of uracil derivatives to thymine deoxyribonucleotides. However, this same level of fluorouracil also caused up to 68% inhibition of the conversion of uracil-2-C^{14} to the uracil or cytosine of the RNA of ascites cells, and the incorporation of orotic acid into the pyrimidine bases of RNA was depressed up to 68% under these conditions.

The preferential inhibition of the incorporation of formate-C^{14} into the thymine of DNA of Ehrlich ascites tumor cells, as compared to that of spleen, liver, or intestine, suggested that the greater capacity of Ehrlich ascites cells for the anabolic utilization of uracil has an enhancing effect on the ability of fluorouracil to cause inhibition. Although the inhibitor was injected intraperitoneally, and came into direct contact with the tumor cells, it will become evident that this 5-fluoropyrimidine exerts a selective effect on tumors, while the 5-fluoro derivative of orotic acid does not exhibit such selectivity. Substantiation of these statements is to be found in the specific concentration of fluorouracil derivatives in a number of experimental tumors of mice and in human cancer tissues following the administration of fluorouracil, whereas such concentration was not apparent following the administration of fluoroorotic acid to tumor-bearing animals.[366] This blockade of pyrimidine metabolism by fluorouracil results in a depression of DNA synthesis, as evidenced by a sharp reduction of phosphate-P^{32} uptake into DNA.[370] An interesting extension of this finding was made through the use of cytochemical techniques. It was noted that the administration of fluorouracil for 7 days to mice bearing Ehrlich ascites cells, in doses sufficient to cause 85% inhibition of tumor growth, caused a comparable reduction in mitotic index and marked alterations in cellular components.[371, 372] Cells from treated animals were considerably larger and contained increased amounts of protein and RNA. The DNA content per cell, however, was reduced to approximately 50% of that found in control cells with little or no change in the average ploidy of the cell. Examination of interphase cells with Feulgen stain disclosed irregular, coarse clumps, and strands of DNA around vacuoles, a phenomenon regarded as charac-

[370] P. Danneberg, B. J. Montag, and C. Heidelberger, *Cancer Research* **18,** 329 (1958).

[371] A. Lindner, *Proc. Am. Assoc. Cancer Research* **2,** 322 (1958).

[372] A. Lindner, *Cancer Research* **19,** 189 (1959).

teristic of early death. Although similar studies with a fluorouracil-resistant subline of this tumor revealed little change in the DNA content of cells exposed to the drug, an unexplained doubling of the amount of RNA and protein in the resistant cells was recorded. A similar depression of the synthesis of DNA with continued production of protein was noted in cultures of *E. coli* exposed to fluorouracil.[373]

The specific sites of action of fluorouracil, though suggested by the above-described studies carried out *in vivo*, are best examined by consideration of the metabolism of this analog and the effects of these metabolites on enzymic reactions *in vitro*. In general, it may be stated that fluorouracil undergoes all the anabolic reactions of uracil, with the obvious exception of methylation at position 5 of the derivative corresponding to 2′-deoxyuridylic acid. Following intraperitoneal injection of fluorouracil-2-C^{14} into mice bearing Ehrlich ascites cells and other tumors, the corresponding ribonucleoside, and its mono-, di-, and triphosphates have been isolated by ion-exchange chromatography.[366] Small amounts of 5-fluorocytidine and its 5′-phosphate have been isolated from the acid-soluble fraction of Ehrlich ascites cells incubated with 5′-fluorouridine[374] (see below); the presence of higher phosphates of these cytidine analogs has yet to be demonstrated.

Minor amounts of diphosphate derivatives of fluorouridine, probably analogous to the uridine-containing coenzymes involved in carbohydrate metabolism, were also detected. Following ion-exchange separation of the ribonucleotides in the acid-soluble extract of Ehrlich ascites cells incubated *in vitro* with fluorouracil, 5-fluoro-2′-deoxyuridylic acid was found to comprise 11% of the fluorouridylic peak.[374] The deoxy derivative was identified by enzymic dephosphorylation and rechromatography with a borate buffer to separate the resultant 5-fluorodeoxyuridine from the corresponding ribonucleoside. The presence of this deoxyribonucleotide in the acid-soluble portion of the cell is of considerable importance in explaining the biochemical activities of fluorouracil. Di- or triphosphates of fluorodeoxyuridylic acid were not detected,[374, 375] an observation compatible with the finding that deoxyuridylic acid, unlike thymidylic acid, is not converted to higher phosphates.[376] Incorporation of fluorouracil into the RNA of many mouse and human tissues also occurs and the mixed 2′- and 3′-monophosphates of fluorouridine have been isolated following hydrolysis of the RNA with alkali.[366] The extent of replacement of normal pyrimidines under the con-

[373] J. Horowitz, J. J. Saukkonen, and E. Chargaff, *Biochim. et Biophys. Acta* **29**, 223 (1958).

[374] E. Harbers, N. K. Chaudhuri, and C. Heidelberger, *J. Biol. Chem.* **234**, 1255 (1959).

[375] L. Bosch, E. Harbers, and C. Heidelberger, *Cancer Research* **18**, 335 (1958).

[376] M. J. Bessman, I. R. Lehman, J. Adler, S. B. Zimmerman, E. S. Simms, and A. Kornberg, *Proc. Natl. Acad. Sci. U. S.* **44**, 633 (1958).

ditions employed ranged from 0.5% to 6% of the normal uracil content of the RNA of various tissues; as yet, however, it cannot be said with certainty that fluorouracil specifically replaces any particular base in the RNA. In tobacco mosaic virus a much greater incorporation of fluorouracil into the RNA occurs, despite a reduced rate of synthesis. In this case it can be said with some certainty that up to 30% of the uracil is replaced by fluorouracil with retention of infectivity, despite reduced production of the virus[377]; in another laboratory, a marked decrease in the rate of proliferation of this virus also was noted.[378] No significant incorporation of fluorouracil in the form of fluorocytosine has been detected in either the RNA or the DNA of mammalian or bacterial cells.

The evidence for extensive anabolism of fluorouracil to ribonucleotide and deoxyribonucleotide derivatives, as well as other evidence cited below, prompted a search for methods of preparing the corresponding ribonucleoside and deoxyribonucleoside. Thus, a chemical synthesis of 5-fluorouracil-β-D-ribofuranoside was devised[379] in which techniques similar to those used for the preparation of thymine riboside[380] were employed. Formation of the deoxyribonucleoside of fluorouracil[379] was first accomplished by transdeoxyribosidation from thymidine with resting cell suspensions of *Streptococcus faecalis*, as has been described[381] for the formation of azathymidine (i.e., the deoxyribonucleoside of 3,5-dioxo-6-methyl-1,2,4-triazine). The transfer of deoxyribose from thymidine to fluorouracil by extracts of *E. coli* resulted in a more efficient conversion and is currently employed for the production of the deoxyribonucleoside.[382]

With a few notable exceptions, the ribonucleoside and deoxyribonucleoside of fluorouracil exhibit *in vivo* many of the biological properties observed with the parent analog.[374, 375, 383] Localization of the deoxyribonucleoside in tissues showed an even greater affinity for tumor (sarcoma-180) than did fluorouracil, whereas the ribonucleoside was found in higher concentration in various tissues other than tumor.[374] Intensive metabolic degradation of the deoxyribonucleoside to fluorouracil occurred in ascites cell suspensions[374] and in bacterial systems,[382] but the ribonucleoside appeared to be much more stable.[374] Comparisons of host-toxicity in mice indicate that fluorouridine is considerably more toxic than fluorouracil, whereas the

[377] M. P. Gordon and M. Staehelin, *J. Am. Chem. Soc.* **80,** 2340 (1958).

[378] C. I. Davern and J. Bonner, *Biochim. et Biophys. Acta* **29,** 205 (1958).

[379] R. Duschinsky, E. Pleven, J. Malbica, and C. Heidelberger, *Abstr. 132nd Meeting Am. Chem. Soc., New York,* p. 19C (1957).

[380] J. J. Fox, N. Yung, J. Davoll, and G. B. Brown, *J. Am. Chem. Soc.* **78,** 2117 (1956).

[381] W. H. Prusoff, *J. Biol. Chem.* **215,** 809 (1955).

[382] S. S. Cohen, J. G. Flaks, H. D. Barner, M. R. Loeb, and J. Lichtenstein, *Proc. Natl. Acad. Sci. U. S.* **44,** 1004 (1958).

[383] C. Heidelberger, L. Griesbach, O. Cruz, R. J. Schnitzer, and E. Grunberg, *Proc. Soc. Exptl. Biol. Med.* **97,** 470 (1958).

deoxyribonucleoside is less toxic. The most marked inhibitory effects on tumors *in vivo* usually (but not always) were obtained with fluorodeoxyuridine.[383] Bacterial inhibition studies[382, 384] indicated a similar order of activity. It has been shown that fluorodeoxyuridine causes a "thymineless" death of *E. coli*; however, there is a limitation on the duration of action because of cleavage by deoxyribonucleosidases, with the release of fluorouracil. In general, the ribonucleoside, though an inhibitor of growth, is not an active bacteriocidal agent, presumably because both it and fluorouracil not only evoke a thymineless status in the cell, but also limit other aspects of pyrimidine metabolism, thereby preventing an expression of the type of lethal effect which results from a deprivation involving only the precursors of DNA thymine.[382]

With this background information on the biological and biochemical effects of fluorouracil and its derivatives *in vivo* and *in vitro*, certain sites have now been established with cell suspensions or soluble enzyme preparations. Confirmation of the blockade of formate incorporation into DNA thymine has been obtained with glycolyzing Ehrlich ascites tumor cells in which an inhibition of 50% was produced by 5×10^{-5} *M* fluorouracil and 7×10^{-9} *M* fluorodeoxyuridine. At these and somewhat higher concentrations little effect of the fluoropyrimidines on the conversion of uracil or orotic acid to acid-soluble ribonucleotides or the utilization of formate for the biosynthesis of purines was observed. However, inhibition of the incorporation of orotic acid and uracil into the thymine of DNA paralleled that seen with formate incorporation.[374, 375] These general results have been confirmed and extended by using cell cultures of a human epithelioma, "H.Ep. # 1,"[385] and slices of this and other human tumors grown in X-irradiated rats.[386] That this does not represent inhibition of the conversion of uridine-5′-phosphate to deoxyuridine-5′-phosphate is apparent from studies with ascites tumor cell suspensions, in which profound blockade of the incorporation of randomly labeled deoxyuridine into DNA thymine was effected by fluorodeoxyuridine.[374] A more precise localization of the site of action of fluorodeoxyuridine has been accomplished with partially purified cell-free extracts of *E. coli* B infected with T6r$^+$ phage.[382] Properly supplemented, these extracts converted deoxyuridylic acid into thymidylic acid, and deoxycytidylic acid into 5-hydroxymethyl deoxycytidylic acid, by tetrahydrofolic acid-dependent condensations with formaldehyde. Both naturally formed and synthetic[387] fluorodeoxyuridylic acid at 2×10^{-6} *M*,

[384] J. M. Scheiner and R. Duschinsky, *Federation Proc.* **17**, 305 (1958).

[385] M. A. Rich, J. L. Bolaffi, J. E. Knoll, L. Cheong, and M. L. Eidinoff, *Cancer Research* **18**, 730 (1958).

[386] M. L. Eidinoff, J. E. Knoll, and D. Klein, *Arch. Biochem. Biophys.* **71**, 274 (1957).

[387] W. G. Farkas, L. C. Iacono, and R. Duschinsky, *Abstr., 4th Intern. Congr. Biochem., Vienna* p. 6 (1958).

caused 50% inhibition of the activity of thymidylate synthetase, whereas fluorodeoxyuridine and fluorouridylic acid were inactive even at 500 times this concentration. The hydroxymethylation of deoxycytidylic acid was insensitive to all fluoro derivatives tested, including 5-fluorodeoxycytidine-5′-phosphate.[387a]

Another enzymic site at which fluorouracil derivatives act is uridine phosphorylase.[388] The partially purified enzyme from Ehrlich ascites tumor cells is inhibited by fluorouracil to the extent of 50%, when its activity on the phosphorolysis of uridine is tested at a molar ratio of 1:1. These enzyme preparations also catalyze the formation of fluorouridine from ribose-1-phosphate and fluorouracil and phosphorylate the ribonucleoside in the presence of ATP and Mg^{++} to form the 5′-phosphate. Furthermore, in a cell line of this tumor selected for resistance to fluorouracil,[389] the presence of uridine phosphorylase activity could not be detected. This apparent shut down of preformed pyrimidine utilization also involved deoxyuridine, since its phosphorylase was not found in the fluorouracil-resistant subline, although it was present in the sensitive line. The effects of fluorouridine on uridine kinase have not been reported, but fluorouracil is inactive as an inhibitor; however, fluorouracil-resistant cell lines of Ehrlich ascites tumor and leukemia L-1210 possess much lower levels of this enzyme. In a different study[389a] with Ehrlich ascites tumor cells which have been selected for resistance to fluorouracil, the analog failed to inhibit the incorporation of formate into DNA thymine, but still partially blocked the incorporation of uracil into RNA. These results have been interpreted to indicate unimpaired formation of fluorouracil ribonucleotides and a change in the affinity of thymidylate synthetase for the deoxyribonucleotide of fluorouracil.

Since fluorouracil or fluorouridine, but not fluorodeoxyuridine, inhibits the incorporation of orotic acid as well as uracil into RNA pyrimidines, an enzymic site of action at the ribonucleotide level beyond uridylic acid is suggested, but this has not yet been demonstrated.[390] However, it has been shown through the use of a mutant of *E. coli* that fluorouracil did not inhibit accumulation of total proteins, although it completely blocked the induction of β-galactosidase activity, presumably by blocking synthesis of new RNA.[391] In mammalian cells, however, this must not be a prominent

[387a] S. S. Cohen, personal communication (1959).

[388] O. Sköld, *Biochim. et Biophys. Acta* **29,** 651 (1958).

[389] P. Reichard, O. Sköld, and G. Klein, *Nature* **183,** 939 (1959).

[389a] C. Heidelberger, P. B. Dunneberg, G. Kaldor, and O. Ghobar, *Federation Proc.* **18,** 244 (1959).

[390] I. Melnick, A. Cantarow, K. E. Paschkis, *Arch. Biochem. Biophys.* **74,** 281 (1958).

[391] J. Horowitz, J. J. Saukkonen, and E. Chargaff, *Biochim. et Biophys. Acta* **29,** 222 (1958).

action of fluorouracil, since the incorporation of lysine into proteins, under the conditions studied, was not significantly depressed.[374]

In common with uracil, fluorouracil is extensively degraded by mammalian liver.[392] The first two degradative reactions apparently are catalyzed by the same enzymes which catabolize uracil; this results in the formation of 5,6-dihydrofluorouracil and α-fluoro-β-ureidopropionic acid; in man, further degradation of fluorouracil-2-C^{14} is indicated by the excretion of urea-C^{14}, whereas mice excrete much of the radioactivity from carbon 2 as respiratory carbon dioxide. A unique metabolite, believed to be 2-fluoro-3-guanidopropionic acid, has also been isolated from the ascitic fluid of mice bearing Ehrlich ascites carcinoma. More recently, α-fluoro-β-alanine has been identified as a major urinary excretion product of human subjects.[393] Although the first two degradation products, dihydrofluorouracil and α-fluoro-β-ureidopropionic, do not inhibit the growth of microorganisms inhibited by fluorouracil,[384] it seems probable that certain of the toxic effects of fluorouracil in mammals are related to the formation of a fluorinated aliphatic acid which is probably fluoroacetic acid.[394] It is significant that, as with uracil, sarcoma-180 and Ehrlich ascites tumors do not degrade fluorouracil to any significant extent; this finding may account in part for the higher levels of this analog found in tumors, as compared to normal tissues.[392] Furthermore, the toxicities of fluorouracil, fluorodeoxyuridine, and fluorodeoxycytidine (see below) are sharply increased when nontoxic amounts of thymidine are administered with these analogs, a result which suggests inhibition of the degradation of these fluorinated compounds.[395]

5-Fluoroorotic acid has properties somewhat similar to those of fluorouracil, particularly with respect to effects on the synthesis of DNA in microorganisms and mammalian systems.[367] However, as indicated previously, it does not localize preferentially in tumors, but appears in highest concentration in the kidneys,[366] a circumstance which may reflect active secretion by the renal tubular epithelium, as has been observed with orotic acid.[396] Fluoroorotic acid, unlike fluorouracil, inhibits the synthesis of orotidine-5′-phosphate from orotic acid and pyrophosphorylribose-5-phosphate by a particle-free supernatant of rat liver.[397] In this same system, extensive

[392] N. K. Chaudhuri, K. L., Mukherjee, and C. Heidelberger, *Biochem. Pharmacol.* **1,** 328 (1959).

[393] C. Heidelberger, personal communication (1959).

[394] F. S. Philips, R. Duschinsky, and S. S. Sternberg, *Proc. Am. Assoc. Cancer Research* **3,** 51 (1959).

[395] J. H. Burchenal, E. A. D. Holmberg, H. F. Oettgen, S. C. Hemphill, and J. A. Reppert, *Proc. Am. Assoc. Cancer Research* **3,** 10 (1959).

[396] L. Peters, personal communication (1959).

[397] J. E. Stone and V. R. Potter, *Cancer Research* **17,** 800 (1957).

metabolism of this analog to the corresponding uridine ribonucleotides could be demonstrated.[398] Like orotic acid, fluoroorotic acid is relatively immune to catabolic attack in mammalian systems; however, a small amount of what appears to be 2-fluoro-3-ureidosuccinic acid was excreted in the urine following injection of fluoroorotic acid-2-C^{14}.[392] Fluoroorotic is reduced at a faster rate than orotic acid by dihydroorotic acid dehydrogenase isolated from *Zymobacter oroticum*.[399]

Although 5-fluorocytosine has relatively little biological activity, fluorocytidine and fluorodeoxycytidine[400] have been shown to reduce the rate of growth of experimental tumors.[395, 401, 402] Studies *in vitro* with Ehrlich ascites tumor cells indicate that fluorocytidine does not appear to act primarily as an inhibitor of incorporation of pyrimidines into nucleic acid cytosine; rather, a predominant effect on the incorporation of uracil or cytidine into the thymine of DNA is indicated.[374] Such a result suggests that fluorocytosine functions primarily through deamination to the corresponding uracil derivative; this reaction could occur after conversion to a deoxyribose derivative, since a very active deoxycytidylic acid deaminase has been demonstrated in certain neoplastic and normal tissues.[403]

2. Other Halogenated Pyrimidines

The members of this group of pyrimidines, in which the hydrogen in position 5 is replaced by chlorine, bromine, or iodine, may be considered primarily as analogs of thymine. Their synthesis was accomplished by the direct halogenation of uracil or its corresponding ribonucleoside or deoxyribonucleoside; however, preparation of the corresponding derivatives of the cytosine nucleosides required photocatalysis of the reaction.

The inhibitory nature of chloro-, bromo-, and iodouracil was first demonstrated with *L. casei*, the growth of which was limited by the supply of thymine.[404] In this first report, the stimulatory nature of these compounds on growth under certain conditions was also noted, an observation which has since been of considerable theoretical interest; confirmation of this result has been obtained in other bacterial systems with bromouracil.[239,

[398] R. E. Parks, Jr., J. L. Way, J. L. Dahl, *Proc. Am. Assoc. Cancer Research* **2**, 333 (1958).

[399] H. C. Friedman and B. Vennesland, *J. Biol. Chem.* **233**, 1398 (1958).

[400] J. J. Fox, I. Wempen, and R. Duschinsky, *Abstr. 4th Intern. Congr. Biochem., Vienna* p. 6 (1958).

[401] J. H. Burchenal, E. A. D. Holmberg, and J. J. Fox, *Abstr. 4th Intern. Congr. Biochem., Vienna* p. 185 (1958).

[402] M. L. Eidinoff, M. A. Rich, and A. G. Perez, *Proc. Am. Assoc. Cancer Research* **3**, 18 (1959).

[403] F. Maley and G. F. Maley, *Federation Proc.* **18**, 280 (1959).

[404] G. H. Hitchings, E. A. Falco, and M. B. Sherwood, *Science* **102**, 251 (1945).

[304, 404-406] More recently it was reported that bromouracil deoxyribonucleoside can substitute for thymidine in the reproduction of HeLa cells in culture for short periods under conditions in which thymidine is normally required.[407] However, in general, the growth of cells is inhibited by these halogenated derivatives under conditions in which thymine is the limiting factor.[304, 408-413] It is of interest that chlorouracil was more active than the bromo and iodo derivatives as an inhibitor of the growth of *E. coli* B/r and that its inhibitory action was partially prevented by uracil as well as by thymine, observations which suggest two sites of action for this compound.[411] Similarly, chlorouridine[414] inhibits the proliferation of Theiler's GD VII virus in chick embryo tissues, an action prevented by uridine.[415]

The deoxyribonucleoside of bromouracil[409, 416, 417] has shown greater activity in most bacterial systems than has either the ribonucleoside or free bromouracil and in mammalian systems[418-420] it is the only derivative with striking biological activity, a result which is consistent with the poor utilization of thymine or thymine ribonucleoside in these systems. Similarly, iododeoxyuridine[421] exhibits a potency as a bacterial inhibitor similar to that of iodouracil; however, unlike the free base, this deoxyribonucleoside inhibits the growth of several transplantable neoplasms.[421–423] In *E. coli* the halogenated pyrimidine derivatives create a condition which resembles in many respects nutritional "thymineless" death.[424]

[405] G. H. Hitchings, G. B. Elion, and H. Vander Werff, *J. Biol. Chem.* **174,** 1037 (1948).
[406] W. H. Prusoff, *Proc. Soc. Exptl. Biol. Med.* **85,** 564 (1954).
[407] M. T. Hakala, *Federation Proc.* **17,** 236 (1958).
[408] G. H. Hitchings, G. B. Elion, and E. A. Falco, *J. Biol. Chem.* **185,** 643 (1950).
[409] T. J. Bardos, G. M. Levin, R. R. Herr, and H. L. Gordon, *J. Am. Chem. Soc.* **77,** 4279 (1955).
[410] F. Weygand and A. Wacker, *Z. Naturforsch.* **5b,** 46 (1950).
[411] D. B. Dunn and J. D. Smith, *Biochem. J.* **67,** 494 (1957).
[412] F. Weygand, A. Wacker, and H. Grisebach, *Z. Naturforsch.* **6b,** 177 (1951).
[413] F. Weygand, A. Wacker, and H. Dellweg, *Z. Naturforsch.* **7b,** 19 (1952).
[414] T. K. Fukuhara and D. W. Visser, *J. Biol. Chem.* **190,** 95 (1951).
[415] D. W. Visser, D. L. Lagerborg, and H. E. Pearson, *Proc. Soc. Exptl. Biol. Med.* **79,** 571 (1952).
[416] R. E. Beltz and D. W. Visser, *J. Am. Chem. Soc.* **77,** 736 (1955).
[417] S. Zamenhof and G. Griboff, *Nature* **174,** 307 (1954).
[418] M. L. Eidinoff, J. E. Knoll, and B. J. Marano, *Proc. Am. Assoc. Cancer Research* **2,** 198 (1957).
[419] S. Kit, C. Beck, O. L. Graham, and A. Gross, *Cancer Research* **18,** 598 (1958).
[420] M. L. Eidinoff, J. E. Knoll, B. J. Marano, and D. Klein, *Cancer Research* **19,** 738 (1959).
[421] W. H. Prusoff, *Biochim. et Biophys. Acta* **32,** 295 (1959).
[422] W. H. Prusoff, J. J. Jaffe, H. Gunther, and A. D. Welch, *Proc. Am. Assoc. Cancer Research* **3,** 54 (1959).
[423] A. Mathias and G. A. Fischer, *Federation Proc.* **18,** 284 (1959).
[424] S. S. Cohen and H. D. Barner, *J. Bacteriol.* **71,** 588 (1956).

The most prominent aspect of the metabolism of these halogenated pyrimidines is their remarkable capacity to replace thymine residues in DNA. This substitution was first noted with bromouracil in *S. faecalis*[413] and has been confirmed in *E. coli*,[425, 426] *E. coli* T2 phage, and numerous other microorganisms[427] in which, under certain conditions, over 50% of the thymine of the DNA can be replaced by this analog. Exposure of H.Ep. #1 cells in culture to bromodeoxyuridine resulted in up to 45% replacement of thymidine by this analog with concomitant inhibition of growth.[428] With $T2r^+$ phage in *E. coli* it was reported[429] that close to 100% of the thymine could be replaced by bromouracil and yet 9% of the infectivity remained. This result, coupled with the findings with HeLa cells and the stimulation of bacterial growth by these analogs, strongly suggests that bromouracil can replace thymine with retention of the functional activity of the DNA. However, the DNA obtained from *E. coli* grown in the presence of bromouracil showed a heterogeneous distribution of the analog in the different molecular species separated by chromatography on Ecteola columns.[430] In general, the fractions containing bromouracil appeared more acidic in nature, a finding consistent with the significantly lower pKa of bromouracil as compared to that of thymine. Similarly, the DNA from T4 phage of *E. coli* isolated after exposure to bromouracil exhibited marked heterogeneity when subjected to equilibrium centrifugation, in contrast to the normally homogeneous pattern displayed by DNA from untreated phage.[431] The differences in sedimentation rates were explicable on the basis of the difference between the molecular weight of the methyl group and that of the bromine atom (79.9); the heterogeneity was confirmed, as with the DNA from *E. coli*, by chromatographic techniques. Unlike the situation with RNA formation in cells exposed to azaguanine, it does not appear that bromouracil results in the synthesis of excess "nonfunctional" DNA.[432] Positive identification of the deoxyribonucleotides of bromouracil, chlorouracil and iodouracil following enzymic digestion of DNA from *E. coli* exposed to each of these analogs, respectively, established the nature of the incorporation,

[425] D. B. Dunn and J. D. Smith, *Nature* **174,** 305 (1954).

[426] S. Zamenhof and G. Griboff, *Nature* **174,** 306 (1954).

[427] A. Wacker, A. Trebst, D. Jacherts, and F. Weygand, *Z. Naturforsch.* **9b,** 616 (1954).

[428] M. L. Eidinoff, L. Cheong, and M. A. Rich, *Federation Proc.* **18,** 220 (1959).

[429] R. M. Litman and A. B. Pardee, *Nature* **178,** 529 (1956).

[430] A. Bendich, H. B. Pahl, and G. B. Brown, *in* "The Chemical Basis of Heredity" W. D. McElroy and B. Glass, eds.), p. 378. Johns Hopkins Press, Baltimore, Maryland, 1957.

[431] M. Meselson, F. W. Stahl, and J. Vinograd, *Proc. Natl. Acad. Sci. U. S.* **43,** 581 (1957).

[432] S. Zamenhof, B. Reiner, R. DeGiovanni, and K. Rich, *J. Biol. Chem.* **219,** 165 (1956).

while analysis for thymine clearly indicated that these compounds specifically replaced this normal pyrimidine.[425, 426] The incorporation of chlorouracil into the RNA of *E. coli* 15T⁻ has also been documented by the isolation of 5-chlorouridylic acid; however, only 2% of the uracil residues were replaced by the chloro derivative.[433] Incorporation of iododeoxyuridine has been demonstrated with Ehrlich ascites cells *in vivo*,[434] and with L-5178-Y murine lymphoblasts[423] and H.Ep. #1 cells growing in culture[428]; in the latter case up to 38% of the thymine of the DNA was replaced by the analog. These results are in contrast to the nonutilization of the 5-iodo derivatives of uracil, uridine, and orotic acid in a number of mammalian systems.[435] Enzymic synthesis of the deoxyribonucleosides of chloro-, bromo-, and iodouracil by thymidine phosphorylase and deoxyribose-1-phosphate can be demonstrated, but the yields were low and the products were not isolated.[436] Although the intermediate steps have not been demonstrated in cell-free systems, bromodeoxyuridine triphosphate serves as a precursor in the enzymic synthesis of a DNA-like polymer by the polynucleotide phosphorylase from *E. coli* B. Under growing cell conditions the incorporation of bromouracil into DNA is paralled by the uptake of phosphate-P^{32} into DNA.[437] However, with resting cell suspensions of *E. coli* in which little if any DNA synthesis is occurring, there appears to be an exchange of the pyrimidines, thymine and bromouracil, such that the bromouracil content of the DNA can increase from 8% to 48% of the normal thymine component.[432]

Bromodeoxyuridine and iododeoxyuridine are not only incorporated into DNA, but also block the incorporation of normal thymine derivatives. At present it is difficult to say whether this is because of their replacing thymine residues in the DNA or because they actually inhibit the incorporation of the normal precursors. Evidence obtained with cell suspensions of Gardner's lymphoma and of spleen has indicated that incorporation of formaldehyde-C^{14} into acid-soluble thymine nucleotides was not depressed by the analogs, but that subsequent incorporation of the radioactivity into the thymine (but not the purines) of the DNA of these tissues, *in vivo* or *in vitro*, was markedly depressed.[419, 420] Substantially the same results with respect to DNA thymine have been obtained using Ehrlich cells *in vitro* and exposing them to iododeoxyuridine.[421] Accordingly, the evidence suggests that these compounds block phosphorylation or polymerization of thymidine derivatives and either replace them in what may be a functional

[433] D. B. Dunn, *Trans. Faraday Soc.* **53**, 259 (1957).
[434] W. H. Prusoff, *Federation Proc.* **18**, 305 (1959).
[435] W. H. Prusoff, W. L. Holmes, and A. D. Welch, *Cancer Research* **13**, 221 (1953).
[436] M. Friedkin and D. Roberts, *J. Biol. Chem.* **207**, 257 (1954).
[437] T. D. Price, P. B. Hudson, H. A. Hinds, R. A. Darmstadt, and S. Zamenhof, *Nature* **178**, 684 (1956).

manner or limit by this blockade the synthesis of additional DNA required for reproduction.

Another facet of the biological effects of bromouracil was seen in the accumulation of significant amounts of 6-methylaminopurine in the DNA of *E. coli*,[411] an effect also produced by thymine deficiency or by inhibition with 5-aminouracil. The relationship of this abnormal purine and the replacement of thymine by bromouracil in the fine structure of the resultant DNA will be important to consider as more information accrues concerning the functional role of this macromolecule. Data already at hand would suggest that such incorporation causes an increased rate of mutation in *E. coli* (exposed to iodouracil)[438] and in its T-even phages,[429] particularly when incorporation of the analog is enhanced by limitation of the synthesis of thymine *de novo* with sulfanilamide. The production of antibodies in slices of spleen is blocked by bromodeoxyuridine, an effect which is prevented by thymidine.[439]

Degradation of the 5-halogenated pyrimidines appears to follow a similar course to that for uracil or thymine. Following administration of bromouracil to human subjects or to rodents, one of the urinary excretion products appeared to be β-ureidopropionic acid.[440] It is believed by some that initial formation of the dihydropyrimidine, a reaction demonstrated *in vitro* for bromouracil and iodouracil,[441] is followed by a dehydrohalogenation which yields uracil and, respectively, either bromide or iodide ions.[442] The uracil is then metabolized in a normal fashion. A large number of substituted halogenated derivatives have been studied *in vivo* and in general the results have confirmed this hypothesis.

Biological studies on chloro- and bromocytosine and their corresponding ribonucleosides[443] and deoxyribonucleosides[444] have not been as extensive. It would appear that a portion of the activity of the ribonucleosides may be attributed to deamination to the corresponding uridine analogs.[445] However, the ribonucleosides are more potent inhibitors of the growth of a pyrimidine-requiring mutant of *Neurospora*, a circumstance which is particularly marked when growth is supported by cytidine.[443]

[438] D. Luzzati, *Comp. rend. acad. sci.* **245,** 1466 (1957); cf. footnote 7, Matthews.
[439] R. W. Dutton, A. H. Dutton, and J. H. Vaughan, *Federation Proc.* **18,** 219 (1959).
[440] H. B. Pahl, M. P. Gordon, and R. R. Ellison, *Arch. Biochem. Biophys.* **79,** 245 (1958).
[441] S. Grisolia, J. Caravaca, S. Cardoso, and D. P. Wallach, *Federation Proc.* **16,** 189 (1957).
[442] H. W. Barrett and R. A. West, *J. Am. Chem. Soc.* **78,** 1612 (1956).
[443] T. K. Fukuhara and D. W. Visser, *J. Am. Chem. Soc.* **77,** 2393 (1955).
[444] D. M. Frisch and D. W. Visser, *J. Am. Chem. Soc.* **81,** 1756 (1959).
[445] S. S. Cohen and H. D. Barner, *J. Biol. Chem.* **226,** 631 (1957).

3. 5-Amino-, 5-Mercapto-, and 5-Hydroxypyrimidine Derivatives

Methods have been devised for the synthesis of 5-aminouracil[446] and the corresponding ribose (aminouridine)[447] and deoxyribose (aminodeoxyuridine)[448] derivatives. Formation of the deoxyribonucleoside of aminouracil by condensation with deoxyribose-1-phosphate has been demonstrated using a thymidine phosphorylase from calf kidney.[436] It has been suggested[408] that aminouracil exerts its antimicrobial activity[404] both as an anti-folic acid agent and as an antagonist of thymine. Aminouracil, as well as 2-thiothymine, and also a nutritional deficiency of thymine, each caused up to a fourfold increase in the 6-methylaminopurine content of the DNA of *E. coli* 15T⁻ (an amount equivalent to 15% of the adenine).[449] The significance of this purine, which itself inhibits microbial growth, in the nucleic acids of a number of bacterial species is not understood; certainly, however, this compound must be taken into account in any formulation of the synthesis and function of DNA. Aminouridine inhibits the growth of Theiler's GD VII virus[415] and a pyrimidine-requiring mutant of *Neurospora* (1298),[443] and these effects are nullified by uridine. The compound exhibited slight antitumor activity[450] and inhibited, in a relatively nonspecific manner, the incorporation of radioactive phosphate, formate, and ureidosuccinate into nucleic acid components by slices of rat liver and hepatoma.[322, 323] Aminodeoxyuridine behaved as an antagonist of the pyrimidine deoxyribonucleosides in the growth of *E. coli* K-12, an effect most effectively overcome by thymidine[448]; the findings suggest that the analog inhibited the synthesis of thymine nucleotides. 5-Hydroxyuridine[447] has many properties in common with 5-aminouridine as an inhibitor of the growth of *Neurospora* 1298 and Theiler's GD VII virus on chick embryo tissue.[415] This unnatural ribonucleoside is also a potent inhibitor of adaptive enzyme formation in yeast,[451] and the inhibition of *E. coli* by this compound can be prevented by uridine or cytidine, but not by the corresponding free pyrimidines or by orotic acid.[452] However, the deoxyribonucleoside of 5-hydroxyuracil[416] differs from the corresponding amino derivative in that inhibition of *E. coli* K-12 by this agent is prevented by deoxyuridine and deoxycytidine, but not by thymidine.[448]

A series of thymine antagonists has been prepared by the introduction of 5-mercapto, 5-disulfide, and 5-isothiouronium groups into uracil through diazotization of 5-aminouracil.[453] These compounds, particularly 5-mercaptouracil, effectively inhibit the growth of *L. leichmannii* and their action is competitively reversed by thymine or, more effectively, by thymidine.[409] The rate of growth of *L. arabinosus* and several experimental tumors was only slightly inhibited,[409] but when combined with fluorouracil at subeffective doses for either compound, significant inhibitions of the growth of sarcoma-180 and adenocarcinoma-755 were noted.[454] Although these data have

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[446] H. L. Wheeler and T. B. Johnson, *J. Am. Chem. Soc.* **31,** 603 (1909).

[447] M. Roberts and D. W. Visser, *J. Am. Chem. Soc.* **74,** 668 (1952).

[448] R. E. Beltz and D. W. Visser, *J. Biol. Chem.* **226,** 1035 (1957).

[449] D. B. Dunn and J. D. Smith, *Biochem. J.* **68,** 627 (1958).

[450] D. W. Visser, *in* "Antimetabolites and Cancer" (C. P. Rhoads, ed.), p. 47. Am. Assoc. Advancement Sci., Washington, D. C., 1955.

[451] S. Spiegelman, *in* "The Chemical Basis of Heredity" (W. D. McElroy and B. Glass, eds.), p. 232. Johns Hopkins Press, Baltimore, Maryland, 1957.

[452] I. J. Slotnick, D. W. Visser, and S. C. Rittenberg, *J. Biol. Chem.* **203,** 647 (1953).

[453] T. J. Bardos, R. R. Herr, and T. Enkoji, *J. Am. Chem. Soc.* **77,** 960 (1955).

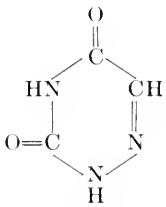
[454] T. J. Bardos, A. Segaloff, and J. L. Ambrus, *Nature* **183,** 619 (1959).

been interpreted as the result of multiple blockade of the synthesis of thymine derivatives *de novo* and the utilization of preformed thymine, further experiments will be required to establish this point.

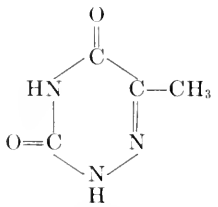
4. Azapyrimidines

Replacement in the natural pyrimidines of one of the ring carbons and its associated hydrogen with the isosteric atom, nitrogen, results in series of symmetric and asymmetric triazines of some biological interest. The symmetric triazine derivative, oxonic acid [6-carboxy-*sym*-triazine-2,4 (1H,3H)-dione], an analog of orotic acid, is prepared as a degradation product of uric acid[455, 456]; it possesses a certain amount of anti-pyrimidine activity.[457] The corresponding uracil analog, oxaidin [*sym*-triazine-2,4 (1H,3H-dione], has also been prepared[455, 456] and found to inhibit the growth of adenocarcinoma-755.[457]

Of greater interest has been the series of asymmetric triazines represented by 4(6)-azauracil [*as*-triazine-3,5(2H,4H)-dione] (XV), 4(6)-azacytosine, and 4(6)-azathymine (XVI). The synthesis of azauracil was described in 1947[458]; however, better methods of preparation, via the semicarbazone of oxomalonic acid, have been reported,[459, 460] and a simplified procedure, which utilizes chloral hydrate in the formation of the semicarbazone of glyoxylic acid, is now used.[461] Azacytosine has been prepared from azauracil, using an intermediate formed with the aid of phosphorus pentasulfide, while azathymine was formed from the semicarbazone of pyruvic acid.[460]



(XV)
4(6)-Azauracil



(XVI)
4(6)-Azathymine

The uracil analog antagonized the growth of microorganisms not only when they were supplied with pyrimidines exogenously, but also when they synthesized their own *de novo*; indeed, growth inhibition was most

[455] H. Biltz, *Ber.* **46**, 3410 (1913).
[456] E. S. Canellakis and P. P. Cohen, *J. Biol. Chem.* **213**, 379 (1955).
[457] S. Bieber, G. B. Elion, H. C. Nathan, and G. H. Hitchings, *Proc. Am. Assoc. Cancer Research* **2**, 188 (1957).
[458] W. Seibert, *Ber.* **80**, 494 (1947).
[459] R. B. Barlow and A. D. Welch, *J. Am. Chem. Soc.* **78**, 1258 (1956).
[460] E. A. Falco, E. Pappas, and G. H. Hitchings, *J. Am. Chem. Soc.* **78**, 1938 (1956).
[461] P. K. Chang and T. L. V. Ulbricht, *J. Am. Chem. Soc.* **80**, 976 (1958).

profound in the latter case.[462] The inhibitory effects of the analog on growth could be prevented by uracil or cytosine, while orotic acid was relatively ineffective in this respect.[462, 463] A number of workers have demonstrated the inhibitory action of this compound on the growth of experimental tumors.[464-468] Results obtained in the combination treatment of adenocarcinoma-755 with urethane and azauracil were particularly impressive.[468]

Further studies in microbial systems revealed extensive conversion of azauracil to the ribonucleoside and the corresponding 5′-phosphates both in *S. faecalis* and in *E. coli*.[469-473] To permit further study of these derivatives a chemical synthesis of the ribonucleoside was devised,[469, 473] but this has been replaced by a more efficient microbial formation, using *E. coli*.[470, 472] Although such anabolic conversion is extensive in several bacterial strains, mammalian cells are relatively inefficient in this respect[467, 474, 475] and, in addition, catabolize the analog (at least to a small extent) to the following urinary products: glyoxylic acid semicarbazone, oxalic acid, carbon dioxide, and an unidentified acid.[474] The biological properties of the ribonucleoside, azauridine, are similar to those of azauracil except that it is approximately 20 times more potent as an inhibitor of tumor growth,[467, 476] is cleaved to a very minor degree even after oral administration,[467, 474, 477] and is rapidly converted in the tissues tested to the 5′-phosphate.[477-480] In a strain of *S. faecalis* selected for resistance to azauracil,

[462] R. E. Handschumacher and A. D. Welch, *Cancer Research* **16**, 965 (1956).
[463] J. Škoda and F. Šorm, *Chem. listy* **50**, 1165 (1956); F. Šorm and J. Škoda, *Collection Czechoslov. Chem. Communs.* **21**, 487 (1956).
[464] M. T. Hakala, L. W. Law, and A. D. Welch, *Proc. Am. Assoc. Cancer Research* **2**, 113 (1956).
[465] F. Šorm, A. Jakubovic, and L. Slechta, *Experientia* **12**, 271 (1956).
[466] J. Sablik and F. Šorm, *Neoplasm* **4**, 113 (1957).
[467] J. J. Jaffe, R. E. Handschumacher, and A. D. Welch, *Yale J. Biol. and Med.* **30**, 168 (1957).
[468] G. B. Elion, S. Bieber, H. C. Nathan, and G. H. Hitchings, *Cancer Research* **18**, 802 (1958).
[469] R. E. Handschumacher, *Federation Proc.* **16**, 191 (1957).
[470] J. Škoda, V. F. Hess, and F. Šorm, *Collection Czechoslov. Chem. Communs.* **22**, 1330 (1957); *Experientia* **13**, 150 (1957).
[471] R. E. Handschumacher, *Biochim. et Biophys. Acta* **23**, 428 (1957).
[472] R. E. Handschumacher, *Nature* **182**, 1090 (1958).
[473] R. E. Handschumacher, *J. Biol. Chem.*, in press.
[474] R. E. Handschumacher and R. J. Davis, *Abstr. Meeting Am. Soc. Pharmacol. Exptl. Therap., Ann Arbor*, p. 17 (1958).
[475] R. Schindler and A. D. Welch, *Biochem. Pharmacol.* **1**, 132 (1958).
[476] F. Šorm and H. Keilova, *Experientia* **14**, 215 (1958).
[477] V. Habermann and F. Šorm, *Collection Czechoslov. Chem. Communs.* **23**, 2201 (1958).
[478] C. A. Pasternak and R. E. Handschumacher, *Proc. Am. Assoc. Cancer Research* **2**, 233 (1958).

azauridine was an effective inhibitor[471]; when tested against the growth of sarcoma-180, or of L-5178-Y lymphoblasts in culture, azauracil was completely inactive, but the growth of the cells was extremely sensitive to inhibition by ribonucleoside.[475, 481]

The accumulation of orotidine[478-480] in tumors inhibited by azauracil or azauridine and the amassing of orotic acid[472, 482, 483] and orotidylic acid[472] in cultures of *E. coli* inhibited by azauracil suggested a blockade in the synthesis *de novo* of pyrimidines, rather than interference with uracil utilization. Independently, orotidine and orotic acid were noted in the urine of animals receiving azauracil.[477] Subsequent studies showed that incorporation of orotic acid into nucleic acid pyrimidines was markedly depressed in mice treated with azauridine, while the incorporation of uridine was even greater than in control animals.[480] Such a blockade by azauridine (but not by azauracil) also has been demonstrated in the conversion of orotic acid to uridine nucleotides by soluble extracts from tumors. This system accumulates orotidylic acid in the presence of azauridine, a result which implies inhibition of orotidylic acid decarboxylase.[471, 480] Preparations of this enzyme from either yeast[479] or tumor tissues[480] were inhibited in a competitive manner by either the naturally formed or the synthetic 5′-phosphate of azauridine (2×10^{-6} *M*), the only metabolite of azauridine found in mammalian tissues; however, neither azauracil nor azauridine, nor the 5′-di- and triphosphates obtained from bacteria, significantly inhibited these enzymes.[484] These results offer confirmation of microbial inhibition studies in which only those organisms which have the ability to utilize exogenous uracil (and hence possess the capacity to form the ribonucleotide of azauracil), but ordinarily depend upon the synthesis of pyrimidines *de novo*, displayed sensitivity to the antimetabolite.[462] Further support for the concept that the same enzymes are involved was afforded by the finding that strains of *S. faecalis* selected for resistance to azauracil were unable to form nucleotides of this analog or to utilize exogenous uracil.[471] Although orotidylic acid decarboxylase appears to be the primary site of the action of metabolically formed azauridylic acid, other sites of inhibition have been observed. In adenocarcinoma-755 the incorporation of uracil over a 16-hour period following treatment with azauracil was depressed, but it is difficult to separate this finding from the inhibition of tumor growth during this period.[468] A similar depression in the uptake of uracil has been ob-

[479] R. E. Handschumacher and C. A. Pasternak, *Biochim. et Biophys. Acta* **30,** 451 (1958).

[480] C. A. Pasternak and R. E. Handschumacher, *J. Biol. Chem.* **234,** 2992 (1959).

[481] R. Schindler and A. D. Welch, *Science* **125,** 548 (1957).

[482] J. Škoda and F. Šorm, *Biochim. et Biophys. Acta* **28,** 659 (1958).

[483] J. Škoda and F. Šorm, *Collection Czechoslov. Chem. Communs.* **24,** 1331 (1959).

[484] R. E. Handschumacher, *J. Biol. Chem.*, in press.

served in hepatomas.[390] Minor effects of azauracil and azauridine on the uridine phosphorylase and kinase activities of extracts of tumors[480] and of bacteria[483] also have been noted. A recent report indicates that azauridine-5′-diphosphate at high concentrations inhibits the polynucleotide phosphorylase of *E. coli*[485]; however, the apparent lack of formation of this diphosphate in mammalian systems would seem to exclude this enzyme as a site of action in animals.[479, 480]

Minor incorporation of azauracil into the RNA of *S. faecalis* has been reported, but none was found in the DNA.[469] As might be predicted, none of this analog was incorporated into mammalian nucleic acids.[475, 480] Azauracil has been reported to increase the levels of hexosamine derivatives in the soluble fraction from *E. coli*[486] (as does penicillin), and to prevent the synthesis of orotic acid in *E. coli* under special conditions through a reported negative feed-back reaction which is also activated by uracil.[487] The deoxyribonucleoside of azauracil has been prepared by transfer of deoxyribose from thymidine to the analog in the presence of suspensions of *S. faecalis* cells[462]; however, this derivative was inactive in several microbial and mammalian systems in which it was tested.[488] In general, the inhibitory activity of 6-azacytosine is not as great as that of the uracil analog; but the growth of adenocarcinoma-755 in mice is remarkably sensitive to this antimetabolite, since azacytosine is effective in doses about one-twenty-fifth those of azauracil.[468]

The methyl derivative corresponding to thymine, i.e., 6-azathymine,[460, 489] is an effective inhibitor of the growth of many microorganisms, an effect which can be prevented in a competitive manner by either thymine or thymidine.[490, 491] This analog prevented the growth not only of organisms supplied with exogenous thymine or thymidine, but also of those which synthesized thymine derivatives *de novo*. Incubation of azathymine with washed cell suspensions of *S. faecalis* and thymidine resulted in the formation of the deoxyribonucleoside, azathymidine, by a transdeoxyribosidation process[381]; this derivative exerts an even greater inhibitory effect on the growth of certain strains of microorganisms.[492] Either azathymine or azathymidine, when added to cultures of *S. faecalis* during the logarithmic phase of growth, creates a lethal condition in the cells which results

[485] J. Škoda, J. Kara, Z. Šormova, and F. Šorm, *Biochim. et Biophys. Acta* **33,** 579 (1959).
[486] Y. Takagi and N. Otsuji, *Biochim. et Biophys. Acta* **29,** 227 (1958).
[487] R. A. Yates and A. B. Pardee, *J. Biol. Chem.* **227,** 677 (1957).
[488] R. E. Davis and R. E. Handschumacher, unpublished results (1958).
[489] J. R. Bailey and L. Knox, *J. Am. Chem. Soc.* **29,** 880 (1907).
[490] W. H. Prusoff, W. L. Holmes, and A. D. Welch, *Cancer Research* **14,** 570 (1954).
[491] G. B. Elion, S. Singer, and G. H. Hitchings, *J. Biol. Chem.* **208,** 477 (1954).
[492] W. H. Prusoff and A. D. Welch, *J. Biol. Chem.* **218,** 929 (1956).

in what appears to be "thymineless" death.[493] However, under these conditions, little or no azathymidine was found in the DNA. Once initiated, the death process usually could not be interrupted by the addition to the culture of massive amounts of thymidine. When added to cultures in concentrations insufficient to abolish reproduction, azathymidine was incorporated into the DNA of *S. faecalis* in an amount equivalent to 18% of the normal thymine content. Clearly, the extent of inhibition and the degree of incorporation into DNA are essentially unrelated, since maximal incorporation occurs under conditions which do not result in lethal effects on the cells.

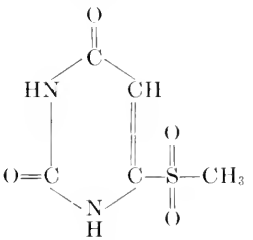
Evidence concerning the mechanism of action of azathymine and its deoxyribonucleoside has been obtained primarily from studies with rabbit bone marrow and with Ehrlich ascites cells *in vitro*.[494-496] The incorporation of formate into DNA thymine in these systems was blocked by azathymidine (but not by azathymine), whereas incorporation of thymidine in ascites cells and bone marrow was relatively insensitive to azathymidine; however, direct comparison of these results is difficult, because in the former case the unnatural deoxyribonucleoside is competing with what is believed to be very small pools of thymine nucleotides. From the urine of mice receiving radioactive azathymine there have been isolated the ribonucleoside of the analog, as well as a catabolic derivative, and also relatively large amounts of uracil.[497] Formation of the ribonucleoside (the structure of which has been proven by chemical synthesis[498]), and of the deoxyribonucleoside by a soluble fraction from liver has been demonstrated[499]; however, these compounds did not influence the formation of the corresponding derivatives of thymine in this system.[499]

A chemical synthesis of the 3′- and 5′-mono- and 3′,5′-diphosphates of azathymidine has been reported,[500] but the biological activity of these derivatives has been tested only in whole-cell systems in which they are less active than the corresponding deoxyribonucleoside. An investigation of the effects of these derivatives on the enzymic steps responsible for the synthesis of thymidylic acid and its subsequent incorporation into DNA would be desirable.

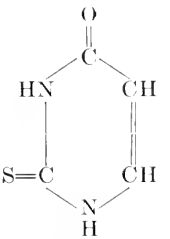
[493] W. H. Prusoff, *J. Biol. Chem.* **226,** 901 (1956).
[494] A. D. Welch, W. H. Prusoff, and L. G. Lajtha, *Trans. Assoc. Am. Physicians* **68,** 112 (1955).
[495] W. H. Prusoff, L. G. Lajtha, and A. D. Welch, *Biochim. et Biophys. Acta* **20,** 209 (1956).
[496] W. H. Prusoff, *Biochem. Pharmacol.* **2,** 221 (1959).
[497] W. H. Prusoff and R. A. Gaito, *Abstr. 131st Meeting Am. Chem. Soc., Miami, Florida*, p. 2C (1957).
[498] R. H. Hall, *J. Am. Chem. Soc.* **80,** 1145 (1958).
[499] W. H. Prusoff and R. A. Gaito, *Federation Proc.* **17,** 292 (1958).
[500] R. H. Hall and R. Hazelkorn, *J. Am. Chem. Soc.* **80,** 1138 (1958).

5. Miscellaneous Pyrimidines

A number of structural analogs of orotic acid have been investigated. Of the 5-halogenated derivatives, 5-fluoroorotic acid has been discussed above; 5-chloroorotic, though not extremely active, caused the accumulation of orotidylic acid in a supernatant enzyme preparation from rat liver. The metabolism of this compound to a nucleotide form also has been suggested. Recently the synthesis of the ethyl ester of 3-diazocitrazinic acid (3-diazo-4-carboxyethylpyridine-2,6-dione) was reported; this derivative of pyridine (or of "de-aza" pyrimidine) inhibits the growth of *L. bulgaricus*, an effect which is prevented by orotic acid.[501] The possibility of "alkylation" by the active diazo function of the enzyme normally responsible for condensation of orotic acid with pyrophosphorylribose-5-phosphate is apparent. Another series of orotic analogs has been prepared by substitution of a sulfonamide or alkyl or aryl sulfone group for the carboxyl group of orotic acid.[502] These compounds, particularly 4(6)-uracil methylsulfone (XVII), are potent inhibitors of the growth of several strains of *Lactobacillus bulgaricus*, the effect of which is noncompetitive with respect to orotic acid and other pyrimidine derivatives; however, crude RNA fractions will overcome the inhibitory effects.[503]



(XVII)
4(6)-Uracil methylsulfone



(XVIII)
2-Thiouracil

Uracil methylsulfone also has been shown to inhibit the conversion of orotic acid to orotidine-5′-phosphate in a competitive manner initially, but the kinetics shortly become noncompetitive.[504] Inhibition of the growth of a number of tumors also has been demonstrated with this uracil derivative[464, 505]; however, recent studies of the metabolic disposition of this agent showed that an exceedingly rapid nonenzymic reaction with sulfhydryl groups occurred, a circumstance which results in the expulsion of the sulfone group and the formation of a 6-uracil-sulfide derivative of the mercapto compound.[506] This finding appears to explain the shift to noncompetitive kinetics in the enzyme study. The effect of uracil methylsulfone on pyrimidine metabolism in tumors is not as clearly related to orotic acid. The uptake by suspensions of Ehrlich ascites cells of orotic acid and formate into the thymine of DNA, but not the incorporation of orotic acid into RNA pyrimidines, was blocked by this analog,

[501] Z. B. Papanastassiou, A. McMillan, V. J. Czebotar, and T. J. Bardos, *Abstr. 135th Meeting Am. Chem. Soc., Boston, Mass.*, p. 11N (1959).
[502] S. B. Greenbaum, *J. Am. Chem. Soc.* **76**, 6052 (1954).
[503] W. L. Holmes and A. D. Welch, *Cancer Research* **16**, 251 (1956).
[504] W. L. Holmes, *J. Biol. Chem.* **223**, 677 (1956).
[505] J. J. Jaffe and J. R. Cooper, *Cancer Research* **18**, 1089 (1958).
[506] J. R. Cooper, *Cancer Research* **18**, 1084 (1958).

whereas the incorporation of preformed thymidine was essentially unaffected.[507] However, using slices of regenerating livers, other workers have reported some effects of uracil methylsulfone on the incorporation of orotic acid into the pyrimidines of the RNA.[508] The high reactivity of this compound, apparently with sulfhydryl sites on several enzymes concerned with pyrimidine metabolism, may make it a useful tool in studying the nature of certain of the reactive centers on these enzymes, and in providing a specific blocking agent for these reactions.

The inhibitory effects of 2-thiouracil (XVIII) on the growth of *L. casei* and *E. coli* were noted as early as 1945 and the specific reversal of these effects by uracil encouraged further study of this analog in other systems.[509] Examples of its effect on the rate of growth of seedlings,[510] protozoa,[511] viruses,[512, 513] and experimental tumors[514, 515] have been added. Although this compound (and its 6-propyl derivative) is considered primarily as an antithyroid drug, its action in most of the systems referred to above appears to be mediated by an effect on nucleic acid metabolism. Incorporation of this analog into the RNA of (*a*) tobacco mosaic virus,[512, 516, 517] (*b*) *Bacillus megaterium*,[518] and (*c*) livers of rats treated with acetylaminofluorene[519, 520] has been described. More complete studies with the first two types of RNA have clearly indicated that the compound was present in normal ribonucleotide form, and that it replaced up to 20% of the normal uracil component.[517, 518] Liberation of this analog from the RNA of tobacco mosaic virus indicated that a large percentage of the compound was present as 2-thiouridine and its 3′,5′-diphosphate.[517] Such a result would suggest that the analog is present in higher concentrations in oligonucleotides or that it may be found primarily at the ends of RNA molecules. It has been proposed that the decrease in growth rate of tobacco mosaic virus exposed to thiouracil, but lack of effect on the total number of infectious particles, is the result of inactivation of some but not all subunits of RNA within the virus; however, this remains only an hypothesis.[516] As with many of the other agents discussed, incorporation into nucleic acids may bear little relationship to inhibition, since 2-thiothymine is a potent inhibitor of *E. coli*, but is not incorporated to any significant degree into its DNA.[432] Although thiouracil is a substrate for the nucleoside phosphorylase from horse liver and yields the corresponding ribonucleoside or deoxyribonucleoside, depending upon the pentose phosphate supplied,[521] little more has been done on the

[507] W. H. Prusoff, *Cancer Research* **18,** 603 (1958).

[508] K. Ogata, T. Shimizu, and K. Togashi, *Biochim. et Biophys. Acta* **29,** 656 (1958).

[509] F. Strandskov and O. Wyss, *J. Bacteriol.* **50,** 237 (1945); *J. Bacteriol.* **52,** 575 (1946).

[510] W. R. Trotter, *Nature* **164,** 63 (1949).

[511] G. W. Kidder and V. C. Dewey, *J. Biol. Chem.* **178,** 382 (1949).

[512] R. Jeener and J. Rosseels, *Biochim. et Biophys. Acta* **11,** 438 (1953).

[513] B. Commoner and F. Mercer, *Nature* **168,** 113 (1951); *Arch. Biochem. Biophys.* **35,** 278 (1952).

[514] K. E. Paschkis, A. Cantarow, and J. Stasney, *Science* **114,** 264 (1951).

[515] J. Meites, *Cancer Research* **18,** 176 (1958).

[516] R. Jeener, *Biochim. et Biophys. Acta* **23,** 351 (1957).

[517] H. G. Mandel, R. Markham, and R. E. F. Matthews, *Biochim. et Biophys. Acta* **24,** 205 (1957).

[518] R. Hamers, *Biochim. et Biophys. Acta* **21,** 170 (1956).

[519] R. J. Rutman, A. Cantarow, and K. E. Paschkis, *Federation Proc.* **12,** 122 (1953).

[520] A. Cantarow, K. E. Paschkis, and R. J. Rutman, *J. Natl. Cancer Inst.* **15,** 1615 (1955).

[521] D. B. Strominger and M. Friedkin, *J. Biol. Chem.* **208,** 663 (1954).

enzymic reactions of this analog at the nucleotide level; therefore, an evaluation of its mechanism of action as an inhibitor of growth must await further investigation. It is of interest, however, that thiouracil inhibited the catabolic release of carbon dioxide-C^{14} from uracil-2-C^{14} in rats previously treated with acetylaminofluorene to induce a "preneoplastic" state in the liver.[519] Although the analog was incorporated into nucleic acids, it did not prevent the incorporation of uracil.[520] Furthermore, this analog suppressed the induction of hepatomas in rats by acetylaminofluorene, an effect which could be overcome by uracil without suppressing the hyperplasia of the thyroid caused by thiouracil.[514] The metabolic alteration of thiouracil in rats results in the formation of minor amounts of 2-methylthiouracil and inorganic sulfate.[522]

VI. Other Agents Which Influence Nucleic Acid Metabolism

1. Urethane

This chemically simple substance, ethyl carbamate ($C_2H_5O—CO—NH_2$), although known as a narcotic agent since 1885, was not recognized until relatively recent years as having the capacity to affect cellular reproduction and nucleic acid metabolism. As a result of studies which showed that urethane produced arrest of mitosis in certain plants, the compound received attention as an inhibitor of the growth of various transplantable neoplasms,[468, 523] and also of spontaneous leukemias in mice.[524] It is used to a limited extent in the therapy of certain neoplastic states in man, particularly myelogenous leukemia and multiple myeloma.[525-527] Urethane has also been shown to induce neoplasms in certain strains of mice and rats, particularly adenomas of the lungs,[528-530] but also tumors of the liver, stomach, skin, and other tissues,[529-531] and to augment, in mice, the leukemogenic activity of X-rays, estrogenic hormones, and methylcholanthrene.[531] Extensive study of the relationship between structure and biological activity has shown that the oncogenic and antileukemic actions in mice of compounds related to urethane are remarkably specific, since even the smallest structural alterations abolish or greatly reduce activity.[532-535] Nevertheless, the

[522] E. J. Sarcione and J. E. Sokal, *J. Biol. Chem.* **231**, 605 (1958).
[523] A. Haddow and W. A. Sexton, *Nature* **157**, 500 (1956).
[524] L. W. Law, *Proc. Natl. Acad. Sci. U. S.* **33**, 204 (1947).
[525] E. Paterson, A. Haddow, I. ApThomas, and J. M. Watkinson, *Lancet* **i**, 677 (1946).
[526] L. Berman and A. R. Axelrod, *Am. J. Clin. Pathol.* **18**, 104 (1948).
[527] J. P. Loge and R. W. Rundles, *Blood* **4**, 201 (1949).
[528] A. Nettleship and P. S. Henshaw, *J. Natl. Cancer Inst.* **4**, 309 (1943).
[529] W. G. Jaffé, *Cancer Research* **7**, 107 (1947).
[530] S. Rogers, *J. Exptl. Med.* **105**, 279 (1957).
[531] S. Kawamoto, N. Ida, A. Kirschbaum, and G. Taylor, *Cancer Research* **18**, 725 (1958).
[532] C. D. Larsen, *J. Natl. Cancer Inst.* **8**, 99 (1947); **9**, 35 (1948).
[533] H. E. Skipper, C. E. Bryan, W. H. Riser, Jr., M. Welty, and A. Stelzenmuller, *J. Natl. Cancer Inst.* **9**, 77 (1948).
[534] H. E. Skipper and C. E. Bryan, *J. Natl. Cancer Inst.* **9**, 391 (1948).

ability of urethane to initiate pulmonary adenomas in mice appears to be caused by a metabolite of the compound[530, 536] or, perhaps less likely, by a substance released from the tissues of animals given even a single injection of urethane.

The nature of the oncogenic substance presumably derived from urethane is unknown, but the parent compound, separately labeled with C^{14} in its alkyl or carbonyl groups, is metabolized rapidly, apparently largely to ethanol, carbon dioxide, and ammonia.[537-539] Studies with the sperm of the sea urchin have indicated that carbon derived from either the carbonyl or the methylene groups of urethane is fixed to nuclear material,[540] while studies with mice showed that, although total uptake was greatest in the nuclear fraction of lung tissue, the highest concentration of C^{14} per milligram of protein occurred in the pulmonary mitochondria.[539] In view of the rapid catabolism of urethane, the significance of these uptakes of radioactive carbon cannot be evaluated.

Recent study of a large number of derivatives of urethane as initiators of lung adenomas and neoplasms of the skin in mice disclosed no substances more active than the parent compound.[535] The modifications in structure of urethane involved changes in the carbamyl and carboethoxy portions, phosphorylated derivatives, and addition compounds with keto acids (for the effect of oxaloacetate, see below) and amino acids. Although less active than urethane, *N*-hydroxyurethane exhibited the most marked activity in inducing lung tumors, while carboethoxyphosphate also appeared to have some activity.

Investigation[468] of the growth of adenocarcinoma-755 in C57BL mice showed that the inhibitory activity of azauracil could be potentiated strikingly by urethane, but this result could not be obtained with other experimental neoplasms.[541] On the other hand, the remarkable sensitivity of this tumor system to inhibition by azacytosine was but little affected by the concurrent administration of urethane.[468]

Important studies[530, 536, 542-544] of the oncogenic action of urethane in

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[535] I. Berenblum, D. Ben-Ishai, N.Haran-Ghera, A. Lapidot, E. Simon, and N. Trainin, *Biochem. Pharmacol.* **2,** 168 (1959).

[536] S. Rogers, *J. Natl. Cancer Inst.* **15,** 1675 (1955).

[537] J. H. Mitchell, Jr., O. S. Hutchison, H. E. Skipper, and C. E. Bryan, *J. Biol. Chem.* **180,** 675 (1949).

[538] H. E. Skipper, L. L. Bennett, Jr., C. E. Bryan, L. White, Jr., M. A. Newton, and L. Simpson, *Cancer Research* **11,** 46 (1951).

[539] I. Berenblum, N. Haran-Ghera, R. Winnick, and T. Winnick, *Cancer Research* **18,** 181 (1958).

[540] I. Cornman, H. E. Skipper, and J. H. Mitchell, Jr., *Cancer Research* **11,** 195 (1951).

[541] G. B. Elion, Personal communication (1959).

[542] S. Rogers, *J. Exptl. Med.* **93,** 427 (1951).

[543] S. Rogers, *J. Natl. Cancer Inst.* **15,** 1675 (1955).

mice have shown convincingly that injection of the agent (e.g., a single dose of 1 mg. per gram of mouse) initiates, rather than promotes, the development of pulmonary adenomas, and that the magnitude of the response (i.e., the number of tumors initiated) is directly related to the rate of growth of the animal at the time of exposure to urethane. This action of urethane was markedly intensified by treatment of the mice with aminopterin and greatly reduced by administration of a hydrolyzate of DNA. Further indications that, in some as yet undisclosed manner, urethane influences the synthesis of nucleic acids, particularly the pyrimidines of DNA, was obtained from ingenious experiments in which lung tissues from week-old mice, prior to implantation in untreated mice, were incubated with the serum of rabbits previously injected with urethane; the effect of other compounds was determined by their addition to this test system. Thus, oxalacetate increased the number of tumors obtained after implantation, while carbamylaspartate and asparagine (but not aspartate) diminished the number. Although these findings might suggest a disturbance in the synthesis of carbamylaspartate, orotic acid, and subsequently formed pyrimidines, the results obtained *in vivo* do not indicate that the explanation is a simple one. Thus, the oncogenic action of urethane, although greatly reduced by the administration to the treated mice of orotic acid, cytidylic acid or thymine, was affected but little or not at all by dosage with uracil, uridylic acid, or deoxycytidylic acid, while thymidine apparently was much less active than thymine, although (as indicated previously) a remarkable effect was obtained with hydrolyzates of DNA.

These results, although confusing, have support, in part, from experiments in which thymine (but not uracil) prevented the production of abnormal mitoses by urethane in the Walker tumor.[545] Similarly, in the studies of the carcinostatic activity of urethane on the 755-tumor, considerable reversal activity was reported for thymine, thymidine, cytidine, glutamine, and asparagine, although with other metabolites the effects observed were less marked or even negligible. Since the growth of adenocarcinoma-755 is fraught with many difficulties and irregular results are sometimes obtained, it may be unwise to draw extensive conclusions from these "reversal experiments."

In view of the fact that thymine is very poorly utilized for DNA thymine synthesis (at least by the rat) and is very rapidly and extensively degraded,[546-549] the possibilities that this pyrimidine may be used in the for-

[544] S. Rogers, *Federation Proc.* **16**, 370 (1957).

[545] E. Boyland and P. C. Koller, *Brit. J. Cancer* **8**, 677 (1954).

[546] G. B. Brown, P. M. Roll, and H. Weinfeld, *in* "Phosphorus Metabolism" (W. D. McElroy and B. Glass, eds.), Vol. II p. 385. Johns Hopkins Press, Baltimore, Maryland, 1952.

[547] W. H. Prusoff, W. L. Holmes, and A. D. Welch, *J. Biol. Chem.* **209**, 503 (1954).

mation of substances other than DNA thymine, or may exert indirect effects (rather than play only a precursory role), should be eliminated. An additional complication is the antagonism by diaminopurine of the inhibition of the growth of *E. coli* by urethane (or by dimethylformamide).[324, 325] Despite the suggestion[550] that urethane influences "the formation of the pyrimidine nucleus from carbamyl phosphate and aspartic acid, the methylation and the amination of the uracil moiety," much more work will be needed to explain satisfactorily the remarkable effects of this compound on nucleic acid metabolism.

2. Miscellaneous

It is evident that the discussion of agents which influence nucleic acid metabolism presented thus far has dealt primarily with structural analogs of compounds intimately associated with the assembly of the nucleotide units. However, in addition to this group there must be a vast number of substances which in one way or another can alter metabolism of the nucleic acids. A few examples, illustrative of certain classes of such agents, will indicate the diversity of these various factors.

It is axiomatic that most general cellular poisons interrupt synthesis of the macromolecules; however, evidence is accumulating which suggests that the so-called alkylating agents, such as the nitrogen mustards[551] may preferentially alkylate the phosphate groups of RNA and DNA and thereby exert their radiomimetic action. Damage to body organs, such as the liver, by carbon tetrachloride, results in a secondary regenerative phase in which nucleic acid synthesis is markedly stimulated preparatory to a sharp increase in mitotic activity within the organ.[552] Another compound, *N*-diethylnicotinamide (nikethamide), has been reported to cause hyperplasia of the liver in rats, an effect accompanied by increased incorporation of phosphate-P^{32} into nucleic acids[553]; whether this represents tissue proliferation in response to injury cannot be stated. The carcinogen, acetylaminofluorene, when fed to rats, creates a preneoplastic state in the liver during which the

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[548] P. Reichard, *Acta Chem. Scand.* **9**, 1275 (1955).

[549] K. F. Fink, R. E. Cline, R. B. Henderson, and R. M. Fink, *J. Biol. Chem.* **221**, 425 (1956).

[550] G. B. Elion, S. Bieber, and G. H. Hitchings, *Abstr. 7th Intern. Cancer Congr., London*, p. 235 (1958).

[551] K. A. Stacey, M. Cobb, S. F. Cousens, and P. Alexander, *Ann. N. Y. Acad. Sci.* **68**, 682 (1958).

[552] J. Hoffman, M. B. Hines, S. Lapan, R. Rizki, and J. Post, *A.M.A. Arch. Pathol.* **59**, 429 (1955).

[553] W. R. Foster and F. G. Brazda, *Cancer Research* **18**, 289 (1958).

incorporation of uracil is increased by tenfold, as compared to that seen in normal liver.[554]

Results which would suggest more specific interference with nucleic acid synthesis have been observed with several antimicrobial agents (in addition to azaserine and DON). Thus, mitomycin C, an antibiotic with carcinostatic activity, has been reported to interfere with the synthesis *de novo* of purine nucleotides by *E. coli*,[555] and, more recently, a selective inhibition by this antibiotic of the formation of DNA as compared to the synthesis of RNA or protein, was demonstrated in this same organism.[556] Extensive investigations of the mode of action of D-erythrochloramphenicol as an antimicrobial agent have recently been reviewed.[557] It appears that this compound causes the formation of an RNA which is abnormal in both physical properties[558] and biological stability,[559] an effect which has been related to the profound influence of this antibiotic on protein synthesis in many bacterial species. Although the L-threo-isomer of this compound is inactive as an antibacterial agent, it reduced by about two-thirds the incorporation of adenosine-5′-phosphate into the nucleic acids of cell suspensions of Ehrlich ascites carcinoma; however, the incorporation of adenine under these same conditions was unimpaired.[143]

Another pair of compounds, ethidium bromide and antrycide, which are potent trypanocidal agents, exhibit apparently selective actions on the synthesis of nucleic acids in *Strigomonas oncopelti*. Thus, ethidium bromide, a phenanthradinium derivative, causes an almost instantaneous inhibition of the production of DNA, while protein and RNA synthesis continue unchanged for some time.[560] In contrast to these results, antrycide, a pyrimidine derivative of a substituted quinoline, interferes with the production of RNA, with only delayed effects on DNA synthesis.[561] More specifically, antrycide prevents the utilization of preformed adenine or guanine without interfering with the synthesis *de novo* of purines from glycine or the utilization of uracil.

The nicotinamide antimetabolite, 2-ethylamino-1,3,4-thiadiazole, has

[554] A. Cantarow, T. L. Williams, I. Melnick, and K. E. Paschkis, *Cancer Research* **18,** 818 (1958).

[555] H. C. Reilley, J. G. Capuccino, and D. M. Harrison, *Proc. Am. Assoc. Cancer Research* **2,** 338 (1958).

[556] S. Shiba, A. Terawaki, T. Taguchi, J. Kawamata, *Nature* **183,** 1056 (1959).

[557] E. F. Gale, *in* "The Strategy of Chemotherapy" (S. T. Cowan and E. Rowatt, eds.), p. 212. Cambridge Univ. Press, London and New York, 1958.

[558] A. B. Pardee, K. Paigen, and L. S. Prestidge, *Biochim. et Biophys. Acta* **23,** 162 (1957).

[559] F. C. Neidhardt and F. Gros, *Biochim. et Biophys. Acta* **25,** 513 (1957).

[560] B. A. Newton, *J. Gen. Bacteriol.* **17,** 718 (1957).

[561] B. A. Newton, *J. Gen. Bacteriol.* **19,** ii (1958).

been shown not only to suppress the "adaptive" formation of diphosphopyridine nucleotide in rats, perhaps by forming the corresponding analog nucleotide, but also to increase by fourfold the output of uric acid in man.[562, 563] The data indicate that this is a true stimulation of purine synthesis and that the effect could be nullified by nicotinamide.

Finally, it would be a grave omission to neglect the multitude of factors which, operating through compounds normally present in cells, control some of the many biosynthetic processes involved in the formation of nucleic acids. Certain examples may be mentioned, but space does not permit a complete discussion of these metabolic regulators. The observation that partial hepatectomy of one of a pair of parabiotically joined rats gives rise to an increased mitotic index in the liver of the partner,[564] as well as to increased uptake of phosphate-P^{32}, suggests the participation of a humoral factor which triggers cell division and, thus, must first initiate an increase in the synthesis of nucleic acids.[565] The role of many factors affecting growth, and hence nucleic acid synthesis, has been reviewed recently[566] and the effects of growth hormone, testosterone, and hypothyroidism on the incorporation of uracil into the RNA of rat liver have been presented.[554] Similarly, a factor has been reported to be present in normal liver which suppresses the incorporation of formate into the DNA thymine of bone marrow.[567] More specific examples may be found in the "negative feed-back" effects on the synthesis of orotic acid in *E. coli* of uracil in whole cells and of cytidylic acid in cell-free preparations.[568] A similar phenomenon has been reported for purine synthesis with resting-cell suspensions of this organism.[569] A more recent report has established the feed-back control by adenine and guanine nucleotides of a partially purified preparation of amido transferase from pigeon liver which forms ribosylamine-5-phosphate from pyrophosphorylribose-5-phosphate and glutamine.[570]

It is apparent that a proper consideration of the effects of the agents discussed in this chapter must include an evaluation of their effects on many intrinsic control mechanisms within the cell, as well as any direct effect which they may exert on the enzymes involved in the synthesis of nucleic acids.

[562] I. H. Krakoff and G. M. Magill, *Proc. Soc. Exptl. Biol. Med.* **91**, 470 (1957).
[562a] I. H. Krakoff and M. E. Balis, *J. Clin. Invest.* **38**, 907 (1959).
[563] J. E. Seegmiller, A. I. Grazzel, and L. Liddle, *Federation Proc.* **18**, 321 (1959).
[564] N. L. R. Bucher, J. F. Scott, and J. C. Aub, *Cancer Research* **11**, 457 (1951).
[565] J. L. Van Lancker and D. G. Sempoux, *Arch. Biochem. Biophys.* **80**, 337 (1959).
[566] K. E. Paschkis, *Cancer Research* **18**, 981 (1958).
[567] J. S. Dunning and L. Wiles, *Science* **129**, 336 (1959).
[568] R. A. Yates and A. B. Pardee, *J. Biol. Chem.* **221**, 757 (1956).
[569] J. Gots, *J. Biol. Chem.* **228**, 57 (1957).
[570] J. B. Wyngaarden and D. M. Ashton, *Nature* **183**, 747 (1959).

VII. Concluding Remarks

Demonstration of the activity of many agents which alter the synthesis of the nucleic acids was accomplished through tests made with living organisms; however, assignment of a direct role in the metabolism of nucleic acids has required further elucidation of their action on isolated systems and ultimately with purified enzymes. Such an approach, as empirical and perhaps "therapy oriented" as it may seem, has nonetheless made many contributions to our understanding of the normal pathways involved in the over-all synthesis of the nucleic acids. Pertinent examples are to be found in the accumulation, in the presence of certain antimetabolites of folic acid or of glutamine, of intermediates in the biosynthesis of purine nucleotides. Indeed, detailed study of the sites of action of these and other inhibitors has given impetus to the elucidation of the enzymic reaction mechanisms which these inhibitory compounds influence, and even the chemical nature of the active centers of the enzymes affected, since the bonds between the antagonists and the enzymes may be exceedingly firm. Gradually information is being provided which eventually could permit a much more rational design of compounds constructed for the purpose of specific reaction with selected enzymes. To attain such ends it is evident that there is need for expansion of our knowledge of the physicochemical properties of analogs and their corresponding metabolites, and particularly of the active centers of enzymes. Thus, the pKa values of many of the effective pyrimidine antimetabolites are much lower than those of the normal metabolites which they resemble. Unlike the normal compounds, such analogs exist, at physiological pH values, to a significant extent as negatively charged ions, and such modifications of ionic structure must alter markedly the affinity of these compounds for certain enzymes; yet, in some cases, their extensive anabolism to nucleotide derivatives is permitted; in turn, the affinity of the latter for appropriate enzymic loci is distorted markedly.

It remains to be established that the action of a number of the agents discussed is directly on the synthesis or function of nucleic acids. Although many cases may be cited in which these compounds cause alterations in nucleic acid metabolism, such observations do not always permit these effects to be distinguished from corresponding changes in other parameters of cellular function. Similarly, it is easy to confuse a given mechanism of resistance, deduced by studies either *in vivo* or *in vitro*, with the mechanism of action of the compound. This is particularly apparent with such compounds as 5-fluorouracil and 6-azauracil, which are relatively inert until converted to nucleotide derivatives, and which, therefore, must experience several enzymic alterations prior to exerting their primary inhibitory activity. Thus, a major reduction in the efficiency of any one of these enzymic reactions can render the analog inactive, but disclosure of this enzymic de-

ficiency, or of the absence of the product of the reaction, need not necessarily imply that this is the site of action of the antimetabolite or, for that matter, that it represents a reaction essential to the inhibitory activity of the compound.

Another aspect of the study of antimetabolites which requires careful interpretation is the effect of "reversing" agents on growing cell systems. Certain of the possible pitfalls were recognized early in the study of the pyrimidine antimetabolites when nonphysiological compounds such as bromouracil were shown to prevent the inhibitory action of other pyrimidine analogs. Thus, prevention of the inhibitory action of an agent by another chemical is by no means a certain indication that the latter is a natural substrate, the formation or further metabolism of which is blocked by the compound under consideration. Also, data which indicate either competitive or noncompetitive inhibition by an antimetabolite of growth on the corresponding metabolite do not always reflect the actual mechanism of the inhibitory activity of the agent on individual enzymes.

One of the potentially most rewarding areas for further exploration is the importance of multiple pathways leading to certain key intermediates in the biosynthesis of nucleic acids; thus, more than one route to uridylic acid, thymidylic acid, inosinic acid, adenylic acid, guanylic acid, and other nucleotides is either known or suspected, but the factors influencing the relative degrees of operation of these routes are but little understood. Knowledge not only of the homeostatic factors which control these pathways, but also of ways to influence these alternate routes *in vivo*, for example, by means of analogs of the normal homeostatic regulators, will have practical as well as heuristic value.

Finally, it is anticipated that in the next few years much more will be discovered concerning the mechanisms by which macromolecules of nucleic acid participate in protein synthesis and in the transfer of genetic information. This entire area of function, as contrasted to synthesis, has been virtually unexplored from the standpoint of antimetabolites and may in the future represent a most rewarding field of study.

CHAPTER 40

The Effect of Radiations on Nucleic Acid Metabolism

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I. Introduction

There are several reasons why, during the last decade, an increasing amount of work was directed towards the study of radiation effects on nucleic acid metabolism. It has been known for a long time that ionizing radiations produce gene mutations, chromosome damage, inhibition of mitosis, and cell death. Indeed the radiation-induced death of higher organisms is usually attributed to depopulation of certain fast growing tissues, such as bone marrow and intestinal epithelium; and radiotherapy of malignant disease is often thought to be based on the differential radiosensitivity (in terms of cell death) of fast growing tissues as opposed to slow- or nongrowing tissues. As there is good evidence for the nucleic acid nature of genes and chromosomes, and as there is active nucleic acid synthesis in growing tissues, the study of radiation effects on nucleic acid metabolism—in particular of deoxyribonucleic acid (DNA) synthesis—offered possible answers to a number of important problems, the most acute of them being the mechanism of radiation-induced cell death.

However, there is an increasing amount of evidence indicating that gene

mutations, chromosome damage, and inhibition of mitosis are not primarily related to nucleic acid *metabolism*. While it is true that in most diploid cells mitosis does not take place without previous DNA synthesis, it is known that mitosis can be inhibited without affecting DNA synthesis. Also, while it is increasingly evident that chromosome damage is a main cause of radiation-induced cell death it is known that doses of radiations which will not produce a measurable effect on DNA synthesis will cause a significant amount of chromosome damage.

It has been clear for some time that the study of radiation effects on nucleic acid metabolism will not give the direct answers originally hoped for, but it was thought to give some basic information on fundamental processes connected with nucleic acid synthesis. The purpose of this chapter is to investigate the validity of this last assumption.

II. Factors Influencing Deoxyribonucleic Acid Specific Activity

Even a brief survey of literature shows that a great variety of experiments have been performed under the title "effects on nucleic acid (or DNA) metabolism (or synthesis)." It is obvious from the apparent contradictions of certain experimental findings that unless the term "nucleic acid metabolism" is better defined it will remain so broad as to be meaningless.[1-4]

In the following, it will be attempted to define certain changes produced by radiation, which may be reflected in nucleic acid content and specific activity, although be essentially independent from the process of nucleic synthesis.

It may be mentioned here, that there is no good evidence which would indicate that the specific biochemical process of ribonucleic acid (RNA) synthesis is affected by doses of radiations which leave other biochemical cell functions intact. Consequently the work surveyed in this chapter will be concerned with DNA synthesis.

1. Population Changes

Differentiating cells have a finite life span or suffer random destruction (or both), and in a steady state population cell production and cell removal balance each other. Inhibition of mitosis, while not greatly affecting the processes of differentiation, inhibits production thus resulting in a depopu-

[1] G. Hevesy, *Revs. Modern Phys.* **17**, 102 (1945).

[2] A. Howard, *in* "Ciba Foundation Symposium on Ionising Radiations and Cell Metabolism" (G. E. W. Wolstenholme and C. M. O'Connor, eds.), p. 196. Churchill, London, 1956.

[3] L. S. Kelly, *Progr. in Biophys. and Biophys. Chem.* **8**, 144 (1957).

[4] M. G. Ord and L. A. Stocken, *in* "Mechanisms in Radiobiology" (M. Errera and A. Forssberg, eds.), Vol. 1. Academic Press, New York, 1960, in press.

lation. In most organs (e.g. lymph nodes) it will be the transient (i.e. dividing-differentiating) population which will be thus affected, leaving the long-lived stroma reticulum cells behind. The result will be a decrease in nucleic acid content of the organ (compatible with loss of cells), and a decreased uptake of DNA label when measured by the specific activity of DNA (compatible with loss of dividing cells). Loss of small cells (e.g. small lymphocytes) may produce a disproportionately large loss of DNA content compared with the decrease in the wet or dry weight of the organ.

2. Population Shifts

In certain cell populations significant shifts in distribution of cells may occur, even before significant changes in absolute cell counts. In a bone marrow sample for example, most of the cells belong to "late" differentiated forms which are either totally or partially incapable of DNA synthesis and division. The proportion of "early" precursor cells is relatively small, but these are the very cells which multiply and synthesize DNA rapidly.[5] Inhibition of mitosis will allow maturation of these early cells into late forms, thus resulting in a population shift and, consequently, a decreased uptake of DNA label as measured by specific activity determination, without, however, significant change in the total DNA content of the population, or, indeed in the number of cells.

Apart from the shifts produced by mitotic inhibition, interphase cell death may remove actively dividing cells, producing a decreased DNA specific activity. As the death rate of the cells may be faster than their removal rate from the tissue, decreased DNA specific activity may be found without the total DNA content of the tissue being diminished, and, of course, without any effect on the process of DNA synthesis in any surviving cell.

3. Cell Cycle Changes Due to Mitotic Delay

It has been shown that DNA synthesis only occurs during part of interphase.[6, 7] Consequently, in any population of cells the specific radioactivity of DNA will depend on the proportion of cells synthesizing DNA at the time of administration of label, and on the rate at which each cell synthesizes DNA. Inhibition or delay of mitosis, even in an exponentially growing population, may delay or inhibit the onset of the subsequent cell cycle, thus decreasing the proportion of cells synthesizing DNA, without necessarily affecting the process of DNA synthesis in any one cell.[2]

[5] L. G. Lajtha, *in* "Kinetics of Cellular Proliferation" (F. Stohlman, ed.) p. 174. Grune & Stratton, New York, 1959.

[6] A. Howard and S. R. Pelc, *Heredity* **6**, Suppl., p. 261 (1953).

[7] L. G. Lajtha, R. Oliver, and F. Ellis, *Brit. J. Cancer* **8**, 367 (1954).

As RNA synthesis occurs throughout the cell cycle, and indeed, is not inhibited by inhibition of mitosis, no depression of RNA synthesis (or protein synthesis) would be expected either.

It is clear from the examples above, that inhibition of mitosis alone may cause a depression of "DNA synthesis" as measured by the specific activity of DNA. It is also clear that a depression of specific activity of DNA does not necessarily mean that the process of DNA synthesis is in any way affected in any cell remaining alive or not being held up in mitosis after irradiation. Although work at the cellular level (autoradiography of single cells) would avoid most of the above mentioned pitfalls, unfortunately a great bulk of early work involved the use of DNA specific activity as an index of DNA synthesis after irradiation; consequently their results need reinterpretation in light of more recent experiments.

However, the above cited changes, shifts, and delays need a certain time to develop and it has been suggested that if experiments are limited to an observational period not exceeding 2 hours after irradiation, there will not be sufficient time for significant population effects.[4] In short term experiments a depression of DNA specific activity may well measure depression of the biochemical process of DNA synthesis. It should be noted nevertheless, that while a 2-hour period may be too short in a large mammal to register population effects, it may be a borderline time in a small rodent, and too long a time for certain organisms with fast cell cycles.

4. Changes Observed within One Interphase

The scheme of events in respect to DNA synthesis in an average interphase of an average cell may be illustrated in Fig. 1.

It should be noted that the times spent in each period may vary greatly with different cell types. The length of the S period may vary from 6 hours (*Vicia faba*)[6] to 10–12 hours (human bone marrow cells *in vitro*,[7] the length of the G_1 period from being undetectable (Ehrlich ascites cells of the mouse)[8] to over 20 hours (human bone marrow cells *in vitro*),[7] and the G_2 period from less than 2 hours (mouse intestinal epithelium)[9] to about $7\frac{1}{2}$ hours (Ehrlich ascites cells of the mouse).[8]

If short term experiments are being performed, i.e. if the label is administered immediately after irradiation and the sample is obtained within 1–2 hours of labeling, then almost all cells which will show labeling will have been in their S period at the time of irradiation. Such experiments can measure the effect of radiation on the DNA synthetic period (*S effect*). In cells which have a suitably long G_1 period, the label may be administered 10–12 hours after irradiation, by which time all those cells which at the time of

[8] S. Hornsey and A. Howard, *Ann. N. Y. Acad. Sci.* **63**, 915 (1956).
[9] H. Quastler and F. G. Sherman, *Exptl. Cell Research* **17**, 420 (1959).

irradiation were in their S period will have completed it (assuming that the dose of radiation did not hold up or delay cells in the S period). In the subsequent short labeling period (1–2 hours labeling) the cells which will collect the label will have been in their G_1 period at the time of irradiation, and such experiments can measure the radiation effect on the presynthetic period (G_1 *effect*).

In such experiments, however, it is vitally important that there should be no cell death, and/or cell removal, especially selective cell removal. No *in vivo* experiments would satisfy the necessary conditions, as emigration and immigration of cells may occur (apart from efficient local destruction of cells) without being detected. Under such *in vitro* conditions as presented by cell suspensions, however, sufficiently accurate total cell counts and absolute differential counts can be made to exclude such changes.

5. Pool Changes

Irradiations may produce cell death within minutes. Cells in mitosis, and probably in interphase too, may be destroyed (especially with doses of a few thousand roentgens); it is known that even after small doses of whole body irradiation the lymphocyte count drops quickly (which may be the result of trapping or destruction of these cells). Also it is known that nucleases (deoxyribonuclease, ribonuclease) are liberated by irradiation. Although the exact fate of DNA and RNA breakdown products is not known, there is good evidence that there is an efficient recirculation of the purine components[5]; consequently, there is a good possibility for a temporary increase of precursor pool size whenever cell destruction takes place. Especially experiments in which very high specific activity label is used (such as thymidine-H^3 with specific activities ranging from 360 μc./μM to 1.9 mc./μM) the possibility of pool size changes must be considered. The point may be illustrated by the following example: If thymidine-H^3 (360 μc./μM)

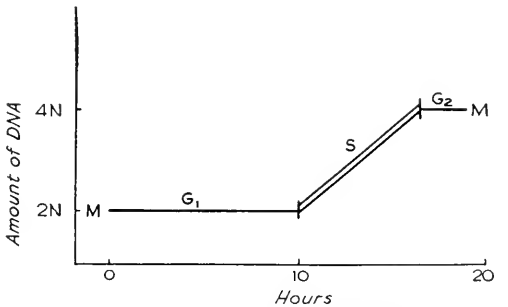

Fig. 1. Scheme of cell cycle with respect to DNA synthesis. S represents the period of DNA synthesis; G_1, the first (presynthetic) gap; G_2, the second (postsynthetic) gap; M, mitosis.

is given to mice in doses of 0.1 μc. per gram body weight, then in a 20-gm. mouse the total amounts to 2 μc., i.e. 5.6×10^{-3} μM. The average diploid cell of the mouse contains about 5×10^{-9} μM thymidine in DNA; therefore, the administered dose of thymidine-H^3 is equivalent to the DNA thymidine content of about 10^6 cells—less than 1 mg. tissue in a 20-gm. mouse. In most experiments where labels with low specific activity (1–10 μc./μM) are used, the amount of material administered is usually large enough to "swamp" natural pools. One is well advised, however, to take into consideration instead of a general pool, individual organ pools, as there may be great variations in this respect between different organs, and indeed between different cells of the same organ.

It is evident from the points mentioned above—as also has been pointed out by excellent previous reviews[2, 3, 4]—that the whole concept of radiosensitivity of DNA synthesis needs critical reappraisal. In the following sections evidence will be examined to establish whether it is legitimate to speak of radiation effects on DNA synthesis, i.e. affecting DNA synthesis while leaving other biochemical cell functions more or less intact.

For the reasons mentioned above only such experiments will be included in this survey where changes within one interphase were investigated. In biochemical experiments (specific activity, DNA content) this means investigations not later than 4 hours after irradiation, in autoradiographic work it may mean a time somewhat shorter than the total intermitotic cycle, i.e. 12–20 hours, depending on the tissue investigated.

III. Radiation Effects within One Interphase

1. Effects in Tumors *in Vivo*

In his pioneer work Hevesy has shown[10, 11, 12] that within about 2 hours of irradiation P^{32} uptake into DNA is depressed in the Jensen sarcoma of the rat. This was confirmed by Holmes[13, 14] who also made the important observation that Nembutal (which was used to sedate the animals during irradiation) in itself is a powerful suppressor of P^{32} incorporation into DNA (and into RNA also), so much so, that it may mask the effects of radiation.

The work was repeated and confirmed in several laboratories, using C^{14} as well as P^{32} as the label, in several types of solid tumors.[15-20] A particularly

[10] H. Euler and G. Hevesy, *Kgl. Dansk Videnskab. Selskab* **17**, 1 (1942).
[11] H. Euler and G. Hevesy, *Arkiv Kemi* **17**(30) (1944).
[12] L. Ahlstrom, H. Euler, and G. Hevesy, *Arkiv Kemi* **19**, (1945).
[13] B. E. Holmes, *Brit. J. Radiol.* **20**, 450 (1947).
[14] B. E. Holmes, *Brit. J. Radiol.* **22**, 487 (1949).
[15] G. Hevesy, *Nature* **163**, 869 (1949).
[16] L. S. Kelly and H. B. Jones, *Proc. Soc. Exptl. Biol. Med.* **74**, 493 (1950).
[17] H. Vermund, C. P. Barnum, R. A. Huseby, and K. W. Stenstrom, *Cancer Research* **13**, 633 (1953).

interesting feature of the findings was that the depression of DNA specific activity rarely exceeded 50%, even with doses up to 9000 r. The variability of experimental conditions does not allow the drawing of a "retrospect" dose response curve based on these experiments, but the observation that in the dose range of 1000–5000 r. the depression remained of the same order of magnitude presented a new problem. A proposition to solve this "50% depression" problem was that radiation depresses DNA synthesis, but not DNA "turnover." However, in the tissues concerned there is no evidence of DNA "turnover" and a more likely explanation of the "50%" phenomenon will be discussed later in this chapter. It is also interesting that in two solid tumors with similar growth rate and mitotic index a significant difference in respect of P^{32} incorporation following 800 r.TBR (total body irradiation) was found: about 20% depression in a mammary carcinoma, and about 50% in a lymphosarcoma—both in the same strain of mice.[18] Holmes, investigating a "radioresistant" and a "radiosensitive" strain of solid Ehrlich carcinoma found a similar depression of P^{32} uptake in both—about 50% $1\frac{1}{2}$ hours after 1500–2000 r.[20]

The ascites tumors appear to be more radioresistant in respect of DNA synthesis than the solid tumors mentioned above. Forssberg and Klein found a depression of about 25% (glycine-C^{14} incorporation into DNA) within the first 2 hours following 1250 r. in mouse Ehrlich ascites cells,[21] Kelly *et al.* found no significant depression of P^{32} incorporation into DNA of their Ehrlich cells in their strain of mice, following 800 r.,[22] and Harrington and Lavik, investigating the effect in different types of ascites tumors found a depression of about 40% in the Ehrlich tetraploid carcinoma and in the 6C3HED lymphosarcoma, but essentially no effect in a diploid Ehrlich tumor and in the TA3 adenocarcinoma.[23] Similarly in the L2 lymphoma, no effect of 300 r. was detected on the P^{32} incorporation.[3]

The apparent discrepancy between net DNA synthesis and glycine-C^{14} incorporation into DNA reported by Forssberg and Klein[21, 24] could be explained perhaps on the basis of timing differences. They found a 66% depression in the rate of net DNA synthesis, but only a 25% depression of glycine-C^{14} incorporation into DNA (Ehrlich ascites, after 1250 r.). How-

[18] L. S. Kelly, J. D. Hirsch, G. Beach, and A. H. Payne, *Radiation Research* **2**, 490 (1955).

[19] R. Backman and E. Harbers, *Biochim. et Biophys. Acta* **16**, 604 (1955).

[20] B. E. Holmes, *in* "Ciba Foundation Symposium on Ionising Radiations and Cell Metabolism" (G. E. W. Wolstenholme and C. M. O'Connor, eds.) p. 225. Churchill, London, 1956.

[21] A. Forssberg and G. Klein, *Exptl. Cell Research* **7**, 480 (1954).

[22] L. S. Kelly, J. D. Hirsch, G. Beach, and N. K. Petrakis, *Proc. Soc. Exptl. Biol. Med.* **94**, 83 (1957).

[23] H. Harrington, D. Rauschkolb, and P. S. Lavik, *Cancer Research* **17**, 34 (1957).

[24] G. Klein and A. Forssberg, *Exptl. Cell Research* **6**, 211 (1954).

ever, the net DNA synthesis was measured in a 50-hour postirradiation period, the first point being at about 2 hours, while the glycine incorporation was measured only for the first 2-hour period, and the reported 25% depression is only the mean figure whereas the actual depression increases from 5.7% at 60 minutes to 44.9% at 135 minutes; thus the discrepancy is not much beyond the experimental error, especially considering that the two experiments were not performed simultaneously.

The general conclusion which can be drawn from the tumor experiments is twofold: First, *in vivo* irradiation can depress P^{32} or C^{14} incorporation into DNA within 2 hours—a period well within one interphase for these cells. The degree of depression varies somewhat with the dose of radiation, but rarely exceeds 50% in the 1000–5000 r. range. Second, it appears that there are differences in radiosensitivity in respect of P^{32} or C^{14} incorporation into DNA between solid and ascites tumors and even between different types of solid or ascites tumors, some ascites tumors not showing significant depression of isotope incorporation into DNA even after 5000 r.

2. Effects in Normal Tissue *in Vivo*

All "radiosensitive" organs—intestinal epithelium, bone marrow, and lymphatic tissues (spleen, thymus, appendix)—have been investigated extensively.

a. Small Intestine

A decrease of ultraviolet absorbing materials (2654 Å.) was reported in the small intestine at 4 hours following 600 r. whole body radiation in rats.[25] Kelly *et al.*[18] found that 300 r. TBR produced a slight, and 800 r. a 50% depression of P^{32} incorporation into DNA of mouse small intestine. Similar observations were made with adenine-C^{14} incorporation[26] with glycine-N^{15} uptake into rat small intestine following 2000 r.,[27] and with thymidine-C^{14} uptake 1 hour after 400–800 r.[28] However, it has been pointed out that within the first 1–2 hours following such doses of radiation there are morphological signs of cell death in the intestinal epithelium.[29, 30] This observation becomes particularly significant if one considers the structure of the tissue sampled[31, 32] in which the cells capable of division and DNA synthesis

[25] J. O. Ely and M. H. Ross, *Cancer Research* **8,** 285 (1948).
[26] E. L. Bennett, L. S. Kelly, and B. Krueckel, *Federation Proc.* **13,** 181 (1954).
[27] J. N. Toal, J. C. Reid, R. B. Williams, and J. White, *J. Natl. Cancer Inst.* **21,** 63 (1958).
[28] O. F. Nygaard and R. L. Potter, *Radiation Research* **10,** 462 (1959).
[29] W. Montagna and J. W. Wilson, *J. Natl. Cancer Inst.* **15,** 1703 (1955).
[30] R. B. Williams, J. N. Toal, J. White, and H. M. Carpenter, *J. Natl. Cancer Inst.* **21,** 17 (1958).
[31] H. Quastler, *Radiation Research* **4,** 303 (1956).
[32] H. Quastler and F. Sherman, unpublished results (1958).

(crypt cells) are in a very small minority compared with mucosal and submucosal cells. If cell death is occurring among the crypt cells—as indeed it does—all the findings could be explained on selective death of cells capable of DNA synthesis, without direct interference with the process of DNA synthesis itself.

b. Bone Marrow

A significant depression of glycine-C^{14} incorporation into rabbit bone marrow following 800 r. TBR was found at 5 hours after irradiation,[33] and of P^{32} incorporation 2 hours after 1000 r. TBR[34]; similarly in mice incorporation of both P^{32} and adenine-C^{14} was depressed after 300 r. TBR.[18, 26]

However, the youngest bone marrow cells—the population with the highest proportion of DNA (and RNA) synthesizing cells—are the most radiosensitive in respect of cell destruction, and within 3 hours their number drops to about 50% of normal.[35, 36] This is in agreement with earlier findings of a 40% drop in DNA content of rabbit bone marrow 3 hours after 1500 r.[37] Consequently, the same criticism as applied to intestinal tissue is valid here: cell population changes alone could explain the experimental findings.

c. Spleen and Thymus

More detailed information is available on the effect of radiation on DNA synthesis in lymphatic tissue, spleen and thymus in particular. Both in rabbit appendix[31] and rat thymus and spleen[38] the incorporation of P^{32} was found to be depressed by 50–70% within 2–3 hours after irradiation (1000 r. abdominal to rabbits, 100 r. TBR to rats). However, Thomson *et al.*[39] have shown in rat thymus, that although 3 hours following 800 r. TBR the DNA P^{32} uptake is depressed by about 50%, by that time also the total DNA content has dropped 15%. As this 15% loss of DNA may have meant selective loss of the most actively growing cells, the situation is rather similar to that with the intestine and bone marrow.

In the spleens of mice receiving 300 r. TBR, P^{32} incorporation into DNA was depressed by about 50%.[18] Kelly, however, points out that changes even within the first 2 hours after irradiation may reflect popula-

[33] R. Abrams, *Arch. Biochem. Biophys.* **30,** 90 (1951).
[34] R. M. S. Smellie, G. F. Humphrey, E. R. M. Kay, and J. N. Davidson, *Biochem. J.* **60,** 177 (1955).
[35] E. V. Hulse, *Brit. J. Haematol.* **3,** 348 (1957).
[36] E. Harris, *Strahlentherapie* **38,** Suppl. III, p. 6 (1957).
[37] C. Lutwak-Mann, *Biochem. J.* **52,** 356 (1952).
[38] L. S. Kelly and E. L. Bennett, *Radiation Research* **5,** 485 (1956).
[39] J. F. Thomson, W. W. Tourtelotte, and M. S. Carttar, *Proc. Soc. Exptl. Biol. Med.* **80,** 268 (1952).

tion changes, as in the mouse spleen the DNA content also drops quickly after irradiation.[3]

Perhaps the most crucial experiment on *in vivo* radiation effects was that made by Ord and Stocken,[40] in which they have shown that within 3 minutes after 1000 r. TBR P^{32} incorporation is depressed by about 50% in rat thymus. The degree of depression was about the same during the first 2 hours following irradiation. The importance of this observation lies in the fact that it is the first attempt to investigate immediate radiation effects on DNA synthesis *in vivo*, and that a depression of DNA specific activity was found. They also made a comprehensive study of P^{32} and glycine-C^{14} incorporation into histone, and of lysine-C^{14} into residual DNA protein, and found that they are all affected at 2 hours after 1000 r. TBR.[41]

d. Resting Liver

Liver is a "radioresistent" organ, in so far as there is very little histological damage detectable shortly after large doses of radiation.[42] Nevertheless Hevesy found a 50% depression of incorporation of C^{14} (from acetate) into DNA 6 hours after exposure of rats to 950 r.[15] Less consistent depressions were found 2 hours after 300–2500 r. using P^{32} incorporation as the index of DNA synthesis.[18] The situation, however, is complicated by the observation of Richmond *et al.*[41] who, while confirming a depression of P^{32} incorporation 2 hours after 1000 r., found no effect of the same dose on the incorporation of glycine-C^{14} into DNA of rat liver. As liver cells are particularly rich in phosphorus compounds, a possible alteration of the local phosphorus pool must be taken into consideration.

To conclude: it is very difficult to collect good evidence for the interference of radiation with the process of DNA synthesis in small intestine, bone marrow, spleen, thymus, and liver *in vivo*, using DNA specific radioactivity as an index. All these organs (with the exception of liver) have a fast cell turnover and show population changes within 1–2 hours after irradiation. The best evidence for an effect on DNA synthesis is the demonstration of a depression of P^{32} incorporation in the thymus 3 minutes after irradiation.[40]

e. Indirect Effects

There are some reports in the literature referring to an indirect effect of radiation on DNA synthesis, i.e. depression of precursor incorporation in shielded parts of the body.[12, 14, 16, 17] However, Holmes has demonstrated[43]

[40] M. G. Ord and L. A. Stocken, *in* "Advances in Radiobiology" (G. de Hevesy, A. G. Forssberg, and J. D. Abbott, eds.), p. 65. Oliver & Boyd, Edinburgh, 1957.

[41] J. E. Richmond, M. G. Ord, and L. A. Stocken, *Biochem. J.* **66,** 123 (1957).

[42] S. Warren, M. W. Holt, and S. C. Sommers, *Am. J. Clin. Pathol.* **22,** 411 (1952).

[43] B. E. Holmes and L. K. Mee, *Brit. J. Radiol.* **30,** 305 (1957).

that even such mild shocks as tying down an animal may affect P^{32} incorporation into DNA. The mechanism of this shock effect is not quite clear, but it is not due to simple pool dilution. In this respect the experiments of Smellie *et al.*[34] are particularly interesting. They found no depression of P^{32} uptake into thymus of rabbits—whose abdominal viscera only were irradiated with 1000 r.—during the first 2 hours after irradiation. However, 1 and 2 days after irradiation the uptake was depressed by about 50%. While this late effect is almost certainly a depopulation effect, it is interesting that it develops so late after irradiation. The interest in heightened by the fact that apparently, in their experiment the animals did not suffer acute shock during irradiation as the 2-hour P^{32} uptake was unaffected in the thymus (excluded from the field of radiation) while the irradiated bone marrow and appendix have shown a 50% depression. This late indirect effect merits further investigation.

Another aspect of indirect radiation effect on DNA synthesis was studied by Harrington and Lavik.[44] They have irradiated mouse Ehrlich ascites cells *in vitro*, and then injected the cells into unirradiated or previously whole body irradiated recipient mice together with some P^{32}. They have measured the depression in P^{32} uptake into DNA compared with that in unirradiated controls and have reported that 1000 r. TBR to the recipient animals, although itself without effect on DNA synthesis in the subsequently injected tumor cells, enhances the effect of *in vitro* radiation in these cells. The number of animals involved in their experiments, however, was not large enough for an experiment of this nature and the work therefore needs confirmation.

3. Effects in Regenerating Liver

Regenerating liver is a very attractive system for the study of radiation effects on DNA synthesis *in vivo*. The tissue behaves in a near synchronized fashion for 30–40 hours both in respect of DNA synthesis and mitosis (Fig. 2).

Consequently, radiation effects well within one cell cycle, and even within parts of the cell cycle can be studied. A great deal of work has, in fact, been done[45-53]; the most detailed information coming from the work of Holmes[49]

[44] H. Harrington and P. S. Lavik, *Cancer Research* **17**, 38 (1957).
[45] J. F. Thomson, M. S. Carttar, and W. W. Tourtelotte, *Radiation Research* **1**, 165 (1954).
[46] B. E. Holmes and L. K. Mee, *Acta Radiol.* Suppl. 116, p. 694 (1954).
[47] B. E. Holmes and L. K. Mee, *in* "Radiobiology Symposium" (Z. M. Bacq and P. Alexander, eds.), p. 220. Butterworths, London, 1955.
[48] L. K. Mee, *in* "Progress in Radiobiology" (J. S. Mitchell, B. E. Holmes, and C. L. Smith, eds.), p. 12. Oliver & Boyd, Edinburgh, 1955.
[49] D. B. Cater, B. E. Holmes, and L. K. Mee, *Acta Radiol.* **46**, 655 (1956).
[50] L. S. Kelly, *Proc. Am. Assoc. Cancer Research* **1**, 24 (1954).

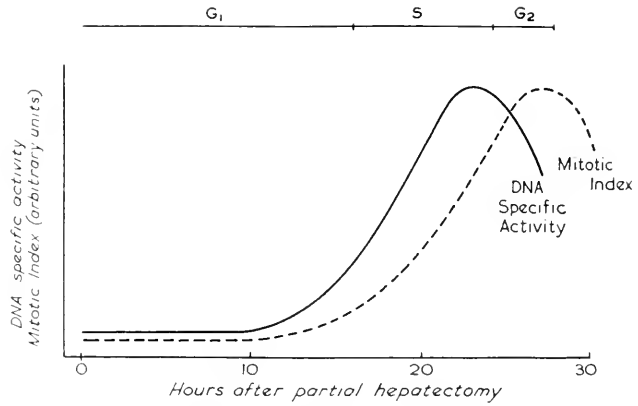

FIG. 2. Scheme of events in regenerating liver.

and Kelly.[51] The essence of the findings is that relatively small doses of radiation (150–450 r.) given within the first 10–12 hours after partial hepatectomy (i.e. before the onset of increased DNA synthesis) delay the onset of DNA synthesis, while once DNA synthesis has begun, much larger doses are required (1000–2000 r.) to produce any measurable depression. The degree of depression in the middle of synthesis following 2200 r. is of the order of 50%.[48] A certain amount of disagreement between different laboratories (as to the degree and optimum time for depression) may be caused by the difference of the time scale of events after hepatectomy in different strains of animals, and also in differences in technique, i.e. surgical partial hepatectomy or carbon tetrachloride poisoning, used to initiate liver regeneration.

Work on regenerating liver was to some extent initiated by the pioneer observation of Howard and Pelc that in bean root cells small doses of radiation may delay the onset of DNA synthesis in individual cells, while not affecting synthesis in cells in which it has already begun.[6] This observation, which has a great significance in understanding radiation effects on DNA synthesis, will be discussed at length later in this chapter.

4. Effects of Irradiation *in Vitro*

It has been held for a long time, that radiosensitivity of tissues, in terms of morphological damage or cell death, is less pronounced under *in vitro* than under *in vivo* conditions. Several factors have been held responsible for this observation, prominently among them is the lower oxygen tension *in vitro* than *in vivo*. This, in most conditions, is undoubtedly true, and the

[51] L. S. Kelly, J. D. Hirsch, G. Beach, and W. Palmer, *Cancer Research* **17,** 117 (1957).
[52] C. D. Jardetsky, C. P. Barnum, and H. Vermund, *J. Biol. Chem.* **222,** 421 (1956).
[53] R. E. Beltz, J. V. Laicker, and R. V. Potter, *Cancer Research* **17,** 688 (1957).

role of oxygen in potentiating radiation effect is well known from the classic work of Gray and his associates.[54] To this, however, another factor should be added: the general natural biochemical milieu, which is rarely, if ever, reproduced *in vitro*. Most freshly explanted cells—if one is permitted to use the term—are not "happy" *in vitro*. For the first few hours after explantation one is usually dealing with a predominantly "unhappy" population of cells; later, by selection and adaptation, new and in many respects different cell lines will develop. Some of these lines may become "happy" even in completely synthetic media, but extrapolation from such cells to *in vivo* cell behavior should be done only with caution.

This is perhaps the reason why, in spite of being a system with simpler parameters, relatively little has been done, until lately, with *in vitro* cell systems in studying radiation effects on DNA synthesis.

The importance of milieu was demonstrated by Ord and Stocken, who have shown that thymus and spleen cells *in vitro* in Krebs Ringer phosphate plus 0.3 *M* glucose show no constant significant depression of P^{32} incorporation into DNA following 2000 r. (*in vivo* in the same experiment 1000 r. produced a 60% depression),[55] however, in a special medium originally made for isolated nuclei[56] they found a 30% depression following 950 r.[57] It is clear that even this medium is not yet ideal, but the demonstration of the importance of choosing the right medium is obvious.

This perhaps explains the lack of any significant depression of P^{32} incorporation *in vitro* after 10,000 r. *in vitro* or *in vivo* in Ehrlich ascites cells as reported by Harrington and Lavik.[44] They kept the cells in their own ascitic fluid during a 3-hour incubation period, but although it was "fortified" with 0.8 mg./ml. glucose, ascites fluid is a very poor medium for these cells *in vitro*. *In vivo* its composition may be kept constant by the host animal, but its reserves are quickly exhausted by the cells, even after 1–2 hours *in vitro*.[58]

In bone marrow of rats, Lutwak-Mann has reported that in short term experiments 10,000–25,000 r. *in vitro* did not affect the nucleic acid phosphorus content[59]; but in view of Trowell's finding on the paradoxical resistance of thymocytes to high doses of radiation,[60] and of a similar tendency

[54] L. H. Gray, *in* "Lectures on the Scientific Basis of Medicine." Athlone Press, London, 1959.

[55] M. G. Ord and L. A. Stocken, *Biochem. J.* **63,** 3 (1956).

[56] D. W. H. Barnes, M. P. Esnouf, and L. A. Stocken, *in* "Advances in Radiobiology" (G. de Hevesy, A. G. Forssberg, and J. D. Abbott, eds.), p. 211. Oliver & Boyd, Edinburgh, 1957.

[57] M. G. Ord and L. A. Stocken, *Biochem. J.* **68,** 410 (1958).

[58] E. Hell and L. G. Lajtha, To be published.

[59] C. Lutwak-Mann, *Biochem. J.* **49,** 300 (1951).

[60] O. A. Trowell, M. J. Corp, and W. R. Lush, *Radiation Research* **7,** 120 (1957).

occurring in bone marrow,[35] her observations are not surprising. In any case in short term experiments *in vitro*, unless large enough doses are given to depolymerize significant amounts of DNA *in situ* (requiring doses of the order of 10^5–10^6 r.), even cell death would not decrease the amount of DNA present.

Investigating formate-C^{14} incorporation into DNA, Totter found a 50% depression in rabbit bone marrow cells *in vitro*,[61] 3 hours after 3000 r. and even greater depressions were reported in cultures of human bone marrow cells after 5000 r., using P^{32} or adenine-C^{14} incorporation as the index of DNA synthesis.[7] These latter investigations, based on autoradiography of cells, indicated a "complete inhibition" of uptake. In view of later experiments it should be emphasized that this was an overstatement. In autoradiographic studies cells showing significant grain counts above background are scored; if the grain counts are not very high then a 50–70% depression of uptake per cell may depress the grain count to near background level and the cells may be scored "negative." Later investigations with better labels (P^{32} is a poor label for any autoradiographs and adenine-C^{14} is not ideal for DNA work as cells have to be treated with ribonuclease prior to autoradiography) indicated that the depression of uptake is in fact of the order of 50% after 2000–5000 r.[62]

5. The Question of Differential Effects of Radiation on DNA Synthesis

Harrington and Lavik suggested that there may be a differential effect of radiation on the incorporation of different precursors into DNA.[63] They have injected P^{32}, orotic acid-C^{14} or formate-C^{14} into rats 30 minutes after 100 r. TBR and isolated DNA from thymus 24 hours later. The incorporation of both P^{32} and orotic acid-C^{14} was depressed by 50–60%, and so was formate-C^{14} incorporation into thymine and guanine. However, adenine-C^{14} incorporation was unaffected, and formate-C^{14} incorporation into adenine showed inconsistent depression.

These experiments were repeated in the Donner laboratory[64, 65] but the findings of Harrington and Lavik were not confirmed. Incorporation of both P^{32} and adenine-C^{14} was depressed by about 40%, under experimental conditions similar to those of Harrington and Lavik.

In chick embryos suspended in saline *in vitro*, Passonneau and Totter

[61] J. R. Totter, *Radiation Research* **1**, 232 (1954).
[62] L. G. Lajtha, R. Oliver, T. Kumatori, and F. Ellis, *Radiation Research* **8**, 1 (1958).
[63] H. Harrington and P. S. Lavik, *Arch. Biochem. Biophys.* **54**, 6 (1955).
[64] E. L. Bennett and B. Krueckel, Univ. Calif. Radiation Lab. Rept. No. 2827/28 (1955).
[65] L. S. Kelly and E. L. Bennett, *Radiation Research* **5**, 485 (1956).

reported an inhibition of uptake of formate-C^{14} and glycine-C^{14} into DNA 2 hours after 2000–5000 r., but the same doses left adenine-C^{14} uptake unaffected.[66, 67] The obvious criticism of their experimental conditions is that it is unlikely that chick embryo cells are "happy" in saline; a fact suggested by the rather poorer uptake of precursors than would be expected under normal growth conditions. This is also signified by the experiments on chick embryos *in vivo* where much smaller doses of radiation (400–450 r.) produced an approximately 50% depression in uptake of both formate-C^{14} and cytidine-C^{14} into DNA.[68]

Similar experiments performed on human bone marrow cells *in vitro* in a more physiological medium indicated the same degree of depression of uptake of both formate-C^{14} and adenine-C^{14} after 1000 r.[69] In these experiments careful consideration was given to the behavior of cells *in vivo* as compared with that *in vitro*, and indeed comparable doses delivered to the sternum *in vivo* gave the same depression of formate-C^{14} uptake as that found after irradiation *in vitro*.[70]

This apparent controversy could, perhaps, be explained on the basis of differences in local pool sizes. Obviously natural pools would be severely altered in dying cells, such as cells in an unsuitable medium. The *in vivo* findings are more difficult to explain but Nygaard and Potter have shown that relative pools for thymidine-C^{14} are greatly different in the thymus, intestine, or spleen—the specific activities of DNA being 2, 4, and 10 times, respectively, that of the liver.[28] With P^{32}, however, the thymus shows at least 6 times the specific activity of liver DNA, even in a young animal with a growing liver,[41] and obviously more than that in the older animals used by Nygaard and Potter. These observations indicate that DNA precursor pools may vary not only from organ to organ, but also that the relative pools for individual precursors may be different in the same organ.

These local pools may vary in different strains of animals under different laboratory conditions, and until they can be measured with reasonable accuracy, differential incorporation studies, and even more, studies of differential radiation effects, are highly inaccurate. At present there is no convincing evidence that radiation acts on any particular step in DNA biosynthesis. On the other hand, as will be discussed in the next section, radiation may interfere with certain basic processes which, under certain conditions, may affect different metabolic functions to different degrees.

[66] J. V. Passonneau and J. R. Totter, *Radiation Research* **1**, 557 (1954).
[67] J. V. Passonneau and J. R. Totter, *Radiation Research* **3**, 304 (1955).
[68] P. S. Lavik and G. W. Buckaloo, *Radiation Research* **1**, 221 (1954).
[69] L. G. Lajtha, *Nature* **180**, 1048 (1957).
[70] L. G. Lajtha, R. Oliver, and F. Ellis, *in* "Advances in Radiobiology" (G. de Hevesy, A. G Forssberg, and J. D. Abbott, eds.), p. 54. Oliver & Boyd, Edinburgh, 1957.

IV. The Analysis of the Mechanism of Radiation Effect on DNA Synthesis

As has been pointed out in Section II and in more detail elsewhere,[71] determination of a mere specific radioactivity is a poor measure of DNA synthesis as population changes and cell cycle changes may be overlooked. For accurate analysis, work at the cellular level is required.

The first important step in this direction was the elegant work of Howard and Pelc[6] who in their autoradiographic study of bean root cells (*Vicia faba*) first established the concept of the cell cycle in respect of DNA synthesis (illustrated in Fig. 1) and have shown that moderate doses of radiation decrease the proportion of cells synthesizing DNA. Their observations were confirmed in human bone marrow cells *in vitro*.[7] They also have shown that within the range of 35–200 r. the depression in the proportion of DNA synthesizing cells is about the same (30–40 per cent), but cells which were in the process of synthesis at the time of irradiation remained unaffected by these doses.[72] The importance of this observation was the establishment of the concept of differential sensitivity in terms of DNA synthesis of cells in different parts of their intermitotic cycle. It was this work which gave impetus to studies on regenerating liver (see Section III, 3) which, being a nearly synchronous population of cells, lent itself for the study of the presynthetic and synthetic periods. Work with regenerating liver also confirmed the findings of Howard and Pelc, and thus the concept was established that relatively small doses of radiation (50–150 r.) delivered before the beginning of the period of DNA synthesis, can delay the onset of synthesis, whereas once synthesis has begun much larger doses are required to interfere significantly with the process (> 1000 r.).

The nature of this "G_1 depression" (as the effect on the presynthetic period was called) was extensively investigated in human bone marrow cells *in vitro*.[62] The conclusion from this work was that small doses (100–200 r.) of radiation slow down the progress of cells through the presynthetic period (G_1 period) with a consequent slower feed into, and depopulation of, the synthetic period (S period) which is unaffected by these doses. It was suggested that there is an indication in the cells for "a system connected with but not identical with DNA synthesis (? trigger mechanism) which is more radiosensitive than the process of DNA synthesis." In this respect the finding of Potter's group is of particular interest: thymidine and thymidylic kinases—enzymes necessary for DNA synthesis, and which are present in regenerating liver at about 18 hours after partial hepatectomy (i.e. at the end of the G_1 period)— are markedly reduced after 375 r. irradia-

[71] L. G. Lajtha, *Nature* **181,** 1609 (1958).
[72] S. R. Pelc and A. Howard, *Radiation Research* **3,** 135 (1955).

tion if the radiation is given 6 hours post heptatectomy (early G_1 period).[73] Once the enzymes have been formed, however, irradiation with 1500 r. has no effect. Apparently a process which normally results in the "triggering off" of such kinases is destroyed or delayed by radiation.

The question emerges: what is this radiosensitive process? In 1942 Mitchell reported an increase in ultraviolet absorbing material in the cytoplasm of irradiated cells,[74, 75] an observation later confirmed by others.[76] This observation, on the face of it, only indicated that RNA synthesis, at least, is not inhibited by radiation (later confirmed in tracer studies) and that in irradiated cells RNA accumulated. Later, an increase in soluble deoxypolynucleotides[77, 78] and soluble deoxyribonucleotides and ribonucleosides[79] was found in irradiated spleen, appendix, and thymus, which could have been the result of either DNA breakdown or accumulation of precursor compounds or both. However, the observation of Ord and Stocken,[80] that there is an increase of deoxyribonucleoside triphosphates in irradiated thymus (compounds very unlikely to result from DNA breakdown) gives a strong indication that synthesis of DNA precursors goes on after irradiation, and that "the inhibition of deoxyribonucleic acid synthesis after irradiation is not due to lack of precursors but rather to failure of the polymerisation or to a breakdown in essential energy-generating mechanisms in the nucleus." In search of such radiosensitive mechanisms Creasey and Stocken found that the intranuclear phosphorylation of purine and pyrimidine monophosphates to triphosphates[81] is a process which is completely and immediately inhibited by 100 r. TBR and depressed by 50–80% even after 25 r.[82]

At the same time an analysis has been made of the mechanism of radiation effect on the process of DNA synthesis (S period). The dose response curve on the rate of DNA synthesis during the process of synthesis gave a polyphasic pattern both in human bone marrow cells *in vitro*[83] and in rat thymus *in vivo*[84] (Fig. 3).

[73] F. J. Bollum, J. Anderegg, A. F. Brumm, and V. R. Potter, personal communication by Dr. V. R. Potter (1958).
[74] J. S. Mitchell, *Brit. J. Exptl. Pathol.* **23,** 285, 296, 309 (1942).
[75] J. S. Mitchell, *Brit. J. Radiol.* **16,** 339 (1943).
[76] M. L. Menten, E. T. Feldsted, and M. Willms, *Exptl. Cell Research* **7,** 83 (1954).
[77] L. J. Cole and M. E. Ellis, *Radiation Research* **7,** 508 (1957).
[78] R. K. Main, L. J. Cole, and M. E. Ellis, *Nature* **180,** 1285 (1957).
[79] C. W. Bishop and J. N. Davidson, *Brit. J. Radiol.* **30,** 367 (1957).
[80] M. G. Ord and L. A. Stocken, *Biochim. et Biophys. Acta* **29,** 201 (1958).
[81] S. Osawa, V. G. Allfrey, and A. E. Mirsky, *J. Gen. Physiol.* **40,** 491 (1957).
[82] W. A. Creasey and L. A. Stocken, *Biochem. J.* **69**(2), 17P (1958).
[83] L. G. Lajtha, R. Oliver, R. Berry, and W. D. Noyes, *Nature* **182,** 1788 (1958).
[84] M. G. Ord and L. A. Stocken, *Nature* **182,** 1787 (1958).

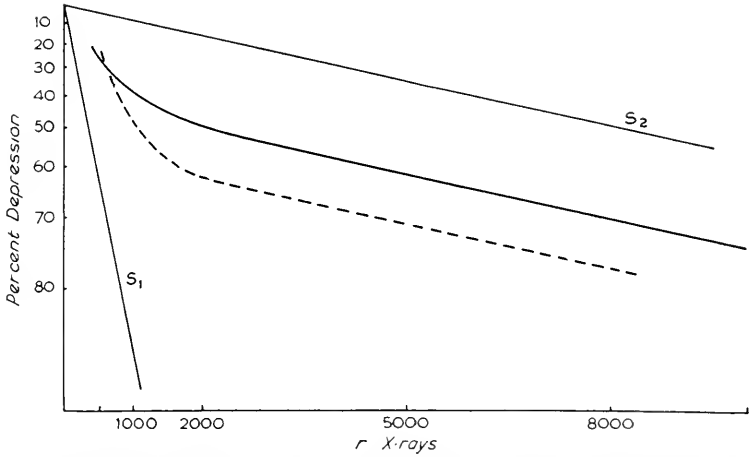

FIG. 3. Dose response curve for X-ray effects on the process of DNA synthesis (S period). Solid line: human bone marrow cells *in vitro* (formate-C^{14} uptake).[83] S_1 and S_2 : slopes of the two main components of the curve. Dotted line: rat thymus *in vivo* (P^{32} uptake).[84] (Note that for the thymus experiment the dose scale at any point is 1/5 of that for human bone marrow.)

The results in bone marrow cells indicated that even after 2000–5000 r. *in vitro*, DNA synthesis proceeds in cells which at the time of irradiation were in the process of synthesis (S period) but at approximately half the normal rate. The shallow slope of the curve after 2000 r. perhaps explains the curious 50% inhibition found by so many workers. It was concluded from these observations that the rate of DNA synthesis (defined as rate of precursor uptake during the S period) depends on at least two factors: S_1 and S_2. Of these factors, S_1 is obviously much more radiosensitive than S_2 (the 37% dose being 500 r. for S_1 and 13,000 r. for S_2 in bone marrow cells *in vitro*). In the cells so far investigated the S_1 factor is responsible for about 50% of the rate of synthesis, consequently if it is eliminated by radiation, a 50% depression in precursor uptake—as usually found—would be expected. There may be cell types, however, in which the S_1 factor is responsible for a smaller or larger share in the rate of DNA synthesis; consequently, the more resistant S_2 curve may start at a 15 or 75% depression.[83]

Obviously the S_1 and S_2 factors are, at present, mere postulates. So far, investigations on the relative biological efficiency of X-rays and α-particles indicate an S_2 "vulnerable volume" (under these conditions a better term than "target") rather similar to the DNA molecule, and the S_2 effect indeed may be the result of direct gradual destruction of the DNA matrix by increasing doses of radiation.[83] As to the nature of the S_1 factor, it is very tempting to correlate it with the radiosensitive triphosphorylation process.[82, 84]

To conclude: there is some evidence which may explain, at least in general terms, the mechanism of radiation effect on DNA synthesis within one interphase (mitotic inhibition excluded).

According to this evidence the following scheme may be put forward: intranuclear triphosphorylation—a very radiosensitive function—is responsible for the normal rate of processes during the presynthetic period (G_1 period), and also for the normal rate of DNA synthesis during the synthetic period (S period). If this function is destroyed, both the G_1 "progress" and the rate of DNA synthesis will be depressed. In most cells so far investigated it is responsible for about 50% of the processes involved.

It should be emphasized that not all cells may behave in a similar fashion. In particular, long term tissue culture cells capable of sustained growth in semisynthetic or fully synthetic media may be rather special exceptions; their G_1 "progress" may not depend on this nuclear process, as indicated by observations on HeLa S_3 cells,[85] although even in these cells some of the findings could be explained on the basis of a sensitive S_1 factor. In cells which do not depend greatly on this radiosensitive function, DNA synthesis may not be delayed greatly, as indeed is indicated by studies with NCTC strain L fibroblast.[86] The dose of radiation employed in both these experiments was 500 r. and 800 r., respectively—doses not likely to affect the S_2 factor (? DNA matrix) significantly.

There may also be some cell types or experimental conditions in which there is no observable S_1 effect. This may be due to intrinsic absence of the S_1 factor in the cells, or loss of it under the particular experimental conditions. Ehrlich ascites tumor cells appear to be an example for the latter.[58]

V. General Conclusion

As was pointed out in the introduction, the study of radiation effects on nucleic acid metabolism was, to a great extent, initiated in the hope that radiation induced disturbances in nucleic acid metabolism might give the explanation to the primary lethal actions of radiations in the cell.

It has been indicated from the foregoing that is is not the case. First, most (although not all) primary lethal actions of radiations are due to inhibition of mitosis, chromosome damage, or gene mutations. These can be produced by smaller doses of radiation than would measurably affect the process of DNA synthesis. Second, radiation induced changes in DNA synthesis are themselves, to a great extent, results of more sensitive underlying biochemical processes, and, contrary to early beliefs, the process of DNA synthesis itself is rather radioresistant.

Nevertheless, radiation has proved to be an extremely useful tool for the

[85] R. B. Painter and J. S. Robertson, *Radiation Research*, in press (1959).
[86] M. Dickson, J. Paul, and J. N. Davidson, *Biochem. J.* **70**(4), 18P (1958).

study of fundamental events in the cell; events which, in turn, may lead to the understanding of the cellular mechanism of radiation effects.

The scheme put forward in this chapter, in an attempt to explain radiation effects on DNA synthesis, is merely a working hypothesis; experiments to test its validity are in progress. However, as a commendable principle it may be opportune here to quote for those engaged in this type of research the concluding remarks of a review written in 1953, that "... no finding, whether biochemical, physiological or pathological, be accepted as primarily due to radiation unless it is established that it does not arise as a result of some other or earlier lesion."[87]

[87] M. G. Ord and L. A. Stocken, *Physiol. Revs.* **33**, 356 (1953).

Author Index

Figures in parentheses are reference numbers. They are included to assist the reader in locating a reference when a work is cited, but the authors' name is not mentioned.

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