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# THE VIRUSES

Volume 1

GENERAL VIROLOGY



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# THE VIRUSES

Biochemical, Biological, and Biophysical Properties

*Edited by*

F. M. BURNET

*The Walter and Eliza Hall  
Institute of Medical Research  
Melbourne, Australia*

W. M. STANLEY

*Virus Laboratory  
University of California  
Berkeley, California*

Volume 1

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### Contributors to Volume 1

- G. H. BERGOLD, Laboratory of Insect Pathology, Sault Ste. Marie, Ontario, Canada
- F. M. BURNET, Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria, Australia
- SEYMOUR S. COHEN, The Departments of Biochemistry and Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania
- E. A. EVANS, JR., Department of Biochemistry, University of Chicago, Chicago, Illinois
- H. FRAENKEL-CONRAT, Virus Laboratory, University of California, Berkeley, California
- S. GARD, Karolinska Institutet Medical School, Stockholm, Sweden
- S. E. LURIA, Department of Bacteriology, University of Illinois, Urbana, Illinois
- O. MAALØE, University Institute of Microbiology, Copenhagen, Denmark
- HOWARD K. SCHACHMAN, Virus Laboratory, University of California, Berkeley, California
- WERNER SCHÄFER, Max-Planck-Institut für Virusforschung, Tübingen, Germany
- CARLTON E. SCHWERDT, Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California
- W. M. STANLEY, Virus Laboratory, University of California, Berkeley, California
- ROBLEY C. WILLIAMS, Virus Laboratory, University of California, Berkeley, California



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## Preface

Experimental biology at the present time is approaching a particularly interesting stage. It is probably true to say that in recent years biochemical concepts have dominated all general thinking on the fundamental problems of biology. There has been an uninterrupted succession of important discoveries and there is as yet no sign of any drying up of the flow of discovery. It is orthodox to believe that the way is now open to a comprehensive understanding of the basic living process in terms of biochemistry, with perhaps only an inspired continuation of progress along present lines being necessary. There are some, however, who are more impressed with the mounting difficulties of applying the methodology of chemistry to the complex macromolecules of living systems and their interactions than with the inevitability of their being overcome.

Clearly the crux of biological thought today is the applicability of chemical and physical approaches to the typical macromolecules, protein and nucleic acid, of living substance. In this context virology seems to occupy a key position among the biological sciences. Viruses are the smallest biological units which manifest all the essential characteristics of life and many are now known to be built up only of nucleic acid and protein. With the development of new biochemical techniques useful in attacking the problems of macromolecular structure viruses have become the material *par excellence* for fundamental study. The very great discovery that nucleic acid preparations possessing virus activity can be obtained from virus-infected tissues and from pure viral nucleoproteins has focused attention on nucleic acid as the key material in virus activity, in genetic activity, and in the synthesis of proteins and of nucleic acids. It would appear, therefore, that nucleic acid structures contain the codes for the fabrication of every individual of every species. Since some viral nucleic acid preparations can be obtained quite pure, chemically as well as genetically, in lots of hundreds of milligrams, it is obvious that the viral nucleic acids offer an especially favorable and perhaps unique possibility of breaking the code and of approaching the synthesis of a replicating structure. These represent great challenges in virology and are, of course, of the greatest importance to science and to mankind.

There have been many systematic compilations of knowledge on viruses as agents of disease in man, in animals, or in plants. To our knowledge, however, the present work is the first to be published in English in which a systematic attempt is made to cover the significance of experimental work on viruses for general problems within and on the borderlines of biochemistry, biology, and biophysics. Since it would be impossible for one or two persons

to write authoritatively about all of the important aspects of virology, it has been necessary to seek the assistance of experts in different areas of virology. The editors have been quite fortunate in securing contributions from 34 of the leading virologists in 8 different countries. Almost two-thirds of the contributors are from American laboratories and this mirrors with reasonable accuracy the relative activity in virology during the past several years. However, there is presently a great upsurge of research activity in virology in several countries and significant new information is now almost world-wide with respect to source.

The plan of "The Viruses" has deliberately followed that of the works on "The Proteins," "The Nucleic Acids," and "The Enzymes" published also by Academic Press. In this treatise on "The Viruses" we are concerned essentially with the chemical and physical characteristics of viruses and with the processes associated with their multiplication in the cell. In general we are not concerned with manifestations of viral infection in multicellular organisms or in populations of susceptible hosts except insofar as they provide information about processes at the cellular level. It is manifestly impossible to treat comprehensively of viruses without consideration of their behavior at the genetic level. Particularly with the bacterial viruses there is now much information on record on recombination between viruses and on interaction between the genomes of virus and host cell. In this region we may well find the material from which will come eventually an understanding of the relationships between the genetic and chemical approaches. The discovery of infectious nucleic acid preparations represents a major start in this direction.

It would not be realistic to separate the academic approach to virology sharply from the clinical. In the last analysis the prevention or cure of virus disease will depend on properties of virus and host cell. In the past, success in control has depended almost exclusively on the use of procedures at the immunological or epidemiological levels, but if these are to be refined and fully understood much use will have to be made of the information provided by the essentially theoretical studies which the present work has been designed to systematize and display. Consider, for example, the new immunological problems that one may encounter if, as may emerge, infectious nucleic acid moves directly from cell to cell. This treatise provides the information and the interpretation of this information that will be necessary for a rational experimental approach to such new problems.

One of the main difficulties the editors have encountered has been one inherent in all attempts at biological generalization, namely, the diversity of the material that is available for study and the widely varying intensity and success with which different sections of that material have been studied. It will be found, for instance, that a large proportion of each of the three

volumes is concerned with the properties of one plant virus (TMV), one bacterial virus (T2) and three animal viruses (vaccinia, influenza A, and poliomyelitis viruses). There are large numbers of other viruses in each of the main categories and undoubtedly many show or will show highly significant differences in behavior from those which for one reason or another have been chosen as prototypes. Perhaps one of the important functions of the work will be to show where such significant differences can most hopefully be looked for. Clearly there is no lack of scope for further work in virology.

*Melbourne, Australia*

F. M. BURNET

*Berkeley, California*

WENDELL M. STANLEY

*December, 1958*



## Chapter I

### The Problems of Virology

F. M. BURNET AND W. M. STANLEY

*Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria,  
Australia*

*and*

*Virus Laboratory, University of California, Berkeley, California*

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#### I. INTRODUCTION

The present work was designed to provide a relatively comprehensive account of current knowledge of viruses regarded, not as agents of disease, but as biological entities whose properties can be studied in the laboratory by the methods of experimental biochemistry, biology, and biophysics. We have interpreted this as broadly limiting our topics to the chemical, physical and, where relevant, genetic structure of the infective units and to what is known of the interaction between virus and the cell it infects. Except where it is necessary to allow definition of some intrinsic quality of a virus, no consideration is given to the pathogenesis of virus diseases nor to clinical, epidemiological, or ecological aspects of such diseases.

Even with this limitation, the field to be covered is enormous and peculiarly difficult to organize satisfactorily. The first difficulty concerns the range and diversity of viruses. If we confine attention to the infective form, we have units ranging in complexity from a relatively simple system of association between two types of macromolecule (tobacco mosaic virus) to large units of complex composition which are more nearly like organisms (vaccinia and psittacosis viruses). Some, such as the smaller plant and animal viruses, possess RNA (ribonucleic acid) only, the bacterial viruses contain DNA (deoxyribonucleic acid) in one group of a unique chemical structure, and some of the larger animal viruses may be found to contain both types of nucleic acid. No direct evidence can be obtained of the ways by which viruses have evolved. It is doubtful whether any biologist would even suggest that all types of virus had a remote common ancestor. Many have suggested, on the other hand, that among the viruses we include a widely heterogeneous group of many different evolutionary origins, the single common feature

being their adoption of the role of strict intracellular parasite. On this view we may find that any treatise on general virology will eventually be reduced to a discussion of the ways by which a living cell can have its metabolic activities diverted to allow the replication of material of alien pattern. As a matter of fact, we have given considerable space to this general area, as may be seen from an examination of the following chapter in this volume. It remains necessary, however, to attempt to discuss at least representative examples of each main group of viruses.

This leads to the second difficulty—the unevenness with which topics bearing on general aspects of virology have been dealt with in the different groups. Following what Sir Charles Harington (1957) has characterized as “the intellectual shock that was administered” to science by the discovery of crystalline tobacco mosaic virus more than 20 years ago, there occurred a great surge of activity in virology. There has, for instance, been a great concentration of biochemical and biophysical work on TMV (tobacco mosaic virus), a moderate amount on some of the other “macromolecular” plant viruses, and virtually none on the larger and less stable plant viruses. Similar work on the animal viruses has lagged behind that on the plant viruses. Details of the process of infection and of the interference by virus with the metabolism of the host are known in much more detail about bacterial viruses, particularly T2 with *Escherichia coli* B as host, than about any other combination. Genetic work is almost limited to a few bacterial viruses and influenza A virus. It is already clear that generalizations about virus behavior cannot safely be drawn on the basis of one or a few well-studied examples. We have, in fact, considerable sympathy with one or two potential collaborators who declined the task on the basis that the time was not yet ripe to undertake a general treatise on viruses. They felt that the study of the bacterial viruses had perhaps gone far enough to give a reasonably clear picture of the process of infection and that attempts to provide a comprehensive description of the processes involved in infection by representative animal, plant, and insect viruses should be deferred until knowledge of these had reached much the same level of completeness as exists for bacterial viruses. The reason for going on with the project is simply our belief that it is of the very nature of science that knowledge must always be incomplete and that the present is a better time to attempt our task than 5, 10, or 20 years ago. We believe, too, that the same two remarks will be made with equal cogency at any time in the foreseeable future.

## II. THE DOUBLE APPROACH

### *A. The Infective Particle*

Virus research is a segment of experimental biology and inevitably encounters the same methodological and interpretative difficulties that beset

every phase of fundamental biology. It is axiomatic that all available techniques are being, and must be, used to define those qualities of viruses which are capable of being usefully expressed in terms of physical and chemical concepts. This approach is directly applicable to the specification of the infective units. There is now such a wide range of chemical and physical techniques for the purification or isolation of molecules, macromolecules, and larger aggregates that the obtaining of smaller or larger quantities of "pure" virus is no longer the formidable task that it was only a few years ago. As in any other type of biochemical work, perhaps the most important single factor is a reasonably convenient means for the bioassay of fractions. A large enough supply of crude starting material and facilities for carrying out the necessary manipulations are, of course, necessary. For most viruses the ultracentrifuge is probably the most important tool for purification but will always need to be assisted by other types of manipulation. Where small viruses must be separated from particular fractions of host cells of similar size and density, the problem of purification may be very difficult and may require the use of all the empirical artifices that the ingenious chemist can devise. The progress of purification will normally be followed by assessing the biological activity in relation to the amount of material in the fraction (measured as dry weight, N content, or in some other fashion). Where viruses have biological activity other than their power to infect and cause demonstrable lesions, this is often more convenient to use in combined chemical-biological work. Influenza viruses will act as hemagglutinins, and many types of virus can be detected and titrated by complement fixation tests with appropriate antisera. In all cases it must eventually be established that the property chosen is one specific for the infective particle and not, for example, associated with some nonviral product of infection, such as a soluble hemagglutinin or complement-fixing antigen.

With purified material it is possible to use a variety of physical methods to define size, shape, and density of the infective units and, if necessary, to elaborate in regard to electrostatic charge, electron density of different regions, and adsorption to various types of surface. Some of the physical methods are applicable to unpurified virus preparations; the first reliable estimate of the range of sizes presented by bacteriophages and animal viruses was obtained by Elford's use of graded collodion membranes in filtration experiments with relatively crude materials (Elford, 1932).

Many of the smaller plant viruses and two of the animal viruses have now been crystallized or at least been shown to take up regular two-dimensional arrays. It is doubtful whether this means more than that the virus particles are nearly or completely uniform in size and composition and are geometrically capable of regular packing. The possibility of crystallization, however, does open up an important new approach to deeper understanding of virus

structure, since it allows the application of the method of X-ray crystal analysis. This has had its most striking success in regard to tobacco mosaic virus, for which Franklin's structure, based almost wholly on X-ray diffraction, is now generally accepted (Franklin, 1955; Franklin *et al.*, 1957). It should perhaps be stressed that the accepted structure is simply a description of how two types of complex macromolecules are associated to produce the hollow rods seen in electron micrographs.

Although they have not been studied as intensively as tobacco mosaic virus, some of the "spherical" plant viruses, such as tomato bushy stunt virus and turnip yellow virus, appear also to be simple geometrical arrangements of protein and nucleic acid macromolecules. Crick and Watson (1956) have discussed the theoretical aspects of symmetry in such structures and Caspar's (1956) X-ray diffraction studies have indicated twofold and threefold symmetry axes in bushy stunt virus. Kaesberg (1956), from an examination of the shadows of heavily shadowed electron microscopic preparations, believes that these viruses are probably very close approaches to the regular icosahedron, a figure bounded by 20 equilateral triangles and with 12 corners. Caspar has suggested that there may be 60 subunits, a group of 5 forming each of the corners.

The physical study of purified virus has always and necessarily been associated with a chemical approach at whatever level was available at the time. More than in any other aspect of virus work the range of viruses that have been chemically studied is very small and there may well be surprises in store when this is extended. The adenoviruses, with their curious type of crystal structure within the host cell, and the larger less stable plant viruses are two groups in which unexpected findings may emerge. However, it is probable that what has been determined for the few prototype viruses, tobacco mosaic and bushy stunt, phage T2, vaccinia, influenza A, and polio, is broadly applicable in each case to a wide range of viruses of much the same general quality. Perhaps the most important generalization that has emerged is that all viruses contain nucleic acid and protein—the smaller animal and plant viruses probably containing only these components. The remarkable and very important work of Fraenkel-Conrat (1956) and of Gierer and Schramm (1956) on infectious nucleic acid from tobacco mosaic virus nucleoprotein indicates that the nucleic acids are the essential elements in the sense of being the carriers of the genetic codes by which new virus is reconstructed. There seems now to be no alternative to an acceptance of this view for tobacco mosaic virus and there are only minor reservations in regard to the bacteriophage T2. Preliminary work on the smaller animal viruses, while not yet advanced to the point of certainty, seems to be pointing in the same direction. The new concept of infectious nucleic acid seems destined to become of the greatest importance to virology. Influenza and related viruses

have been extensively studied but suffer from the disadvantage that the ribonucleic acid present is only about 1 % of the dry weight of the infective particle. In addition, the virus particle is a rather loosely organized entity which appears to contain relatively large amounts of host cell material whose precise relation to the specifically viral components is still not established. There is nothing inconsistent with the view that RNA is the carrier of the genetic qualities of influenza virus but equally there is nothing to exclude the possibility that viral protein may be partly or wholly concerned in providing the patterns to which the new generation of virus particles is built.

Possibly the most important question for the future development of theoretical virology is the direction in which research on nucleic acids will develop. The methods by which the proximate constituents, purine and pyrimidine bases and sugars, can be identified and quantitated are well established. Where pure virus has been obtainable these quantities have been recorded. Perhaps the most interesting findings have been (1) the identification of hydroxymethylcytosine and glucose in the DNA of the T-even bacteriophages by Wyatt and Cohen (1953); (2) the finding of a significant difference in the bases present in viral RNA from A and B strains of influenza virus (Ada and Perry, 1956); and (3) the observation by Reddi (1957) that analysis of RNAase (ribonuclease) digests can demonstrate significant differences in the structure of nucleic acids from different strains of TMV. The next step will probably be to develop methods analogous to those that have been used in protein chemistry with the ultimate objective of establishing the sequence of bases in the polynucleotide. The technical difficulties are extreme. In the first place it is necessary to establish whether or not the nucleic acid in a virus particle is in the form (a) of a single nucleotide structure (single chain, double helix, or something else) of essentially unique character, (b) of a series of 1, 2, or more types of nucleotide structures, each present in certain constant or variable numbers. In other words, if the nucleic acid could be extracted from a mass of purified virus essentially in the form in which it is present in the infective particle, how many molecular species would be found? Once means have been found for obtaining each natural molecular species in a pure form, it will obviously be necessary to establish the general pattern of structure. In the case of tobacco mosaic virus, the preliminary evidence favors a single polynucleotide chain following the helical pattern of the protein subunits. In the case of a DNA virus, it will be necessary to determine whether the Watson-Crick formulation applies and what qualifications need to be introduced in each specific case. The recent work of Levinthal (1956) and Stent and associates (Stent and Jerne, 1955; Stent *et al.*, 1958) with heavily labeled phage represents a current approach in this direction, making use, however, of the biological attributes of DNA to allow chemical deductions, rather than to use chemical and physical methods to

obtain a direct solution. The final step will be to build up a detailed account of the sequence of nucleotides (and any other minor components which may emerge from future work) that build up each type of macromolecule. At the present time such a program may appear to be out of the question but recent success in elucidating the amino acid sequence of some of the simpler proteins suggests that another decade may well see the publication of what we should now regard as a full chemical description of the protein component and perhaps also of the nucleic acid of tobacco mosaic virus.

If insulin has been the protein par excellence for the unraveling of a structure which can serve as a model of other proteins of greater biological significance, so we can feel confident that the logical choice for nucleic acid is the RNA of tobacco mosaic virus or possibly that of one of the small spherical viruses, such as tomato bushy stunt or turnip yellow mosaic. The current hypothesis that in some way each type of RNA must carry the "information" eventually expressed in the structure of the protein associated with it may be qualified and elaborated, but it is inconceivable that there is no significant relationship between the two types of macromolecule. Tobacco mosaic or some other readily available plant virus has the enormous advantage of providing a system with, as far as we know, one species each of protein and of RNA in association. The fact that the RNA can induce the formation of additional molecules of its own kind and can in addition induce the formation of a highly specific protein with which the RNA eventually combines offers a direct avenue of study of the synthesis of polynucleotides and polypeptides and of their interrelationships. If the chemical significance of the general association of protein and RNA is ever to be elucidated, here is the least complex system we are likely to find for its study. Investigators at the Virus Laboratory in Berkeley have already launched a long-range program of study of this system. A substantial part of the amino acid sequence of the 164 amino acids comprising the protein subunit of TMV has already been worked out and comparisons with the amino acid sequence of TMV strains have revealed significant differences (Gish *et al.*, 1958; Ramachandran and Gish, 1958; Niu and Fraenkel-Conrat, 1955; see Knight, Chapter 3 of Volume II). Work on the polynucleotides of TMV and certain of its strains has yielded indications of structural differences (Reddi, 1958). Eventually a point-to-point structural relationship between polynucleotide and the polypeptide it induces may be revealed.

If, as we believe, the nucleic acids are the essential carriers of all genetic codes in the smaller viruses, we can adopt an analogy with the old view that the mammalian body is a mere temporary carrier of the potentially eternal "germ plasm." It is the nucleic acid of the virus which must be brought into an appropriate environment where it can replicate its pattern. The rest of the virus is mere machinery to allow this to be accomplished. This at least is

a convenient way of generalizing the situation until increasing knowledge allows a more subtle appreciation. In the case of tobacco mosaic virus, the specific viral protein seems to have the quality of building up a helical structure to enclose ribonucleic acid threads of almost any type, even the semisynthetic polynucleotides produced by Ochoa's methods (Hart and Smith, 1956). However, such products are far less stable than the structures formed with homologous protein and nucleic acid. Although it seems unlikely, it may be that the only functionally important character of TMV protein is its geometrical form and what other attributes are necessary to allow orderly aggregation into the characteristic hollow helices. If this is so, less may be obtained from strictly chemical studies of amino acid content and sequence than from physical and topological analysis of the secondary coiling and organization of the polypeptide chain. It is still open, however, that the protein surface of a plant virus has a specific function in facilitating entry of virus into cells not previously infected. In the case of the bacterial viruses, the organization of the protein coat seems to be much more elaborate than in a typical plant virus and a number of functionally distinct regions are recognized. It is now generally accepted that the tip of the tail is a highly specialized structure the terminal fibers of which serve as a specific anchoring mechanism while another portion is exposed or activated so as to act enzymatically on components of the bacterial wall. There are hints that the smaller animal viruses may have components, presumably protein, with specific adsorptive qualities for the cells they infect. The polio-like virus GD V11 agglutinates human red cells. Theiler's virus is inhibited by a polysaccharide from mouse intestine. These may be accidental findings of no biological significance, but they point in a direction where further study may be fruitful.

The larger viruses are clearly beyond the macromolecular level and for the present at least must be regarded as primitive organisms. Chemical study will therefore be limited to the analysis of proximate constituents without any serious hope of providing a meaningful picture of the virus particle at the purely chemical level. Basically the same methodology must be applied as to the morphological and chemical study of any small microorganisms. In the larger virus types there will automatically be greater opportunities for relatively unmodified host materials to be present in the particles. It is probably too naive to use terms like "pure virus" or "contamination with host material" at this level. If one view of the structure of influenza virus is correct, essentially unmodified host components form a very significant part of the virus surface. In each case the nature of components which are not identifiable as of specifically viral quality will have to be judged on what biological and other evidence is available.

The question of enzymes in relation to viral particles has been widely discussed. Only in the influenza and related viruses of the myxovirus group has

the position been resolved by the recognition of neuraminidase as an essential component. Here the problem was simplified by two factors: (1) that the substrate of the enzyme has a functional relationship to the entry of the virus particle into its host cell and (2) the absence of any enzyme of similar action in host tissues. Where an enzyme well-known to be present in host tissues is shown to be present in or on conventionally purified virus particles, an almost insoluble problem of interpretation is presented. A good example of this difficulty is discussed in relation to the virus of erythroblastosis by Andervont in Volume III. Recent work on the attachment of bacterial viruses to their hosts points strongly to the existence of enzymatically active groups at the end of the tail. There are, however, only preliminary indications of the nature of the enzyme(s) or substrate(s) concerned.

### *B. The Virus-Host Cell Relationship*

The characters of the infective virus particle represent only a minor part of the significant information to be gained from the laboratory aspects of virology. The interactions of the virus and host cell, which are of the utmost importance insofar as the true expression of viral activity is concerned, are, however, much more difficult to observe and interpret than the physical and chemical qualities of the infective particles. For example, there are viruses which cause cancer in several kinds of animals, yet almost nothing is known concerning the mode by which a virus can urge cells toward wild and unorganized growth. This problem and its possible relationship to the human cancer problem have been discussed by one of us (Stanley, 1957, 1958). The difficulties are common to all aspects of intracellular function and, in fact, provide an important area of controversy in regard to the objectives of biological investigation and the form and legitimacy of generalization. In most technical publications in virology it is sufficient to conform to the accepted canons of scientific investigation without expressed question as to the basis of the conventional approach. It seems to us, however, that in a general treatise some attempt should be made to discuss somewhere not specific interpretations and hypotheses but the type of ideas in terms of which general statements may appropriately be made. This demands a brief discussion of the objectives of scientific work in the biological field.

In general, biological research has two immediate motivations. Usually in the background, but sometimes dominant, is the objective of providing ways by which felt and expressed human needs may be satisfied. The more important motivation of scientific work can perhaps be expressed as *to experience the various emotional and intellectual satisfactions and social rewards of effective work within the accepted canons of scientific investigation*. As a result of three centuries of modern science, a body of experimental and logical procedures has been developed. This will inevitably change with increasing

knowledge and there will be variations in its application in different fields of science, but in general and at the level of normal laboratory thought, the important principles may be stated as follows:

1. To determine the conditions under which an observed phenomenon can be reproduced with regularity.
2. To define the parameters which determine its quantitative expression.
3. To express its relation to other phenomena in the simplest acceptable form.
4. On the basis of these results and deductions, to examine whether new phenomena occur as would be expected in other relevant fields.

In a general treatise, it is conventional and necessary to exclude description of the technical detail needed in every experimental manipulation to ensure reproducibility of results. Quantitative results will also be limited to an occasional illustrative protocol. With some obvious qualifications, the essential task of a comprehensive treatise is to express in the simplest acceptable terms the relationship between the phenomena observed within the chosen field of study.

There can be no question that the most acceptable form of simplification is the generalized mathematical expression that, within stated limitations, allows a quantitative statement of the expression of the phenomenon. Even in the most exact of the experimental sciences any statement of this type will need to be qualified by some indication of the range of variability introduced by inadequacies in the technique of observation. In biological phenomena this tends to be so large that there is still a grave distrust among experimentalists of attempts to derive a mathematical relationship directly from a set of observations. The other procedure—to derive a working hypothesis from the general pattern of results and to show that the results are not inconsistent with the quantitative implications of the hypothesis—is more usual. Except at a rather superficial level, even this is rarely attempted in virology, where it has not often been possible to isolate systems from disturbing environmental and intrinsic circumstances sufficiently to allow quantitative reproducibility. Even in the reproduction of bacteriophage, individual infected units show a wide range of yield and time of lysis. The destruction of the functional activity of a virus population by such accurately measurable agents as heat, various types of ionizing radiation and, with some qualification, immune serum, is sufficiently regular to allow a satisfactory quantitative formulation. Outside of this special and limited field there has been little success in providing quantitative formulations of virus behavior. Virtually all current work on the process of viral infection is in a sense quantitative, but the characteristic statement of the results is in the form of a scatter diagram sufficiently consistent to indicate a qualitatively significant regularity but quite unsuited for any more refined mathematical handling.

For many of the most humanly important aspects of virology we must be content at present, and perhaps for many years, with providing concepts that have provisional value in making it easier for students of the phenomena to predict the result of changing circumstances. Some, but by no means all, of these concepts can in principle be expressed quantitatively and the general objective will be to bring more and more phenomena into a quantitative mold.

Probably all experimental biologists will agree that this is the most desirable approach and, to a very considerable extent, the topics chosen for study are chosen because it seems likely that they will be amenable to quantitative study. Many biologists will, however, suffer serious doubts about the adequacy of such a view and even experience at times a sense of the futility of a vast amount of quantitative biological research. A small amount of virus is inoculated into a susceptible system—mouse brain, allantoic cavity, or tissue culture. In some hours' time there is present in the system much more virus than was added to it. This phenomenon presents a multitude of aspects, but at the simplest level we can ask what are the factors which determine the yield of virus in relation to time since inoculation. To be more specific, let us take the example of influenza virus inoculated into the allantoic cavity—a minimal infective dose increases to say  $10^{11}$  infective doses after 48 hours at  $35^{\circ}\text{C}$ . This process has been extensively investigated at a high level of technical skill but the final result may be fairly expressed in the statement that “after a variable lag of a few hours an approximately logarithmic increase of virus proceeds for about 20 hours followed by a slow final rise which is eventually balanced by thermal degradation of virus.”

The best way of exemplifying our third objective, of expressing the relation of the phenomenon studied to other phenomenon in the simplest acceptable form, is to list some of the more “interesting” aspects of this same process of influenza virus multiplication in the allantoic cavity. An interesting aspect might be defined as one which, in addition to providing an experimental situation for which parameters can be defined with at least some precision, also appears capable of logical relation to other experimentally accessible aspects of the whole process.

The chief interest of such a list is the opportunity it offers to consider the extremely wide range of concepts in which provisionally valuable scientific generalizations are being made. Under each of the aspects listed a brief comment is given on the type of provisional generalization that has emerged.

1. Adsorption to the cell surface and entry into the cell, of which the first phase is mediated by adsorption of a virus constituent of enzymatic character, a neuraminidase according to Gottschalk (1957), to its substrate in one of the prosthetic groups of a cell surface mucoprotein molecule with, in addition, a variety of ionic and other adsorptive forces playing a part in the attachment.

This leads to a typical enzymological formulation in which the functional activity of a protein or of portion of an organized surface is defined in terms of the range of chemically defined substrates that are destroyed or modified. As far as the virus is concerned this is a functional and biological way of describing what are presumably special protein configurations.

2. The eclipse phase associated with a partial disappearance of infectivity. Currently this is interpreted as meaning the liberation of genetic material from the crust of somatic material that surrounds it in the infective particle.

The eclipse phase with its indications of somatic and genetic aspects of the virus particle is an intensely biological concept. Its closest analogy is perhaps with the old concept of the continuity of the germ plasm.

3. The appearance of soluble antigen in the infected cell, macromolecular material containing protein and nucleic acid, the protein having an immunological structure specific for influenza virus.

The recognition of soluble antigen by immunological means is a function of mutually reactive pattern in macromolecules. Here both patterns are defined biologically. The nucleic acid is defined as ribonucleic acid by chemical means and in a relatively crude way it may be differentiated from some ribonucleic acids by the relative amounts of the different nucleotides composing it.

4. Reversible inhibition both of virus multiplication and of accumulation of soluble antigen under the action of RNAase, both suggesting strongly that free RNA plays an essential part in the process of multiplication.

The use of RNAase serves essentially to bring the phenomena into relation with other examples of protein synthesis.

5. The genetic phenomena of heterozygosis and recombination, indicating a phase in which the genetic material of two or more infecting particles is free to mix and interact.

Here we are dealing with experimental results wholly in genetic concepts and it is immaterial whether the genetic determinants are nucleic acid, protein, or something else.

6. Interference in virtue of which preinoculation of inactive virus can almost completely inhibit the multiplication of active virus subsequently introduced. The details of this phenomenon suggest strongly that a limited number of "points of contact" within the cell are specifically blocked and denied to the after-coming infective virus.

Here interpretation is still problematical, but most attempts make use of ideas basically similar to the interactions of patterned surfaces of macromolecules.

7. The key part played by the host cell surface in the emergence of new virus particles and the bearing of this on the morphology of the particles or filaments, and on the phenomenon of phenotypic mixing of serological character.

The concept of the cell surface more or less modified by infiltration with viral components, being used to form the outer surface of the virus is in most respects a simple spatial and morphological one.

As each reproducible phenomenon is recognized and attempts made to define the parameters that influence its manifestations, new aspects susceptible to a greater or lesser degree of quantitative investigation will emerge. This potentiality of infinite elaboration, once any new phase of biological study is initiated, is at once an opportunity and an obstacle. The opportunity arises when some aspect of the biological phenomenon appears clearly capable of analysis by chemical or physical techniques to give an interpretation expressible in terms of established concepts. In the case of influenza virus, the recognition by Hirst (1941) that "receptors" on the red cell were destroyed by what must be an enzyme associated with the virus, led after some years and a tortuous course of investigation to Burnet and Stone's (1947) recognition of the existence and usefulness of the receptor-destroying enzyme of *Vibrio cholerae*, and eventually to the synthesis of neuraminic acid and the demonstration that purified influenza virus will quantitatively split the acetylgalactosamine neuraminic acid compound (Gottschalk, 1957; Cornforth *et al.*, 1958).

It seems unlikely that the enzymatic action of influenza virus will now attract many other virologists to its investigation at the physicochemical or enzymological levels. Yet it is still perfectly evident that there is an infinitude of work to be done on this tiny facet of influenza virus activity, *at this particular level*. What, for instance, is the nature and distribution of the neuraminic acid compounds on the red cell surface, what is the basis of the receptor gradient, of its association with change in the electrophoretic mobility of cells treated by virus? What change occurs in the virus enzyme to produce the "indicator" state on heating virus preparations, what allows irreversible union to the cell under certain circumstances, why do Newcastle disease and mumps viruses give rise to hemolysis apparently by a similar or the same enzymatic mechanism? These points refer only to the model reaction between virus particle and red cell. The surface of the susceptible cell is clearly more complex and labile and, in the allantoic cavity at least, plays demonstrably important roles both in allowing the occurrence of infection and in the final fabrication and liberation of the new brood of infective units.

Perhaps it will underline the complexity and the difficulty of a general approach to recall that all these problems of the interaction between influenza virus and cell surface have emerged only because the special type of hemagglutination shown by influenza and related viruses provides a uniquely favorable tool for the technical approach. Every other type of virus probably has an equally complex and subtle approach to the surface of the cell

it infects but no "breakthrough" has yet appeared to initiate the phase of infinitely elaborate investigation.

Any experimental biologist could provide a similar picture for his own particular field of study. The potentialities are infinite. To some this may provide a happy picture of an endless stream of experimental biologists finding intense and subtle enjoyment through centuries to come. To those concerned with the problem of how to present the current state of knowledge in such a field as the interaction of virus and host cell, it presents overwhelming difficulties.

Perhaps the most modest and practical approach to adopt is to regard the function of the generalization, the textbook and the treatise, as simply to provide an adequate index and guide to available knowledge. Knowledge of any aspect of the universe may be needed and it is the function of the expert in both academic and practical worlds to be able to find and supply what is known of his subject as rapidly and completely as possible. It may be that in biology truth is a particularly elusive concept but there may be many approximations to truth—incomplete or speculative generalizations—which can assist the intelligent search for and utilization of available knowledge.

In virology, as in other biological domains, the current effort can perhaps be stated as to seek out a small series of model systems for which there can be sketched out a set of working hypotheses which are not inconsistent with the facts available, and which provide a means of marshalling and indexing factual data as well as suggesting lines for further study. Any approach to other systems—to the prevention of a hitherto unknown virus disease, for instance—will then be guided by what is the accepted picture for the model system which appears most relevant to the new situation.

This point of view carries the implication that it is desirable and necessary at every stage in the development of a biological science that reviews and textbooks should state, with all necessary qualifications, those generalizations which appear at the current stage to provide help to understanding. The form in which generalizations are to be stated is quite immaterial provided it is helpful in the sense we have used.

For obvious reasons, generalizations within a given biological field must not be incompatible with the concepts which are accepted as valid for some broader inclusive field. Throughout the history of science, special value has been placed on the discovery of concepts of wide application or on the recognition of how in a new field old concepts with or without modification can still be shown to be valid. It is a special feature of present-day biology that almost all the special fields find a point of common convergence in the problems of the synthesis of specific nucleic acid and protein macromolecules. The study of virus multiplication in the host cell may throw light on

the ways by which virus protein is synthesized, but any generalizations propounded at a biochemical level must be looked at very carefully in the light of facts about adaptive enzymes, of protein synthesis in glandular cells, and a dozen other situations. It is equally possible that useful generalizations may be suggested, not at the biochemical but at the genetic level. Then, having due regard to differences in the systems being compared, what is suggested must not be at variance with what is accepted as covering all other genetic systems.

In our opinion there is no possibility, even in principle, of producing a complete account of what happens when a virus invades and multiplies within a susceptible cell. Each chapter, each review must be something of a work of art as well as of logic. Basically the problem is to select from the available studies those facts and interpretations which help best toward understanding—understanding being used at the pragmatic level of something that will facilitate the rapid and effective marshalling of knowledge needed for some human purpose, whether practical or academic.

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## Chapter II

### Structural and Chemical Architecture of Host Cells with Special Reference to the Synthesis of Polymers

SEYMOUR S. COHEN

*The Departments of Biochemistry and Pediatrics, University of Pennsylvania  
School of Medicine, Philadelphia, Pennsylvania*

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## I. INTRODUCTION

A virologist must have cells to practice his discipline and he should be a cellular biologist before approaching problems of virus infection. He must understand cell parts, their composition, functions, and interactions, as well as the duplications of the parts which result in the multiplication of cells. All synthetic processes relevant to these biological phenomena are relevant to the virologist.

This chapter should therefore be a text of cellular biochemistry, although written with an eye to the special interests of virologists. In a summary of the biochemistry of cells and cell parts, the virologists would presumably wish to learn of the synthetic reactions that can be of particular interest in analyzing the behavior of infectious particles that are synthesized within cells. This is the task that has been assumed, i.e., to summarize the present state of cellular biochemistry, with particular reference to the syntheses which may be involved in the production of viral components and viruses. However, a number of relevant areas have been neglected in recognition of the restriction of space.

### A. *Historical Notes*

Early chemical virology was confined to the study of the properties of isolated viruses. Following the crystallization of tobacco mosaic virus by Stanley (1935) and the detection of nucleic acid in purified preparations of this virus by Bawden *et al.* (1936), work developed around the isolation and the chemical and physical characterization of other plant viruses. In addition, the isolation and characterization of purified preparations of a bacteriophage, vaccinia virus, rabbit papilloma virus, and other viruses were soon reported.

Special attention was given to the problem of the size and shape of infectious particles, a problem whose solution was subsequently greatly facilitated by the use of the electron microscope. Chemical effort concentrated on the detection, identification, and estimation of virus nucleic acid, and studies were initiated on the physical characterization of viral fragments, proteins, and nucleic acid. Systematic amino acid analysis was also begun from the point of view of distinguishing genetically different but related viruses. These were the initial steps toward a chemical genetics, operating on the view that proteins were probably the critical elements in determining inheritable specificity.<sup>1</sup> Clues to viral parasitism and virus multiplication were sought in the analysis of virus preparations for intermediate metabolites, vitamins, and a few enzymes for which a then primitive biochemistry was capable of testing.

By the early 1940s the biophysical analysis of the viruses became as sophisticated as the biophysics of the era, and indeed contributed in no small part to its development. On the other hand, biochemical virology was pursued as an exercise in analytical chemistry.<sup>2</sup> In addition, having adopted the approach of obtaining maximal analytical information concerning the smallest characteristic particle capable of "self-duplication," chemical virology had become almost divorced from developmental and physiological aspects of biology and from certain major trends then developing in biochemistry, particularly enzymology and the study of intermediary metabolism. The prevailing orientation was, however, ideally suited to vaccine production, a problem dictated by the exigencies of World War II; for a number of years in this period, the biochemists working in this field continued to explore the concentration, purification, and characterization of viral antigens, as well as other practical problems.

### *B. The Metabolic Machinery and Virus Infection*

#### *1. The Host As a Source of Metabolites Necessary for Virus Multiplication*

Nevertheless, the biochemical work of the early period established the theoretical basis which called for the shift to the metabolic study of the host

<sup>1</sup> At one time in this period the author was busily engaged in racing preparations of the polymeric ribose nucleic acid (RNA) of tobacco mosaic virus to the analytical ultracentrifuge rather than to the greenhouse (Cohen and Stanley, 1942), since it was never imagined that isolated RNA might have been infectious.

<sup>2</sup> Biochemistry was quite unprepared at that time to dissect the elephantine polymers presented by virology. However, after almost a decade, some of the same laboratories returned to prod at the structures of tobacco mosaic virus with the new tools of a decade of progress in chemistry and physics, and the virus revealed its innermost secrets at every new, more sophisticated touch; the particle seemed to be only too eager to show how simply it was put together.

cell in growth and in infection. It appeared to have been demonstrated that the viruses then isolated did not contain the metabolic equipment essential for the provision of energy and of intermediary metabolites essential to virus synthesis (Stanley *et al.*, 1945; Cohen, 1947, 1955; Gottschalk, 1957a). In a negative way, these findings defined the apparent nature of viral parasitism in terms of the metabolic dependence of the virus upon its host.

It had been shown that the smaller viruses contained a more limited variety of substances than do any cells. Thus they possessed only a single type of nucleic acid, either ribose nucleic acid (RNA) or deoxyribose nucleic acid (DNA), in contrast to all cells which contained both nucleic acids<sup>1</sup> (Cohen, 1947, 1955). The viruses lacked an internal pool of intermediary metabolites (Cohen and Anderson, 1946); these had to be provided by the cell in which polymer synthesis leading to virus multiplication occurred. Further, when tests were made for specific enzymes involved in glycolysis, dehydrogenation, respiration, or other of the common degradative paths, these had been negative, although it must be noted that few if any of the enzymes of the biosynthetic paths leading to the amino acids, nucleotides, or combinations of these had ever been sought, nor could they have been before this last decade.

As reviewed on numerous occasions (Cohen, 1949; Gottschalk, 1957a), the host cell appears to provide most of the low molecular building blocks not supplied in the external medium, as well as the energy for their biosynthesis and coupling into larger viral-specific units. It appears evident that a biochemical understanding of the events occurring in the various phases of a multiplication cycle requires a systematic comparative study of the intermediary metabolism of normal and virus-infected cells. It can be hoped that such a comparative study will do much to explain the cytopathological effects of virus infection, and in so doing will provide important clues which might lead to the development of a chemotherapy of particular virus diseases. Such expectations have more or less come to fruition in the chemical analysis of a few bacteriophage systems, in which the biological attributes of the systems have been favorable for chemical study and in which the biochemical approaches have at least been contemporary.

It is true that the biology of animal virus systems are only now becoming similarly amenable to biochemical investigations. Nevertheless, with the exception of the study of the enzyme of influenza virus, to be mentioned

<sup>1</sup> However, as will be seen below, it has occasionally been reported, although as yet unconfirmed, that certain gametes lack one or another of the two main types of nucleic acids. Some organisms, such as the rickettsiae and microbes of the meningopneumonitis-psittacosis group, formerly classified as viruses but more recently excluded from this category, appear to contain two kinds of nucleic acid (Cohen, 1955) and possess other attributes more commonly associated with metabolically active cells.

below, it is scarcely an exaggeration to note that the metabolic approaches to animal virus infection most commonly applied as of this writing are oriented by the biochemistry of the 1930s. Unquestionably, the preoccupation even now with the estimation of oxygen consumption and glycolysis, and the inhibitory effects of cyanide, fluoride, and iodoacetate reflect the classic schooling in biochemistry of our animal virologists. Perhaps the most generally demonstrated and commonly reproduced metabolic studies in this field have shown that (1) the respiration of a host cell is not affected by virus infection, and (2) an inhibitor which interferes with respiration, or alternatively with the development of an energy supply, whether generated aerobically or anaerobically, prevents virus synthesis.

The author does not mean to deprecate the significance of data of this character. However, it seems necessary to stress that, in the last decade, the discipline of biochemistry has proceeded somewhat past this area of investigation. The biosynthetic paths leading to the formation of the amino acids and nucleotides have been delineated in such detail that it has been estimated that approximately one-half of the intermediates in these sequences are already known. However, it may quite properly be asked why it might be more illuminating to compare any of these biosynthetic paths in normal and infected cells than it was to study respiration and energy supply. In some virus diseases, such studies might not be useful. However, the following considerations suggest that there might be a point to such exploration:

(1) The viruses are structurally quite different from their host cells. Their polymers differ from cellular polymers with respect to their specific organization, as manifested in specific antigenicity and genetic continuity. Such organizational and functional differences often reflect quantitative differences in the amounts of particular amino acids and nucleotides employed. Thus, an infected cell may differ from the normal cell with respect to the amounts required of precursors of these units, or of the units themselves, if supplied exogenously. The infected cell may show a shift in ratio of the nucleic acids or of other units, and thereby may reveal properties of the viruses as yet unrevealed by study of the isolated agent.

(2) Many paths of metabolism have alternative routes. Virus infection may affect the balance of these, or even eliminate one completely. Our own studies of the effect of virus infection on paths of glucose utilization in *Escherichia coli* have established the existence of such shifts (Cohen, 1953), and have assisted in the dissection of the controlling mechanisms of metabolism in infected bacteria.

(3) Some viruses have units which are not present in the host, e.g., 5-hydroxymethyl cytosine (HMC), which is present in the T-even phage DNA and not in the host nucleic acids (Wyatt and Cohen, 1953). Such units not only provide specific tags and unusual opportunities for the study of

virus multiplication, but also possible sites for chemotherapeutic attack. Although this particular base has not been found in other viruses, it is far too early to say that structurally and metabolically unique units will not be found in other viruses. For example, the specific hydrolase of influenza virus has not yet been found in the host cells in which the virus multiplies (Gottschalk, 1957a), and the structural basis of this enzymatic activity requires exploration. The C-terminal tripeptide of tobacco mosaic virus and its strains may also be specific unto these viruses and therefore possess unusual metabolic interest.

(4) In addition to the utility of HMC in following viral DNA through its life cycle, the formation of this compound relates precisely to the problems of possible aberrancy of important biosynthetic paths under the influence of virus infection. For example, that the diversion of cell metabolites to virus synthesis does occur is documented in only a few instances, and the data are most complete in the case of infection of *E. coli* by the T-even phages. In this extraordinarily virulent infection, the infected cell stops the synthesis of most of its normal polymers, enzymes, etc. (Cohen and Anderson, 1946; Monod and Wollman, 1947; Cohen, 1948), essential building blocks being entirely diverted to viral synthesis. For this case, the hypothesis has been developed that this qualitative alteration in metabolic emphasis is due to the diversion of cytidylic acid and deoxycytidylic acid, essential for the synthesis of normal nucleic acid and complementary protein, to form 5-hydroxymethyl deoxycytidylic acid, which is uniquely a component of the DNA of the T-even phages (Wyatt and Cohen, 1953; Cohen, 1953). This process is represented in Fig. 1.

One should also like to know if the synthesis of HMC nucleotides affects in any serious way the synthesis of phospholipids, discovered by Kennedy (1957) to be mediated via coenzymes containing cytosine nucleotides. Does the formation of HMC also affect thiamine synthesis and utilization, since this vitamin contains an analogous hydroxymethyl cytosine? Does this relate in any way to the accumulation of pyruvate (Spizizen, 1957) observed with *E. coli* infected by the T-even phages, since thiamine-containing coenzymes are most important in the further metabolism of pyruvate and the other  $\alpha$ -keto acids? Will the effect then extend to  $\alpha$ -ketoglutarate and thereby control the tricarboxylic acid cycle, glutamate production, etc.?

(5) There is so little known that it is difficult to predict what one might find. Will an insect virus contain ortho tyrosine as do some insect cuticles (Dennell, 1956)? Will a virus infecting apple trees contain 1-amino cyclopropane-1-carboxylic acid (Burroughs, 1957)? Do the *E. coli* phages contain diaminopimelic acid, which is present in the cell wall of the bacterium, and if not, what is there about the origin and metabolism of these substances which excludes them from virus protein? Can not a new pathway be developed

for the synthesis of a viral unit, even though a familiar pathway is known to operate for the same substrate in the normal cell?

Despite the evident interest of these problems of intermediary metabolism to the development of biochemical virology, it is not my intention to pursue these questions further, at least not in the form of providing a biochemical map separate from the consideration of biological and chemical problems of polymer synthesis. Such maps and discussions of particular paths may be found in a number of biochemical texts and in the innumerable reviews of advances in these areas.

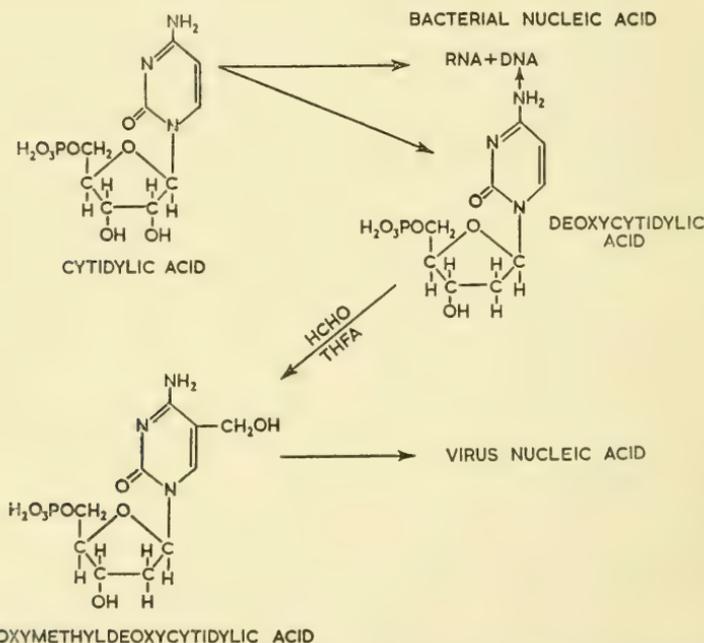
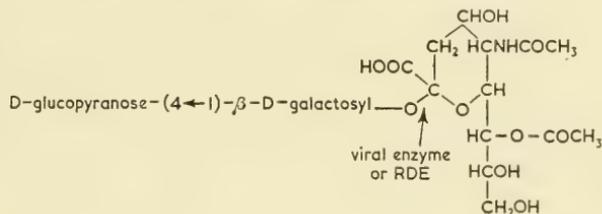


FIG. 1. The production of a virus-specific intermediary metabolite, 5-hydroxymethyl deoxycytidylic acid (Flaks and Cohen, 1957).

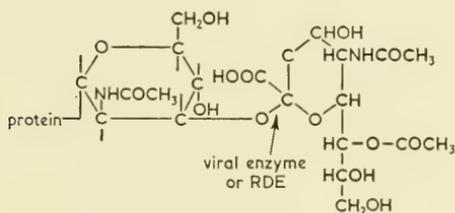
## 2. Viral Enzymes

In recent years some viruses have been shown to have some intrinsic catalytic activity, which may be termed enzymatic, as indicated by the recent studies of the specific hydrolases of influenza virus and the T-even bacteriophages. In the latter instance, the tail of the virus contains an enzyme which can bore holes in bacterial cell walls (Brown and Kozloff, 1957) and liberate fragments containing amino acids (Koch and Weidel, 1956). This activity is apparently important in permitting the penetration of viral DNA into the bacterium. Although the substrates and reaction products of this

reaction are as yet poorly characterized and it cannot yet be stated that the T-even viruses act catalytically in lowering the energy of activation of a specific reaction, it appears to be only a matter of time before the precise character of the hydrolytic activity of these viruses is defined.



(a) Neuramin-lactose



(b) Prosthetic group of the mucoprotein of bovine submaxillary gland

FIG. 2. The mode of action of the neuraminidases of influenza virus and RDE (Gottschalk, 1957a,b).

A somewhat more satisfying chemical position, summarized by Gottschalk (1957a), has been attained with respect to the hydrolytic activity of influenza virus. The virus appears to contain a glycosidase which has not yet been found in any of the cells that support multiplication of influenza virus. A similar soluble glycosidase is produced in cultures of *Vibrio cholerae* and has been called the receptor-destroying enzyme (RDE). The substrate for viral glycosidase and RDE is present at the surface of appropriate erythrocytes and apparently on susceptible cells of mouse and ferret lung. Pre-treatment of these cells with RDE hinders their subsequent infection by influenza virus. In addition, many animal products, e.g., mucins, urinary mucoprotein, etc., possess the ability of interfering with the ability of the virus to agglutinate red cells and may be degraded by virus and RDE. It appears that both the cellular receptors and soluble products contain a common grouping capable of reacting with the glycosidase.

When the virus is incubated with a purified mucoprotein isolated from human urine, an acetyl neuraminic acid (sialic acid) is released (see Fig. 2).

The structure of this compound, has recently been elucidated by a number of workers, among whom Gottschalk has made important contributions. This substance is a previously unsuspected condensation product of a substituted hexosamine and pyruvic acid. Sialic acid resembles in many respects the lactyl ether of hexosamine recently discovered in the cell walls of numerous bacteria, e.g., *B. megatherium* (Strange and Powell, 1954; Park and Strominger, 1957).

The well-defined compound, neuramin-lactose, which has been isolated from rat mammary gland, is cleaved by the viral enzyme and by RDE into diacetyl-neuraminic acid and lactose, as presented in Fig. 2a. The name "neuraminidase" has been suggested for enzymes capable of catalyzing this cleavage.

In the liberation of sialic acid from the mucoprotein of bovine submaxillary gland by RDE, the *O*-glycosyl linkage is cleaved between *N*-acetyl-galactosamine and sialic acid, as presented in Fig. 2b (Gottschalk, 1957b). In the urinary mucoprotein the neuraminyl derivative exists as the terminal end of a small polysaccharide, which also contains hexosamine, galactose, mannose, and fucose.

It is not believed that the neuraminidase is important in viral penetration, as is the hydrolase of the T-even phages. In the case of influenza virus, penetration may be effected by a process more akin to pinocytosis. It has been suggested that in this animal virus infection the enzyme may be involved in the release of virus particles from the surface of the infected cell.

In an important study by Adams and Park (1956) it was shown that a specific strain of phage liberated from an encapsulated strain of *Klebsiella pneumoniae* contains an enzyme for the hydrolysis of the polysaccharide capsule. The synthesis of the enzyme is apparently induced by infection by this phage; on lysis the enzyme is both freely diffusible and attached to virus. It is not clear whether particle-bound enzyme does not arise as a fortuitous accident of adsorption. However, from the point of view of the functional penetration of virus to the cell wall, this question may not be meaningful.

The sole evidences of the metabolic activity of the viruses, then, are confined to the viral hydrolases noted above. These burglar tools are special requirements of an infective cycle which involve the penetration of the tough cell wall or other protective coat of a bacterium or an escape through the less rigid but none the less tough plastic envelope of an animal cell. No evidence has yet been obtained for their existence in plant viruses, although conceivably an enzyme of this character may be found in those viruses which multiply in both plants and their insect vectors.

### *C. Possible Contributions of the Host Cell to the Synthesis of Viral Polymers*

Despite the existence of such virus enzymes, the host is still called upon to provide the metabolic equipment essential to the synthesis of the amino acids and nucleotides, which are then incorporated into the specific viral polymers. How are specific viral polymers synthesized? To what extent do the viruses organize their own duplication? It can be assumed that the models provided by the infecting virus particle in some manner determine the specificity of the viral polymers finally produced. However, it was noted only some five years ago (Cohen, 1952) that biochemists were not then in a position to state that viruses do not contain enzymes for the specific organization of nucleic acid from nucleotides or of proteins from amino acids. At that time we did not know how to test for such enzymes, nor did we know if enzymatic activities were required for the specific organization of these particular polymers. We do not yet have answers to the first of these questions. Nevertheless, it is now known that the biosynthesis of nucleic acids and protein do require specific proteins and it may be anticipated that tests will be made in the near future to determine whether the viruses contain the enzymes for the synthesis of their own polymers, i.e., to see if they are truly "self-duplicating." It will be a major function of this article to detail the rapid growth of biochemical knowledge in the areas relevant to the problem of the synthesis of viral polymers.

Two lines of evidence suggest that the enzymes of the host cell are also used for polymer synthesis:

1. The first relates to the apparently unique case of the production of 5-hydroxymethyl cytosine. As of this writing, the enzyme for the formation of the deoxyribotide of HMC by the hydroxymethylation of deoxycytidylic acid in the presence of 5, 6, 7, 8-tetrahydrofolic acid (see Fig. 1) has been found only in extracts of bacteria infected by the T-even phages (Flaks and Cohen, 1957). Since the enzyme does not appear to be present within virus and hence is probably not introduced with virus DNA, the following possibilities concerning its origin are now beginning to be explored:

a. The enzyme is inhibited in uninfected cells and this inhibition is released upon infection with virus, or

b. The enzyme is synthesized after viral infection:

i. The synthesis of the enzyme is induced by low molecular weight fragments of injected virus DNA containing hydroxymethyl cytosine.

ii. The synthesis of the enzyme is linked to the presence and perhaps the metabolism of a specific polynucleotide sequence derived from the injected viral DNA.

Of the formal possibilities which are indicated above, it can be seen that "bi" and "bii" call for the participation of the host cell in polymer synthesis

essential to the production of a viral-specific intermediary metabolite. Although it has been shown that the early protein synthesis, which occurs before DNA synthesis in the eclipse period, is essential to virus production (Cohen and Fowler, 1947) and to a subsequent DNA synthesis (Burton, 1955; Tomizawa and Sunakawa, 1956; Hershey and Melechen, 1957), it must also be noted that such early protein synthesis is also important for phages which do not contain HMC (Miki and Matsushita, 1956). Nevertheless, it has recently been shown that the appearance of the deoxycytidylic hydroxy methylase induced by infection with T-even phages containing their DNA does require protein synthesis, tending to support hypotheses of the "b" type (Flaks and Cohen, 1958).

2. The other line of evidence suggesting a host synthesis of viral polymers is of a more biological character and is more complete. It derives from the data on lysogeny and the behavior of the prophage. The following attributes of lysogenic systems may be taken to suggest that the enzymes of the host are involved in the synthesis of viral polymers and that the genetic material of the lysogenic viruses may act to tie up these synthesizing capacities.

a. The presence of a prophage produces immunity to superinfection by a mutant temperate phage (Lwoff, 1953), suggesting competition for a specific synthetic mechanism, the numbers of which are limited by the host.

b. The cytological basis of the above is indicated by the findings that (1) the prophage is incorporated into the bacterial genome; (2) it exists in the same number as do the bacterial nuclei; (3) the prophage is localized at a specific site on (although perhaps not in) the bacterial chromosome; and (4) a prophage is duplicated at the rate of duplication of other genetic loci (Jacob and Wollman, 1957).

c. Analysis of the mechanism of induction by ultraviolet irradiation of lysogenic bacteria containing two different prophages has led to the conclusion that prophage development results from an indirect effect mediated through the bacterium, rather than from a direct change in the prophage (Jacob, 1954).

d. In certain lysogenic systems, a partial genetic homology may exist between a phage and its host (Garen and Zinder, 1955). After infection, host material may replace homologous phage substance damaged by irradiation. Such a reactivation cannot occur if the host material has been similarly damaged. Some of the virulent phages, such as T1 and T3, may also undergo cellular reactivation after sustaining irradiation damage. One may imagine that the genetic homology reflects a chemical identity of the polymers involved and that the enzymes of the host are capable of synthesizing polymers which are specific and characteristic of host and phage alike.

In addition, we may note that photoreactivation of ultraviolet-irradiated viruses (including the chemically unique T-even set) is effected by the

application of visible light to the complex of cell and damaged virus and not to the free virus alone (Dulbecco, 1950). Most recently, Rupert *et al.* (1958) have described the increase of activity obtained when an ultraviolet-inactivated transformation agent (DNA) derived from *Haemophilus influenzae* was treated with visible light in the presence of an extract of *E. coli* strain B.

#### *D. On the Origin and Cellular Relations of the Viruses.*

It is possible to imagine that many viruses are degenerate microorganisms and, indeed, that their composition and life cycle reflect such a development. For example, vaccinia virus may be thought of in this way. The virus seems to contain a number of units capable of metabolic activity (flavin adenine dinucleotide, biotin, copper) and it might be supposed, among many possibilities, either that these are evolutionary vestiges of a once extensive metabolism or that the metabolism still exists, but that the biochemists have not sought the proper reactivities. Although this virus contains DNA alone, a nuclear constituent, the DNA of the virus appears to be deposited when the particle is in the cytoplasm. Since the host cell lays down its DNA in its nucleus (suggesting a nuclear localization for the enzymes involved in this synthesis) and some DNA-containing insect viruses do appear to be formed within the chromatin of the infected cell (Smith, 1954), the mode of synthesis of DNA in vaccinia virus suggests the possibility that this virus may possess its own enzyme for such a synthetic achievement. Although it has been reported that the development of vaccinia virus has an eclipse phase, as do the phages, it can be imagined that the virus is masked temporarily, and that we do not know how to demonstrate the complete active particles which may be present. However, that there is a basic dissimilarity in the multiplication of vaccinia virus and true cells is suggested by the electron micrography of vaccinia-infected cells, in which the sections seem to reveal immature particles consisting of apparently empty external membranes and do not expose intact dividing particles (Morgan *et al.*, 1954).

On the other hand, it is considered to be quite significant that all the phages contain DNA alone, that phage infection demonstrably produces effects to a greater or lesser degree on bacterial nuclei and chromatin, and, in the case of lysogenic systems, the prophages exist and multiply in close association with a bacterial chromosome. As noted above, a similar situation may exist in the multiplication of many insect viruses. Data of this type represent perhaps the strongest evidence to suggest that many viruses are aberrant cellular particulates, aberrancies produced by a mutation in polymer synthesis, which confers a selective advantage on the mutated polymer at the expense of the development of normal polymers and particulates. In this context, one might ask if the plant viruses are the colorless

products of the variegation of chloroplasts, even as proposed by Woods and Dubuy (1943). Also, we would wish to know which viruses, if any, have been produced by mutations in the production of microsomes. As is well known, many carcinogenic treatments are also mutagenic, and it is important to know whether tumor viruses, as well as tumor cells, may not arise as the result of such treatments.

As is evident from this discussion, problems of virus origin, specificity, and multiplication have merged with current problems of cytology, development, and genetics and the dissection of mechanisms of inheritance. All of these problems have begun to be formulated in common terms. Fortunately, the biochemistry of polymer synthesis also cuts across all of these fields. Biochemical development in this particular area has also kept pace with knowledge in these biological disciplines and has even begun to contribute significantly to the advancement of biological knowledge. With this realization, we shall discuss the basic data of these biological disciplines, particularly as they relate to biochemical problems of polymer formation, in the hope and expectation that the emerging principles and problems of cellular biochemistry will be of fundamental interest and utility in the development of knowledge concerning the viruses.

## II. CELLULAR ORGANIZATION

### *A. On the Cell in General*

The development of a biochemical cytology, as well as an increasing sophistication of the biochemist with respect to biological phenomena, have emphasized the facts and problems of cellular organization. That cells are not mere bags of enzymes dissolved in a soup or jellied consommé of metabolites has come to be accepted not only by biologically oriented biochemists but by the classic enzymologists as well. This is marked by the development of a literature in which it is no longer sufficient to report that an enzyme has been obtained in cell-free preparation, but also that it sediments as a particle of large particle weight or, alternatively, is "soluble," i.e., not readily sedimentable. In dealing with cells, numerous investigators will also attempt to determine if an enzyme is not present in a nuclear fraction or in one of several separable cytoplasmic fractions, and even if its apparent cellular distribution is not related to its apparent function. Thus, biochemical cytology is rapidly accumulating data on the localization of enzymes and on the metabolic division of labor.

However, in the increasing activity of biochemists to fractionate the cell, this atom of the biological world is frequently treated as an invariant unit of structure. Few biochemists appear to take into account the fact that the

increase of the living world involves the production of more cells and that this rather basic phenomenon calls upon cells to be altering their structures and organization, even their metabolism, as the cycle of growth, division, and growth proceeds. A given cell may be "fixed" by the biologist for the purposes of observation; if it had not been stopped in its tracks, it would almost certainly have been something else soon thereafter. The study of the temporal sequence of biochemical events and their relation to cellular activity is in its infancy; as an example one might point to the dearth of biochemical information concerning the mechanism of cellular division, although this subject is beginning to be explored more actively.

In most instances, however, the existing analyses of cell structure are undefined with respect to the life history of the specific cell being studied; most commonly, various structures will have been isolated from a heterogeneous cell population of which like members will have been in different stages of their division cycle.<sup>1</sup> We are compelled to proceed despite the existence and recognition of this deficiency.

It is possible that the very process of obtaining homogeneous populations of cells in the clonal development of microbes or animal cells tends to emphasize the properties of the culture rather than the attributes of individual cells, which are, after all, the units of infectability of virology. From an inoculum of relatively few cells or even only one, selected to insure genetic homogeneity of the progeny, a large culture may be produced in which the cycle of growth, division, and growth is reproduced many times. In many instances, rapid growth rates involve concomitantly a maximum efficiency in the production, utilization, and assembly of metabolites. One gets the impression of a well-ordered balance in the course of cellular synthesis and duplication, of an extreme interdependence of parts in such experimental systems, in which biological variability has been minimized. This is in contrast to the functional separation of growing parts from dividing parts, which is readily seen in differentiating and differentiated cells. Such cells of this latter type exist in adult tissues or in certain phases in microbial cultures; they are usually difficult to handle and to maintain in these states. Their physiological and genetic variability tend to be maximal; as a consequence,

<sup>1</sup> The recognition that such material is unsatisfactory for many biological purposes cannot help but produce a tremendous growth of biological study on the production of homogeneous cell populations, as virology has already demanded of tissue cultures. This will also extend to the production of synchronous cell division and controlled life cycles, even as effected in Nature during the early development of various embryological systems. That Nature has already provided some materials ready-made for such biochemical studies calls to mind the comment of Sir Frederick Gowland Hopkins, who asserted that in exploring and cultivating the fields of Nature, the chemists were best provided with the machinery for this cultivation, but that the biologists knew best the lay of the land.

sophisticated virologists prefer to avoid such experimental systems. However, until the present, the role and relative independence of cell parts in such matters as polymer synthesis has been explored most successfully in only a few carefully chosen highly differentiated systems, which have not been of experimental utility to virologists. The work of Brachet and Mazia with the giant unicellular alga, *Acetabularia*, and with *Amoeba*, in which the metabolic and particularly the synthetic capabilities of nucleate and enucleate fragments can be compared, emphasize this point. Nevertheless, experiments on the relative independence of cell parts and function in these systems inevitably raise the question, for example, of the necessity for a nucleus in the synthesis of influenza and poliomyelitis viruses and point to the need of developing new biological materials for the solution of such problems.

Finally it may be asked whether, for example, our information on the composition of the resting nucleus of an animal cell tells us anything about a bacterial nucleus or even about the nucleus of a plant cell. Since but a single paper (Spiegelman *et al.*, 1958) bears on the composition of isolated bacterial nuclei, and not many more exist which relate to the composition of isolated plant nuclei, it is evident that extrapolation of one set of data to other biological systems may be fraught with danger.

Having noted a few of the important arguments against accepting any generalized version of a cell, it is nevertheless convenient to present some reference cell. A schematic representation of a parenchymatous cell of rat liver, with currently available detail, is given in Fig. 3. As we shall see, a very considerable portion of the analytical effort on cell structure and composition has been directed at this unusual animal cell, which has been of little interest to the experimental virologist.

We shall begin with a discussion of the chemistry of the nucleus, and consider existing data on nuclear elements, such as chromosomes, nucleoli, nuclear sap, and nuclear membrane. Data on cytoplasmic components, such as mitochondria and other particulates, and the cell sap will then be presented. Following the discussion of data on the composition of these cell parts, we shall consider the problem of the localization of distribution of metabolic function within cells, with particular reference to questions of the localization and control of polymer synthesis, under normal and pathological conditions of growth. Having presented the main relevant biological data, we shall turn to the chemical problems of polymer syntheses which must be answered within this biological context.

### *B. The Composition of the Nucleus*

#### *1. On the Existence of Chromosomes in the Interkinetic Nucleus*

As noted above, chemical work on nuclei has tended to concentrate on the nuclei of adult tissues containing large proportions of interphase nuclei.

Although it is usually impossible to discern chromosomes in such nuclei, it is most frequently assumed that such nucleoprotein structures are nevertheless present. In support of such an assumption it has been asserted that the cellular requirements of exact duplication and consequent genetic continuity demand a continuity of substance.

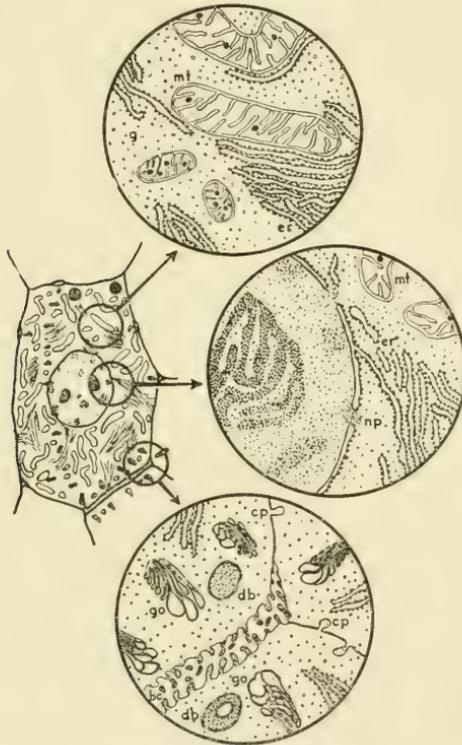


FIG. 3. Schematic representation of a parenchymatous cell of rat liver (Novikoff 1957). The following structures are represented: mitochondria (*mt*); basophilic material (*er*); glycogen (*g*); nucleolus, nuclear membrane, nuclear "pores" (*np*); Golgi apparatus (*go*); "dense bodies" (*db*); interlocking cell processes (*cp*); and bile canaliculus (*bc*).

Of course, it is precisely this kind of reasoning which has been called to question in recent years by the data on transformation agents and on viruses. The life cycle of a nucleoprotein does not require its structural continuity since viral nucleic acid, appropriately placed within its host, is sufficient to direct the subsequent production of not only nucleic acid but also a variety of differentiated proteins as well, and to control their subsequent organization into a protein-associated structure. It is, however, true that biology has not yet developed a hypothesis concerning a mechanism, other than those

involving chromosomes, for the distribution of large numbers of genetic units. Indeed, we should be at a total loss, temporarily at least, if the genetic units present in interphase nuclei should prove not to be organized in a differentiated thread plus an existing procentromere, i.e., point of spindle fiber attachment, so necessary for the activities of the chromosome during mitosis.

The experimental evidence for the presence of chromosomes in such materials is not the strongest. It is considered that the inability to see such structures arises from their imbibition of water, such that the refractive index of the swollen and extended chromosome approaches that of the surrounding nuclear sap. However, when needles have been placed within resting nuclei and wiggled about, it has not been possible to detect the presence of the anticipated resistant structures. On the other hand, it is reported that the insertion of carbon particles into nuclei has resulted in an apparent outlining of ghostlike chromosomes. The isolation of "chromosomes" and chromatin threads from resting nuclei has been described by some workers, as will be described below; such reports have been questioned and the products have been described as artifacts.

Ris (1957) has stated that even in electron micrographs of sections through the interphase nucleus "it is not at once obvious what is nuclear sap and what chromosomal material." However, after an examination of sections of chromosomes, he feels that he knows what to look for and reports the presence of characteristic chromosome fibrils in the sections of interphase nuclei of various plant and animal tissues. Whether such fibrils are truly characteristic of chromosomal material alone and, indeed, reflect the presence and organization of the genetic material in such nuclei will require far more study.

"Heterochromatin" has been defined by some workers as chromosomal regions that remain condensed and darkly staining throughout interphase. The existence of such chromocenters in interphase nuclei are then presumably markers of the existence of chromosomes bearing these differentiated regions. Ris (1957) has observed that sections through heterochromatin in isolated lampbrush chromosomes and the chromocenters of interphase nuclei reveal the same fibrillar structures that may be seen in the sections of the bulk of the chromosomes, the euchromatin.

## *2. The Visualization of Intranuclear Constituents*

In the last 15 years, the cytologist concerned with the relationship of visible structure to cell function has produced a revolution in cytological technique. Abandoning a purely descriptive methodology, he has turned to the chemist and physicist for collaborative assistance in developing quantitative data for the characterization of cell structure. The ordinary light microscope may resolve structures down to 1000 to 2000 Å; phase microscopy and ultraviolet microscopy have pushed this limit to about

200 Å. The electron microscope, when used by the most skillful investigators, is resolving structures down to 10 to 15 Å. Improved methods of calibration of these instruments have facilitated a precise analysis of structural dimension.

It may be noted that a 10-fold increase in the resolving power of the electron microscope would permit an atom-by-atom chemical analysis of a given molecule. However, at present the instrument can distinguish between electron-scattering and non-scattering structures, concentrates of the nucleic acids tending to belong to the former group and proteins to the latter. The development of techniques to magnify these effects and to permit the assignment of chemical composition to electron-dense regions is being actively studied. For example, electron-dense iron can, by binding to nucleates, increase the density of these materials to the electron beam and assist the localization of these materials, as in the demonstration of the nucleic acids within virus particles and in bacteria (Bernstein, 1956). Although a variety of techniques are known to the X-ray crystallographer for associating metallic ions with specific functional groups to determine their position in a protein crystal, e.g.,  $\text{Hg}^{++}$  to protein SH, these procedures have not yet been systematically exploited in staining protein groups for electron microscopy.

In bacteria, rich in electron-dense RNA, the central region possesses a relatively low electron-scattering power. Bradfield (1954) has reported on the electron micrography of bacteria in which the aldehyde group of deoxyribose liberated from DNA deposits metallic Ag from ammoniacal  $\text{AgOH}$ . By this technique, even as with more conventional Feulgen staining (to be discussed below), the DNA is found to be concentrated in these central bodies, which are therefore termed bacterial nuclei. However, sectioning has not revealed nuclear membranes at all comparable to those observed in plant and animal cells. Also, Bradfield considers that these bacterial nuclei divide amitotically. Spiegelman *et al.* (1958) have presented evidence to suggest that the DNA itself may constitute the limiting element of the nuclear body of *Bacillus megatherium*.

The use of ultraviolet microscopy in the microchemical analysis of cells was first exploited by Caspersson and his associates (Caspersson, 1950). Using the marked ultraviolet absorption of the nucleic acids at 2600 Å, as a function of their content of purines and pyrimidines, the Swedish school has made many observations on the distribution of these moieties in nuclear and cytoplasmic structures. Although the qualitative observations of these workers have generally been confirmed and accepted, many difficulties exist in the interpretation of the quantitative data. In addition to the effects of light scattering, superimposed protein absorption, or even the orientation of nucleic acid within a structure, it may occasionally happen that a marked ultraviolet absorption with a peak at about 2600 Å is not due to nucleic acid

at all. For example, the ultraviolet absorption of the mitotic apparatus suggests the presence of 2 to 3 % protein-bound nucleic acid. However, this seems rather to be due to the presence of firmly bound mononucleotide, perhaps adenosine triphosphate (ATP), in this material (Mazia, 1957).

The reactions of cell structures with chemical reagents to give colored products has long been known, as in the use of dyes. Structures which became colored by binding basic dyes, such as rosaniline, hematoxylin, or azure A applied in acid solutions, were termed basophilic. This property reflects the presence of acidic groups and has been particularly important in the development of knowledge of the structural and functional relations of the nucleic acids. The earlier embryological studies of Brachet exemplify such a use of this technique.

Wide use has been made of differential staining techniques, as in the use of a mixture of methyl green and pyronine for the study of the nucleic acids. Brachet (1953) and Kurnick (1955) have discussed various problems associated with the use of these dyes. Methyl green reacts with highly polymerized DNA, as it exists in chromosomes; its reactivity has been observed to be a function of the degree of polymerization of the nucleic acid (Kurnick, 1954). Pyronine reacts with relatively low molecular weight nucleates, such as commercially obtainable RNA. It was originally thought that this reaction was specific for RNA; however, Kurnick (1951) has shown that pyronine will also react with depolymerized DNA. In a well-controlled preparation (Kurnick, 1955), chromosomes are clear green, and nucleoli are bright red. RNA-containing areas of the cytoplasm also stain red.

The Feulgen reaction has provided the most important nuclear stain for the characterization of DNA (Di Stefano, 1948; Swift, 1955). The reaction has also been used successfully on microorganisms to reveal structures which are comparable to the chromatin of higher cells, in that both types of structure contain DNA. The Feulgen stain is a modified Schiff reaction, given generally by aldehydes; it involves the absorption of  $\text{SO}_2$  from a decolorized fuchsin- $\text{SO}_2$  mixture to give a red fuchsin stain. Cellular constituents contain few compounds besides DNA capable of interfering with the specificity of the reaction. Fatty aldehydes may be removed by alcohol extraction after a gentle acid hydrolysis of acetal phosphatides. The hydrolysis also removes purines from DNA, liberating the active carbonyl group in the aldehyde sugar, 2-deoxyribose, which is then capable of binding  $\text{SO}_2$ . The specificity of the stain depends on the much greater tendency of 2-deoxysugars to exist in the straight chain aldehyde form than do furanoses, such as the ribose of RNA, or the pyranoses present in many polysaccharides.

The Schiff reaction has also been modified for the detection of polysaccharides in the cell. Prior incubation with periodate cleaves the glycol configuration to two aldehyde groups, which are then also detectable by fuchsin- $\text{SO}_2$ .

The major components of DNA, base, deoxyribose, and phosphoric acid group can therefore be visualized by the ultraviolet absorption technique, Feulgen reaction, and basophilia staining, respectively. Only the nucleus gives tests for all three of these constituents. The cytoplasm, usually containing only RNA, does not give a Feulgen reaction, with a few rare exceptions, such as the kinosome of some protozoan flagellates. The discovery of Feulgen-staining bodies in the cytoplasm is usually taken to suggest the presence of a microbial parasite or symbiont within the cells examined, although an apparently negative reaction can not be taken as proof positive of the absence of infecting microorganisms (Trager, 1952). The Kappa particles of *Paramecium* were first observed by means of the Feulgen technique (Preer, 1948), which facilitated a direct study of the kinetics of multiplication of this particle within its host. The existence of cytoplasmic inclusions containing DNA in human rectal polyps has been taken to suggest a viral etiology for these lesions (Leuchtenberger, 1954). On the other hand, a cytological confusion of mitochondrion or symbiont within some cells, as in insect tissues, can usually be resolved on this basis since mitochondria lack DNA.

In resting nuclei, as indicated above, lack of condensed chromatin may lead to a very faint Feulgen reaction. It has been claimed that the nucleus of the oocyte of the sea urchin does not give an appreciable reaction for DNA, and, therefore, that this nucleic acid is absent from the egg prior to fertilization (Marshak and Marshak, 1953). However, many workers have taken the position that very faint Feulgen reactions can be seen in these nuclei, and that DNA is really present (Brachet and Ficq, 1956).

A positive basophilic reaction, marked ultraviolet absorption, negative Feulgen test, and red stain with pyronine suggest the presence of RNA in nucleoli and cytoplasm. To prove the presence of this nucleic acid, we can also introduce the method of difference. Several techniques may be used to remove RNA from cell structure; these include specific solubilization under rigorously controlled conditions with ribonuclease, dilute alkali, or perchloric acid in the cold. The loss of ultraviolet-absorbing capacity or basophilia is evidence for the prior presence of RNA in the structure. This technique was first used to demonstrate RNA in bacteria (Dubos and Thompson, 1938; Dubos and Macleod, 1938) and was then extensively exploited by Brachet.

It should be noted that before McDonald (1948) devised a technique of freeing ribonuclease from proteolytic contaminants, even crystalline preparations of the enzyme contained such impurities (Cohen, 1945; Schneider, 1946). The use of such preparations in cytological work opens the way to possible error, although recent results with purer enzyme preparations (Kaufmann *et al.*, 1951) have been concordant with the older findings.

Several reactions, such as Millon's reaction for the phenolic groups of tyrosine, or a modified Sakaguchi reaction for arginine, are also useful in detecting protein. A simple staining procedure with the acid dye, fast green, has been described for basic proteins, such as histones and protamines (Alfert and Geschwind, 1953). This reaction is almost specific for these nuclear proteins, since other proteins with high isoelectric points, e.g., cytochrome c, which also stain, do not occur in cells in significant concentration. The procedure requires the prior removal of DNA. Under the conditions described, nucleoli fail to stain. Only the chromosomes take this stain, a result in accord with other results on the distribution of the histones. These histones are most acidophilic in dividing cells and it has been suggested that interphase nuclei increase their content of a nonhistone protein which combines with the chromosomes in such a way as to restrict the combination of histone with fast green (Alfert, 1957).

The quantitation of these staining reactions for nucleic acids and proteins has been accomplished through spectrophotometric methods applied to intact cells, complete nuclei, or extracts. A detailed discussion of these reactions is given by Swift (1955).

### 3. *The Isolation of Nuclei*

The preparation of nuclei by chemical methods was first accomplished by Miescher (1897), who isolated the nuclei of pus cells after digesting away the cytoplasm with pepsin-HCl. Miescher and Kossel (1928) also isolated the nuclei of fish sperm, and many workers undertook to isolate the nuclei of bird erythrocytes. Nuclei have been liberated from the latter source by such methods as freezing and thawing, or by means of hemolytic agents, such as saponin or lysolecithin. A peculiarly suitable biological source has occasionally been discovered as a source of nuclei; for instance, before cell wall formation the coconut endosperm contains large numbers of syncytial nuclei. These nuclei may be liberated readily and isolated. Cutter and associates (1952) record the isolation of 0.13 gm. of undamaged nuclei from a single coconut of 2 kg. and recommend their procedure for certain other plant nuclei.

Behrens (1932) developed an important method for the separation of nuclei that has been applied to plant and animal tissue. Both this technique and the Feulgen stain were used to demonstrate DNA in plant nuclei, thereby disposing of the old notion that only animal cells contained this nucleic acid (Dounce, 1955). The Behrens procedure consists of drying the frozen tissue, and grinding it to a very fine powder. The anhydrous nuclei are laboriously obtained by repeated centrifugation from mixtures of benzene and carbon tetrachloride of adjusted density. The flotation process therefore separates cytoplasmic constituents as a function of their anhydrous densities. This procedure has been used to avoid the loss of water-soluble autolyzable

constituents, such as proteins, coenzymes, and other metabolites. However, as might be expected, nuclei isolated by this method are altered morphologically and certain enzymes, such as aldolase (Allfrey *et al.*, 1953), are inactivated by this procedure.

In another early procedure, tissues were homogenized in citric acid. Nuclei obtained in this fashion were clearly damaged. Dounce (1950) has modified this technique by using citrate at pH6 to maintain enzymatic activity. However, he has also shown that the protein to DNA ratio of these nuclei is lower than those obtained by means of the Behrens procedure. In addition, Kay *et al.* (1956) have recorded the extraction of an RNA fraction of low metabolic activity from nuclei by the use of citrate.

According to Schneider and Hogeboom (1951), these earlier procedures gave poor yields, led to loss or denaturation, or both, of the proteins, altered the cytological appearance of the nuclei, and were poorly controlled for the extent of contamination by cytoplasmic material. Some of these problems have been resolved by homogenization and subsequent isolation in isotonic or hypertonic sucrose solutions. Nuclei have been isolated in good yield in this way and resemble living nuclei in many respects; however, contamination by intact cells and mitochondria has been a serious problem.

As a result of osmotic lysis, the latter contaminant also tends to release deoxyribonuclease (DNAase), which damages nuclei. Sucrose tends to reduce this damage; the use of 0.3 *M* sucrose + 0.06 potassium glycerophosphate more effectively minimizes this effect (Philpot and Stanier, 1955). It will be recalled that glycerophosphate is also known to protect herpes virus from an inactivating phosphatase during the isolation of the virus (Amos, 1953). Novikoff (1956) has recommended a mixture of 0.25 % *M* sucrose — 7.3 % polyvinylpyrrolidone for homogenization and subsequent isolation steps leading to the electron microscopy of nuclei. Nevertheless, cytoplasmic contaminants are still frequently observed in such preparations.

Hogeboom and associates (1952) isolated cellular fractions from homogenates in 0.25 *M* sucrose containing  $2 \times 10^{-4}M$   $Ca^{++}$  and thereby reduced mitochondria in nuclear preparations (70 to 90 % yield) to less than 0.5 % of their number in liver cells. However, a small number of intact cells and collapsed cell membranes still contaminated such preparations. The isolated nuclei were optically homogeneous and did not appear to be physically altered. Liver cell nuclei isolated in this way, accounted for 12 % of the total nitrogen of the liver, but the extraction of water-soluble compounds by this technique was not ruled out. Nevertheless, these workers demonstrated that the ability to synthesize diphosphopyridine nucleotide (DPN) was completely retained by these nuclei. A modification of the  $Ca^{++}$ -sucrose procedure has recently been applied to the isolation of wheat germ nuclei (Johnston *et al.*, 1957).

Most recently, it has been discovered (Allfrey *et al.*, 1957a) that thymus nuclei isolated in sucrose are equivalent in DNA, protein, and enzyme content to thymus nuclei isolated by the nonaqueous Behrens method. These workers considered such "sucrose" nuclei to be about 90 % pure and suitable for use in studies of protein and nucleic acid synthesis, whose results will be described below.

Parenthetically, it may be noted that virologists have not yet reported an attempt to study the reproduction of intranuclear viruses in isolated infected nuclei.

#### 4. *Some Data on the Composition of Nuclei*<sup>1</sup>

*a. Constituents of Normal Nuclei.* Although the procedure described by Hogeboom and Schneider (1952) is recommended to give high yields of nuclei, it appears that  $\text{Ca}^{++}$  aggregates submicroscopic particles, causing their incorporation into the mitochondrial fraction. At the present time, the use of sucrose solutions in homogenization and in centrifugal fractionation seems to permit the best balance in the separation of tissue components. Such an analysis for liver has been given by Muntwyler *et al.* (1950), comparing the distribution of nitrogen, phosphorus, and the nucleic acids in normal liver and in the liver of animals held under conditions of restriction of dietary protein. Following homogenization, substances are separated according to their mass by the controlled use of defined gravitational force for limited time intervals.<sup>2</sup> Thus, nuclei are deposited at 200*g* for 10 minutes, mitochondria at 8500*g* for 10 minutes, and microsomes at 20000*g* for 1 hour.

As given in Table I, the DNA of the cell is entirely in the nuclear fraction, whereas only 10–15 % of the total RNA was found in this material. This fraction contained 15–20 % of the nitrogen (N) and phosphorus (P) of the cell. Under conditions of dietary protein depletion a small loss of N and P occurred per gram of liver. The major site of such loss was, however, in the microsomal fraction, which had lost 35 % of its nitrogen.

Although RNA decreases markedly in liver, kidney, and muscle under conditions of a prolonged protein fast, the DNA content of these organs remains constant. However, it has been reported that similar conditions markedly reduce the DNA content of pancreas, adrenal, and spleen, as well as their contents of RNA (Mandel *et al.*, 1954).

<sup>1</sup> Spiegelman (1956) has briefly recorded the isolation of bacterial nuclei from *B. megatherium*, after disrupting protoplasts with lipase. Also, see Spiegelman *et al.* (1958).

<sup>2</sup> Instead of relying on differences in sedimentation rate in a liquid of constant density, recent procedures have successfully explored the separation of particulates within homogenates in media of controlled density gradients (Holter *et al.*, 1953; Kuff and Schneider, 1954; Anderson, 1955; Ottesen and Weber, 1955). It can be noted that the technique was introduced in the separation of plant virus particles (Brakke, 1951, 1953) prior to its development for cytological study.

In liver, about 45 % of the N and P and 36 % of the RNA are to be found in the supernatant fluid after sedimentation of the particulate materials. This nonparticulate RNA is also associated with protein, in almost all cells. In general, neither RNA nor DNA exists in the free state in uninfected cells, but only as nucleoprotein. However, the existence of a particle of free RNA (Lindner *et al.*, 1956) has been reported in some plants.

TABLE I  
RAT LIVER CELL FRACTIONS <sup>a</sup>

	Nitrogen		Phosphorus	
	Control	Protein-deficient	Control	Protein-deficient
Homogenate (mg./gm.)	19.8	24.0	3.3	3.1
Nuclear fraction	4.54	4.56	0.56	0.55
Mitochondria	5.14	3.99	0.40	0.36
Microsomes	7.95	5.22	1.18	0.90
Residual cytoplasm	12.95	11.17	1.56	1.63

	DNA		RNA	
	Control	Protein-deficient	Control	Protein-deficient
mg./gm. liver	2.68	3.06	8.39	8.03
Nuclei	2.52	3.26	1.13	0.96
Mitochondria	—	—	0.43	0.56
Microsomes	—	—	4.16	3.49
Residual cytoplasm	—	—	3.04	3.78

<sup>a</sup> Muntwyler *et al.* (1950).

McIndoe and Davidson (1952) have presented data on the distribution of acid-insoluble P compounds of various types in animal nuclei. The nucleic acid and phospholipid contents of the nuclei studied are given in Table II. As can be seen, the ratios of RNA to DNA may vary from 0.05 in the erythrocyte nucleus of a tumor-bearing fowl to 1.2 in the tumor nucleus of the same bird. On the other hand, the ratios of RNA to nuclear mass vary little, i.e., between 0.15 to 0.33.

Osawa *et al.* (1957) have recently studied the nucleotide composition of the acid-soluble fraction of calf thymus nuclei isolated in sucrose or by the Behrens technique. Both types of nuclei have given similar patterns of nucleotide distribution, although some nucleotides tend to be lost by the former method. In addition nonaqueous nuclei contain substantially the

TABLE II  
 MEAN VALUES FOR THE COMPOSITION OF THE SINGLE CELL NUCLEUS <sup>a, b</sup>

	Liver, rabbit	Liver, rat	Liver, rat (77 hr. fast)	Liver, tumor-bearing fowl	Erythrocyte, fowl	Erythrocyte, tumor-bearing fowl	GRCH <sub>15</sub> tumor, fowl
Thymus	0.21	0.70	2.14	0.93	0.19	0.48	10.3
Phospholipid	0.45	1.21	2.88	2.13	0.55	0.25	6.38
RNA	7.30	6.57	9.65	9.85	2.91	2.46	5.09
DNA	28.2	44.4	79.4	66.5	16.5	11.1	217
Nuclear mass							

<sup>a</sup> This table is somewhat condensed from the original of McIndoe and Davidson (1952).

<sup>b</sup> 10<sup>-12</sup> gm./nucleus.

same kinds and amounts of nucleotides present in the whole tissue. Mononucleotides [adenosine monophosphate (AMP)] predominate in the non-aqueous nuclei. However, a rapid isolation of nucleotides from aqueous nuclei have revealed a markedly higher content of triphosphates, e.g., ATP. The quantity of nucleotides amounts to about 1.7 % of the total mass. These workers have also found that nuclei from metabolically active tissues, such as calf thymus or liver, have a higher content of nucleotides than is found in nuclei of chicken erythrocytes or trout sperm, which are metabolically relatively inert.

In wheat germ nuclei isolated by the  $\text{Ca}^{++}$ -sucrose method, about 6 to 7 % of the total nucleic acid plus protein is DNA (Johnston *et al.*, 1957), and about 1.5 % is RNA, which is 2.5 times as concentrated in the whole tissue as in the nuclei.

Analyses of individual nuclei by spectrophotometric methods have revealed the existence of classes of nuclei containing multiples of the DNA content of the most frequent class. Such high values have been ascribed to polyploidy. One approach to the analysis of effects of an increased chromosomal complement has been the study of the relation of DNA content to nuclear volume. Such a correlation exists in the development of polytene salivary gland nuclei of *Drosophila*, whose giant chromosomes have been so important in the development of cytogenetics. The gland nuclei may contain up to 400 times the DNA of the anlage cells and are proportionately larger (Swift, 1950a). However, in a wide variety of developing tissues, the DNA content of the nuclei is quite unrelated to nuclear volume; the latter function appears more closely related to protein content (Alfert, 1954). For example, Alfert and Bern (1951) have reported that the injection of estrogen into ovariectomized rats doubles the volume of the nuclei of uterine gland cells without change of their DNA content. In these instances, volume is presumably determined by non-histone nuclear protein, since the DNA-histone ratio tends to remain constant under conditions in which the variations of other nuclear protein fractions may be considerable (Ris and Mirsky, 1949).

Striking studies of the relation of the number of chromosome sets to cell size and composition have been performed by Ogur, Lindegren, and their collaborators. Increases of ploidy have proportionate effects on the size of yeast cells. In Table III are presented data which demonstrate that a constant relation exists between numbers of chromosome sets, the content per cell of DNA, RNA, and metaphosphate in this organism, and the  $\text{O}_2$  consumption and  $\text{CO}_2$  production per cell in air.

*b. On Nuclear Pathology.* Leuchtenberger (1950) has described cytochemical changes occurring during pycnotic degeneration of nuclei of normal and neoplastic tissues. During this process, the nuclei became spherical and shrunken, nucleoli disappeared, and chromatin became homogeneous and

fused. At this stage, about half of the protein had disappeared and the DNA was extensively depolymerized but not lost from the nucleus, as measured by stainability with methyl green and by the Feulgen method. Later, the DNA also progressively disappeared and it was suggested that the maintenance of histone content is important for preservation of the highly polymerized state of the associated DNA. Nevertheless, it is known that deoxyribonuclease depolymerizes DNA, even when associated with histone. It was concluded that pyknosis involves an initial high proteolytic activity in the nucleus, followed by action of nuclease.

TABLE III  
PLOIDY—DEPENDENT CHARACTERS IN SACCHAROMYCES<sup>a</sup>

	Haploid	Diploid	Triploid	Tetraploid
DNA-P/cell <sup>b</sup>	2.26 ± 0.23	4.57 ± 0.60	6.18 ± 0.54	9.42 ± 1.77
RNA-P/cell <sup>b</sup>	55.6 ± 16.6	96.5 ± 22.4	164.0 ± 33.6	172. ± 6.
Meta-P/cell <sup>b</sup>	100.1 ± 25.8	166.5 ± 31.2	217.8 ± 57.7	362. ± 34.
Dry wt./cell <sup>c</sup>	1.38 ± 0.23	2.70 ± 0.61	3.40 ± 0.67	7.29 ± 0.5
Q O <sub>2</sub> /cell	7.93 ± 2.7	16.5 ± 2.7	24.6 ± 3.3	33.2 ± 3.4
Q CO <sub>2</sub> (air)/cell	19.3 ± 4.2	33.3 ± 6.7	49.9 ± 6.3	77.5 ± 14.2

<sup>a</sup> Ogur *et al.* (1952), Ogur (1954).

<sup>b</sup> Units  $\mu\text{g}$  ( $\times 10^9$  for P data).

<sup>c</sup>  $\mu\text{g}$  ( $\times 10^5$  for dry wt. data).

The depolymerization of the DNA of mesenchymal cells is also a feature of lupus erythematosus (L.E.). The bone marrow of patients with this disease show the L.E. cell, a polymorphonuclear granulocyte whose cytoplasm contains a homogeneous basophilic mass comprised of depolymerized DNA. L.E. sera contain a  $\gamma$ -globulin fraction capable of binding to intranuclear DNA (Holman and Kunkel, 1957). The relation of this reaction to the production of the L.E. cell and the disease entity is being studied in many laboratories.

*c. Mineral Constituents.* Data on the distribution of inorganic constituents in cells have been relatively meagre until the recent study of Thiers and Vallee (1957). Little can be said about their presence within the nucleus. As summarized by Horning (1951), microincineration studies of this structure *in situ* have revealed the presence of fair amounts of ash, probably calcium (Ca) and magnesium (Mg) salts, in chromatin. The presence of these cations is particularly marked in certain states of mitosis, such as late prophase and metaphase. Conversely, the "resting" nuclei of ganglion cells were apparently ash-free, with the exception of the nucleoli. The presence of significant amounts of iron (Fe) in the latter has been claimed.

Williamson and Gulick (1944) have analyzed thymus cells and nonaqueous nuclei for Ca, Mg, and P. Both divalent cations were concentrated in the nuclei.  $\text{Ca}^{++}$  was present in amounts considerably greater than  $\text{Mg}^{++}$  and approached the content of the diesterified phosphate present in DNA.

In addition to an unusually high content of these metals, nuclei contain the largest proportion of the manganese of the rat liver cell, as well as significant amounts of cellular zinc. Such data, as well as a comparison of the cation contents of various cell fractions, are presented in Fig. 4.

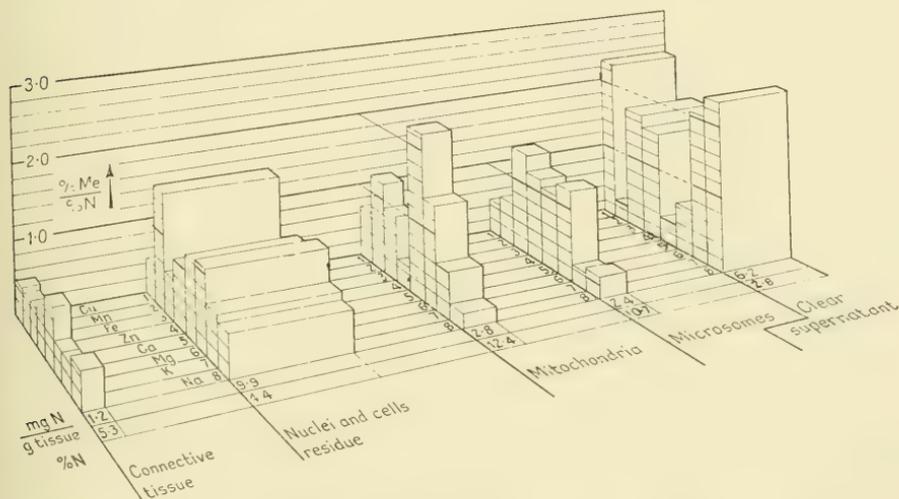


FIG. 4. Sodium, potassium, magnesium, calcium, zinc, iron, manganese, and copper in fractions of rat liver. The height of each bar represents the metal content of each fraction, expressed as a percentage of the total content of that metal in liver, divided by the same parameter for nitrogen. This normalization allows direct comparison between different fractions and metals. The width of each bar represents the nitrogen content of the fraction (Thiers and Vallee, 1957).

Chromosome breakage in *Tradescantia paludosa* has been shown to increase considerably when the plants are grown in decreasing amounts of magnesium (Steffensen, 1953) or of calcium (Steffensen, 1955). These results were consistent with the data given above, as well as that of Bernstein and Mazia (1953), which indicated that the dissociation of chromosomes into constituent deoxyribonucleoproteins was facilitated by citrate or other sequestering agents. Thus,  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  seemed to be implicated in maintaining the structural continuity of the chromosome.

Levine (1955) has reported that altering cationic conditions, particularly with respect to  $\text{Ca}^{++}$ , markedly affects crossing over in *Drosophila melanogaster*. However, King and Rubinson (1957) have summarized considerable

evidence to indicate that this organism contains at most trace amounts of calcium and that the ion is not concentrated in chromosomes to any degree higher than that prevailing in the cytoplasm of body fluids. In recent studies of the deformational changes effected in chromosomes by ethylenediamine-tetraacetic acid (Kaufmann and McDonald, 1957), it was concluded that alterations in the structural nucleoproteins arose from modifications of the general ionic environment of the cell and not merely from the removal of specific divalent cations.

*d. The Protein and Amino Acid Composition of Nuclei.* A number of reviews have appeared on the proteins of the nucleus (Hamer, 1951; Allfrey *et al.*, 1955b; Butler and Davison, 1957). Some of these proteins will be mentioned below in connection with the chemical dissection of chromatin. Soluble proteins possessing the properties of globulins may be extracted from the nuclei of calf thymus and liver with 0.14 *M* NaCl. In addition to these poorly defined and relatively unfractionated materials, basic proteins are prominent constituents of nuclei, associated with DNA in chromosome structure. Relatively low molecular protamines, very rich in arginine and devoid of aromatic amino acids and sulfur-containing amino acids, are found in sperm. More complex basic proteins or histones are present in the somatic cells of the same animal. The histones contain tyrosine and phenylalanine, but lack tryptophan. Although it is commonly stated that histones contain sulfur, the analyses of Hamer (1951) and of Daly *et al.* (1951) indicate the absence of cystine and methionine. Intermediate types of basic proteins have also been described in the sperm of sea urchins, starfish, and squid (Hamer, 1955).

The most recent studies of histones derived from calf thymus nucleohistone suggest that these substances consist of a very complex mixture, whose fractionation is made difficult by their tendency to associate and to aggregate (Butler and Davison, 1957). It has at least been shown that a lysine-rich histone may be separated from an arginine-rich histone in such a mixture.

Sperm nuclei are presumed to contain a genome which is also found in somatic nuclei, although the latter nuclei contain two chromosome sets in contrast to the haploid sperm. The fact that their nuclear proteins are qualitatively so different has been considered to point away from a key genetic role of the proteins. In immature salmon testis, the transformation of somatic nucleohistone to sperm nucleoprotamine was first studied by Miescher (1897) in the 1870's. Nucleoprotamine in the salmon accounts for at least 91 % of the nuclear mass (Pollister and Mirsky, 1946); a replacement of histone by protamine must occur in this change. Alfert (1956) has shown that this replacement does indeed occur during the maturation process, more particularly after completion of meiosis during an advanced stage of spermiogenesis. A comparable result has been obtained for the replacement in bull testis (Vendrely *et al.*, 1957).

Vendrely *et al.* (1956) have studied the ratio of arginine to DNA-P in the nuclei and nucleoproteins of many animals. They have recorded that with a very few exceptions, e.g., carp, the transformation of somatic nuclei to sperm nucleus is accompanied by a marked increase in the ratio of molecules of arginine to atoms of DNA-P, i.e., from 0.33-0.41 to 1.10-1.27.

Estimations have been made of the histone and DNA content of nuclei during different stages of division (Bloch and Godman, 1955). The synthesis of these substances proceeded simultaneously, resulting in their doubling prior to cell division.

*e. Lipids.* Stoneburg (1939) and Dounce (1943) demonstrated the presence of these important structural elements in their preparations of nuclei. A number of workers have studied the fractionation of these lipids (Dounce, 1955). Of particular interest are the findings of the absence of cerebrosides and of significant amounts of neutral fat and phospholipid, consisting mainly of lecithin and cephalin. Stoneburg had earlier reported relatively large amounts of ether-insoluble lipids which were then presumed to be sphingomyelin and cerebrosides. Levine and Chargaff (1952), who also found nuclear lipids insoluble in petroleum ether, suggested that much of the nuclear lipid may exist as polymeric entities. The latter workers found ethanalamine, a constituent of one type of cephalin, in amounts in nuclear lipid equal to that of choline, the base present in lecithin and sphingomyelin. Only small amounts of serine, thought to be derived from serine cephalin, were found.

A study of phospholipid synthesis in regenerating rat liver (Johnston *et al.*, 1954) has shown a high rate of synthesis of phospholipid (cephalin, lecithin, and sphingomyelin) coincident with mitosis. This was not affected by colchicine, which stops development at metaphase. It was suggested, therefore, that lipid synthesis was associated with either interphase or prophase, but not with anaphase or telophase.

*f. The DNA Content of Nuclei.* The following circumstances have led us to believe that DNA plays a particularly important role in the determination of heredity:

1. DNA is a component of all chromosomes in all types of cells.
2. Highly purified DNA is active in bacterial transformation in pneumococci, *Hemophilus*, etc.
3. DNA is the major component (about 97 % of the total) transferred from a phage to the bacterium it infects, and therefore determines not only the synthesis of viral DNA but also of viral protein.

In this context we are led to the propositions that (1) the DNA content of the nucleus is characteristic of its content of genetically active substance; (2) in multicellular organisms, diploid somatic cells should have identical DNA contents; (3) diploid somatic cells will contain twice the DNA present

in the haploid gametes. The possible validity of proposition (1) will be discussed in a later portion of this section.

Boivin and the Vendrelys (1948) and Mirsky and Ris (1949) first estimated the DNA content of nuclei. It was shown that the DNA content of nuclei of different bovine tissues in the same animal were nearly identical and twice that of the sperm (Boivin *et al.*, 1948). Some data on the DNA contents of the nuclei of various fowl tissues are summarized in Table IV. Many workers

TABLE IV  
AVERAGE DNA CONTENT PER NUCLEUS OF FOWL TISSUE <sup>a, b</sup>

Tissue	DNA
Sperm	1.25
Erythrocyte	2.58
Liver	2.65
Kidney	2.28
Spleen	2.63
Heart	2.54
Pancreas	2.70

<sup>a</sup> Allfrey *et al.* (1955b).

<sup>b</sup> Mg.  $\times 10^{-9}$ .

have obtained similar results, e.g., Swift (1950) for plant nuclei, and it has generally been concluded that the amount of DNA was constant for each chromosome set. Indeed, it is now known that the base composition of DNA remains the same from tissue to tissue in the same animal.

Leuchtenberger and the Vendrelys (1951) applied both cytochemical and chemical methods to the analysis of isolated nuclei and concluded that the thesis of Boivin was essentially correct, at least with respect to adult tissues. In addition, they were able to distinguish three classes of liver nuclei whose DNA contents were in the ratio of 1 : 2 : 4—a phenomenon ascribed to the existence of diploid, tetraploid, and octaploid nuclei in liver.

The constancy of DNA per cell has been a very useful base line for many researches. Kurnick (1951) has followed both the average increase in mass and increase in DNA during normal growth of kidney tissue. Since the two maintained a constant ratio, it was concluded that the increase in mass was due primarily to increase in cell number rather than to the growth of individual cells. Davidson and his collaborators (Leslie, 1955) have similarly used DNA content as a standard in investigating the change of cell composition in tissue cultures, a type of material in which suitable base lines had been unusually difficult to establish, prior to the development of suspended cell cultures.

Although sperm do have one-half of the DNA content of the diploid cell, it has been difficult to obtain complete data on ova. Marine invertebrates have provided most of the gametes studied. In general, chemical methods have revealed an apparent deoxyribose content in eggs greatly in excess of that found in the sperm. Frog sperm, for instance, contain  $8.6 \times 10^{-12}$  gm. DNA; the eggs are stated to contain almost 10,000 times as much DNA (Hoff-Jørgensen, 1954). In the frog's egg, large amounts of the excess deoxyribose exist in the cytoplasm (Hoff-Jørgensen and Zeuthen, 1952) and the DNA content of the frog embryo does not increase until about the 5000-cell stage (Hoff-Jørgensen, 1954). Thus, cytoplasmic DNA and deoxyribosides provide a store for the production of nuclear DNA. Frog ova nuclei have recently been isolated; prior to a reductional division to the haploid state their DNA content was twice that of the haploid sperm (England and Mayer, 1957).

Numerous studies have been made on the DNA content of nuclei of tumors. In most instances it was found that, with certain exceptions attributable to polyploidy, the values were essentially identical with those of normal cells. However, in the case of the Ehrlich ascites mouse tumor, the DNA content was about twice that of the normal mouse cells or other mouse tumors. In the development of crown gall tumors of tomato, a marked increase of DNA also occurred prior to the appearance of proliferation (Klein, 1952). This increase in DNA content is followed by cell divisions, however, which reduce this substance to a value approaching that in normal tissue.

The nuclei of malignant tissue show a greater scatter of DNA content than do those of normal tissue, a result attributed to polyploidy and a higher proportion of dividing cells in tumors. Nevertheless, observers have stated that even in populations of presumably normal nuclei, examined cytochemically, the highest value may be 50 % larger than the lowest. This may arise from technical difficulties, such as the existence of optical inhomogeneity of individual nuclei, or to an actual variation of DNA content from nucleus to nucleus. If the latter is the case, we are faced with the problem of deciding whether the observed differences arise from a variation per chromosome set from nucleus to nucleus, or from examining nuclei in different stages in the production of chromosomal substance during the interphase.

Although the apparent anomalies displayed by eggs relative to the principle of DNA constancy seem to be resolved by the discovery of cytoplasmic DNA and the apparent equality of DNA in sperm and egg nuclei, some anomalies in DNA contents during embryological development have not yet been explained. For example, Moore (1952) has studied the DNA content of embryonic tissues and has observed that so-called diploid nuclei exhibit a wide range of DNA content, i.e., 1.5 to 2.5 times that of haploid nuclei. She has concluded that the rule of the constancy of DNA per chromosome set is

not valid for embryonic tissues. As noted above, it is difficult to assess the relationship of this variability to the stage of DNA doubling and differentiation in which the cell has been examined. This case, among others, has been discussed in some detail by Vendrely (1955).

The analyses of these instances of apparent variability has inevitably been tied to the problem of the time of DNA doubling during the mitotic cycle. Most workers (Thorell, 1955) have pointed to the interphase for this event, which, as noted earlier, occurs simultaneously with histone doubling (Bloch and Godman, 1955). However, Vendrely (1955) has recorded a number of exceptions to this time of DNA doubling, involving any time from late prophase in onion tip mitosis to late telophase in several vertebrate cells. It may be noted that a far more complicated series of patterns of DNA synthesis and divisions occurs in the development of the pollen grain (Ogur *et al.*, 1951).

It might be imagined that changes in nuclear composition might occur during intense physiological activity, as in the development of the secretory activity of various glands. In the growth of the salivary glands of adult *Drosophila melanogaster*, the cells double in size but do not divide. In this system, the DNA content and volume of the nuclei do not change, although the RNA and protein contents of the cells increase markedly (Patterson and Dackerman, 1952). Such a pattern appears to be common; however, the following remarkable case has been reported: in the salivary glands of the snail, *Helix pomatia*, the secretion of polysaccharide into the cytoplasm is accompanied by a 30-fold decrease of nuclear DNA and nuclear volume (Leuchtenberger and Schrader, 1952).

That the DNA content of adult nuclei is usually a constant character of the species is supported by considerable evidence. It was hoped that this value would prove a useful taxonomic tool in studying evolutionary relationships. This expectation has not been realized, following the accumulation of data on this parameter in a wide variety of organisms (Allfrey *et al.*, 1955b; Vendrely, 1955). Among the invertebrates, there does seem to be a general drift to more DNA per cell in the higher phylogenetic categories, although numerous exceptions to this trend can be found. Among the vertebrates, not even a drift of this sort can be detected. If anything, the series (Dipnoi, Amphibia, Reptilia, and Aves) is accompanied by a drop in DNA per nucleus. The range of values within certain classes, such as birds and mammals, is not very great. However, among the Amphibia the range may be 20-fold, as in the case of the toad, with  $7 \times 10^{-9}$  mg. DNA per nucleus, to *Amphiuma* with  $168 \times 10^{-9}$  mg. DNA per nucleus. Mirsky and Ris (1949) have concluded that from species to species the DNA content per cell follows the size of the cell more closely than the number of presumed genetic units.

### 5. *The Isolation and Composition of Chromosomes*

Starting with intact tissues or isolated nuclei, nuclear membranes have been disrupted by vigorous mechanical treatment, such as that provided by a Waring Blendor. The extent to which these treatments damage the internal chromosomal structures or produce artifacts is not clear. Sonic vibrations readily depolymerize DNA and the grinding of bacteria with alumina can result in the complete solubilization of deoxyribonucleoprotein. It may be noted that the shearing forces produced in a Waring Blendor readily convert thymus DNA of average molecular weight  $8 \times 10^6$  to a DNA of average molecular weight  $2 \times 10^6$  (Cohen, Hanlon, and Schachman, unpublished observations).

In any case, Claude and Potter (1943) and Mirsky and Pollister (1943) showed that fine chromatin threads containing deoxyribonucleohistone may be isolated from the disrupted resting nuclei of leukemic cells, erythrocytes, and liver. These threads contain over 90 % of the DNA of the cell. If these threads are indeed the chromosomes of resting nuclei, this would be important evidence for the theory of the continuity of chromosomes throughout the life of the cell.

Several workers have challenged the claim that these threads are condensed chromosomes. Lamb (1950) states that various disruption techniques merely draw out the nuclei into threads or fragments that bear little resemblance to mitotic chromosomes. Schneider and Hogeboom (1951) report that the addition of isotonic saline to liver nuclei caused the precipitation of threads within the previously homogeneous nuclei. If nuclei are isolated in sucrose and disrupted, threads were not observed and over 60 % of the DNA was not sedimentable at 60,000 *g*.

However, Mirsky and Ris (1947, 1951) have examined their isolated threads quite closely and consider that they have the visible characteristics of chromosomes. Further, a number of individual types can be seen repeatedly. Polli (1952) and Denués (1952, 1953) have supported this view. A close similarity has been found between the threads from erythrocyte nuclei and the chromosomes observed in the metaphase plates of vertebrate cells. Mitotic chromosomes also correspond closely in length with isolated chromosomes of the same species, which also show characteristic intraspecific differences.

Four types of compounds have been described in the "chromosomes." Nucleohistone, consisting of DNA and histone, has been extracted at low ionic strength, a process which is markedly facilitated by a prior treatment of the chromosome with a chelating agent, such as citrate or ethylenediamine tetraacetate (Mazia, 1954). The components of nucleohistone may be obtained in the dissociated state by extraction in solutions of high ionic strength, e.g., 1-2 *M* NaCl. The viscous solution produced in this way may be

precipitated in isotonic saline to give threads of histone-nucleate. These probably should be regarded as artifacts in which the original spatial relations of histone and DNA no longer exist (Cohen, 1945); in addition, non-histone protein can thus be introduced into the nucleoprotein when the latter is taken from the dissociated state in  $M$  NaCl to  $0.11 M$  NaCl.

DNA and histone comprise a large proportion of chromosomal substance, e.g., about 80 % of calf thymus chromosomes. After extraction of nucleohistone with salt, the remainder of the chromosome exists in the form of small coiled threads, which stain red in methyl green-pyronine and consist of tryptophan-containing protein or proteins and about 10 % RNA (Allfrey *et al.*, 1955b). The proportion of the tryptophan-containing protein in chromosomes is stated to vary directly as the quantity of cytoplasm in the cell. For instance, placing DNA content as a constant, liver chromosomes contain four times as much residual protein as thymus chromosomes.

The careful analysis of chromosome structure in fixed cells by digestion with enzymes of known specificity followed by staining has been described by Kaufmann *et al.* (1950). Although previous investigators had stressed the solubilization of chromosomes by trypsin, attributing the integrity of the chromosome to a particular protein continuum, these workers have shown that the subsequent washes, after trypsin action, served to dissociate the degradation products. Although DNAase removed the deeply staining basophilic substance from the bands of certain chromosomes, this did not dissolve the chromosome. However, successive treatments with proteases and nucleases do disintegrate the chromosomes and it has been concluded that the chromosome represents an integrated fabric in which no single protein or nucleic acid may be regarded as the primary structural component.

However, in studies on isolated liver chromosomes, it has been shown that after extraction of histone at pH 2.8, which leaves the DNA on the still intact structure, treatment with DNAase does disrupt the chromosome into minute coiled threads of residual protein (Mirsky and Ris, 1951). It was concluded, contrary to the conclusions of Kaufmann *et al.* (1950), that the residual protein forms the fundamental thread of the chromosome, whose gross configuration depends on the association of this protein and DNA. It was proposed that in native chromosomes DNA is bound to both histone and other protein structures.

One might expect that the residual protein remaining after removal of nucleic acid and histone consists of many proteins. Unfortunately, this fraction has been difficult to obtain in amounts permitting fractionation and characterization. Nor have immunochemical techniques proven useful since

no components of chromosomes have yet been observed to be antigenic.<sup>1</sup> However, various cytoplasmic structures readily induce antibody production and it would be quite desirable to have data on the cross-reactivity of the chromosomal constituents with these antisera, both as an approach to possible metabolic relationships with other cellular substances and to the problem of possible contamination with cytoplasmic antigens.

### 6. *Properties of Isolated Mitotic Figures*

Preparations of fertilized frog eggs or sea urchin eggs, in which the cells were dividing approximately synchronously, were homogenized in very cold aqueous alcohol to prevent autolysis (Mazia and Dan, 1952). Intact mitotic spindles could be isolated by differential centrifugation and further purified with a detergent solution containing  $H_2O_2$ , which maintained the —S—S— linkages apparently essential to fiber formation. If the initial dispersal of egg material is effected in the presence of ATP, subsequent purification with detergents can be avoided. Isolated mitotic figures can be seen in Fig. 5.

These structures can be isolated in all mitotic stages containing the spindle and appear to consist of characteristic fibers containing embedded attached chromosomes. During the distribution of chromosomes effected by mitosis, the genetic material behaves quite passively. The chromosomes comprise a very tiny portion of the total mass of the mitotic apparatus, which in the fertilized sea urchin egg approaches 12 % of the total protein of the cell. As noted earlier, spindle protein contains about 2 % of adenine nucleotide in a tightly bound form.

Mazia (1957) has reported on the properties of the proteins of the spindle. In the cell taken before division, there is a comparable mass of soluble protein, which aggregates as the spindle forms and returns to a soluble form as the mitotic apparatus disappears. Mazia (1956) has reviewed the biochemistry and biophysics of cell division and has discussed the problem of the origin and role of the spindle structures in some detail.

### 7. *Nuclear Sap*

Direct ultraviolet spectrophotometry of the nuclear fluid in which chromosomes are immersed has not revealed the characteristic absorption of nucleic acid. This sap was removed from oocyte nuclei; analysis revealed a typical

<sup>1</sup> Two recent reports bearing on this problem can certainly be expected to stimulate a careful exploration of this question. The first is the claim that isolated DNA is indeed antigenic (Blix *et al.*, 1954). The second is the study of Billingham *et al.* (1956), which records the observations that the antigens important in the development of skin "transplantation immunity" are confined to nuclei. Furthermore, the antigenic activity of nuclei is unaffected by ribonuclease and by trypsin but is destroyed by treatment with deoxyribonuclease. Isolated histone nucleates, after digestion by trypsin but not before, were found to have perceptible immunizing power.

protein spectrum (Brown *et al.*, 1950). On hydrolysis, many amino acids could be detected. The material was not particularly rich in basic amino acids. Degradation products of the nucleic acids could not be detected.

### 8. *Nucleoli*

These usually spherical bodies are believed to be expansions of special chromosome regions, which are for the most part heterochromatic in their Feulgen stainability. The nucleoli usually disappear during mitosis; possibly they are absorbed into the chromosomal substance. In Fig. 3b the nucleolus has been represented as a coil of linearly arranged particles on a doubled thread, a structure which has been seen as such in electron micrographs of sections of certain nuclei (Novikoff, 1957). Consistent with this representation is the fact that no evidence has ever been obtained for a nucleolar membrane. The absence of such a membrane and the apparent presence and distribution of particles in a plant nucleolus can be seen in the electron micrograph of Fig. 6.

In certain stages of oocyte development, nucleoli may exist in large numbers, detached from the chromosomes at the nuclear membrane. Some workers have stated that they pass intact through the membrane, others that their contents are everted into the cytoplasm. According to Caspersson (1950), they appear to be largest and most dense under conditions of extensive protein synthesis. A role in this important metabolic function has been ascribed to the nucleolus, as a unit of transport between chromosomal sites of synthesis and the cytoplasm. No direct evidence supporting this hypothesis has yet been presented.

Nucleoli do not take the Feulgen stain, although they do absorb ultra-violet light. Since this absorption is diminished after treatment with ribonuclease, they are considered to contain RNA. Caspersson (1950) had interpreted spectrophotometric evidence to imply the presence of histones in the nucleolus. In the nucleoli of the starfish oocyte (Vincent, 1952), it was not possible to demonstrate the presence of basic protein. On the other hand, the isolated nucleoli of liver cell are reported to contain 22 % of histone, or a protein extractable in cold 0.1 N HCl and precipitable with ammonia (Monty *et al.*, 1956). However, the significant DNA content of these preparations suggests the presence of nucleohistone as a result of contamination by chromosomal fragments.

The isolated nucleoli of the starfish oocytes (Vincent, 1952) were spherical refractile bodies containing one or more vacuoles. They swelled in alkali and were broken down by trypsin. They were solid or semisolid and contained 30 % protein and 2-5 % RNA. The composition of the latter was different from the RNA of cytoplasmic granules in that the former contained more guanine and less uracil. Their enzyme content will be discussed below.

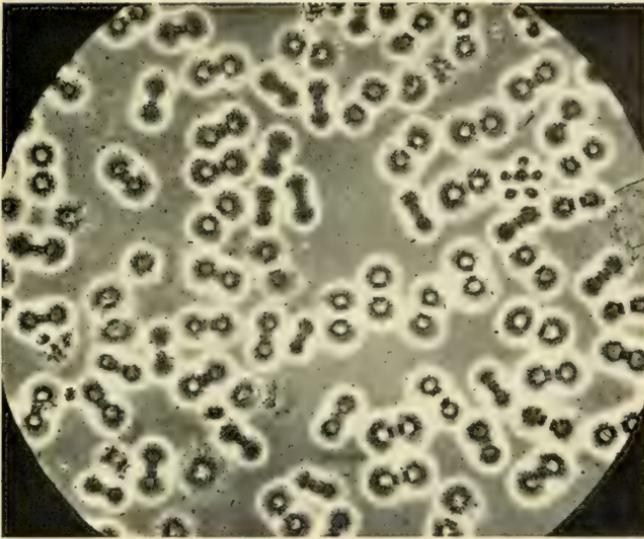


FIG. 5. Mitotic figures isolated from fertilized sea urchin eggs (Mazia and Dan, 1952; Mazia, 1956). Upper photograph: most figures are in metaphase or anaphase. Lower photograph: high-power view of isolated mitotic apparatus at anaphase.

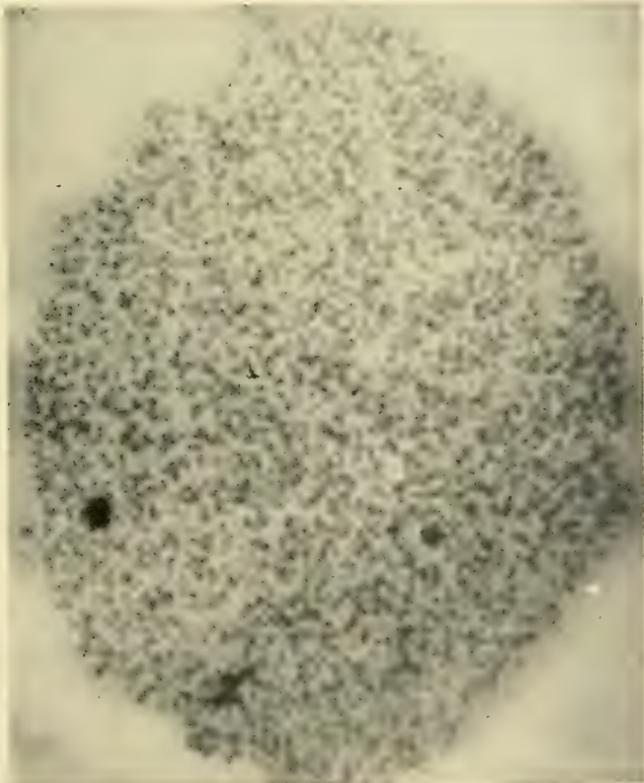


FIG. 6. Electron micrograph of isolated nucleolus from isolated nuclei of pea embryo (Johnston *et al.*, unpublished).



## 9. Nuclear Membrane

The ready penetration of enzymes into isolated nuclei has been stressed by many workers, e.g., Anderson (1953), who have pointed to the apparent porosity of the nuclear envelope. Recent electron micrographs of interphase nuclei (Gall, 1954; Watson, 1955) have clearly revealed the existence of a double nuclear membrane in which circular pores or annuli are formed by continuities between inner and outer membranes (see Fig. 3). Similarly, porous nuclei exist in a wide variety of tissues and the ease of penetration of proteins into nuclei is readily explained by the physical existence of holes. Nevertheless, the properties of the retention of protein, nucleotide, and  $K^+$  by the nuclei of thymus lymphocytes (Allfrey *et al.*, 1957a; Osawa *et al.*, 1957) raise questions concerning the nature of this particular membrane.

In addition to this direct contact of nucleus and cytoplasm, involving perhaps one-tenth of the nuclear surface (Watson, 1955), the remaining area is in indirect contact through membrane-enclosed cavities of the endoplasmic reticulum, which, in some cells, at least, appear to be continuous with the outer nuclear membrane (see Fig. 3). It has been suggested that, whereas large molecules may pass through the pores, inorganic ions and small molecules may be exchanged by way of the endoplasmic reticulum. It is thus supposed that there are two distinct pathways of exchange between nucleus and cytoplasm, related to the two structurally distinct points of contact between these parts of the cell.

### C. Cytoplasmic Structures

#### 1. Historical Notes

Until about 15 years ago the isolation of cytological structures and their subsequent characterizations were being attempted exclusively by biologists, who also provided much of the chemical data. The anatomists, Bensley and Hoerr (1934a,b), first separated mitochondria and reported on their content of protein and lipid (Bensley, 1942; Lazarow, 1943). Claude attempted the isolation of Rous tumor virus by means of differential centrifugation, which had proved so useful in the isolation of various viruses. In the course of these studies, Claude (1939, 1940) discovered the presence of antigenic submicroscopic particulates, which he called microsomes, in many normal tissues; indeed, these were found to be extensive contaminations of his virus preparations. Simultaneously, Brachet, who was studying the correlation of basophilia and RNA content during embryological development, deduced the existence of a small particle fraction rich in RNA, and with his collaborators proceeded to the isolation of cytoplasmic particulates in yeast, etc. Although many biochemists had observed the dependence of various enzymatic activities upon organized cellular structure and had pointed to the

importance of studying these integrated structural and enzymatic relations (Warburg, in 1913; Quastel, in 1930; Peters, in 1937), it can be seen that the most important impetus and guidance in this field has come from anatomists, cytologists, embryologists, and virologists.

## 2. *On Cytoplasmic Staining*

Two types of basophilia, important in the differentiation of cytoplasmic structure, have been distinguished. In the common test for basophilia, a basic dye reacts with and is absorbed by the acidic substance organized in visible or submicroscopic particulates. With most basic dyes, the peak of absorption is shifted only slightly toward longer wave lengths and the cell is stained with a color comparable to the color of the dye in solution. The existence of submicroscopic particles containing RNA in the apparently clear cytoplasm of cells particularly active in protein synthesis is revealed by a strong basophilia of this type, which can be removed by pretreatment with ribonuclease. This type of basophilia usually parallels the observed ultraviolet absorption of this region of the cell.<sup>1</sup>

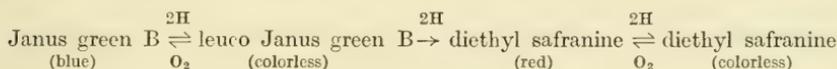
Earlier, Paul Ehrlich had described metachromasy as a special staining reaction of certain dyes, such as thionine or toluidine blue. In these instances, the color of the adsorbed dye is quite different from the color of the dye solution itself. The relatively rare compounds that are stained in this way are polymeric sulfuric acid esters such as agar, heparin, chondroitin sulfuric acid, mucin, and the metaphosphates, which may exist in cells as volutin granules. Other acidic polymers, such as the ubiquitous nucleic acids, will not shift the color of bound toluidine blue from blue to pink or purple, as will agar. The shift in color and in the absorption spectrum was shown by Michaelis (1950) to be a function of the dimerization and polymerization of this dye, an aggregation which was readily reversible. The shift is also a function of the nature of the absorbent and it is believed by some workers (Walton and Ricketts, 1954) that the extent of metachromasia is mainly dependent on the solubility characteristics of the dye-substrate complex. Nevertheless, it is still not clear why the nucleic acids should not influence the dye, yielding the spectrum of the monomer, whereas a substance such as agar does influence the spectrum, as though the dye were highly polymerized.

<sup>1</sup> In a remarkable monograph (Hedberg, 1953) it is reported on the basis of ultraviolet absorption measurements that the RNA content of the human ovarian oocyte is less than 0.1 %, being too low to be determined by this method. Despite the apparently negligible concentration of RNA, it should be noted that the oocyte accomplishes an enormous increase of protein unaccompanied by any significant increment in RNA content. Hedberg poses the question of whether this protein has been synthesized by the oocyte or by the surrounding follicular epithelium.

Similarly, one may note the report (Bhargava, 1957) of the absence of RNA in bull sperm, which are capable of incorporating radioactive amino acids into protein.

However, it may be mentioned that rosaniline exhibits metachromasy in the presence of DNA (Lawley, 1956).

Michaelis (1900) had recommended the supravital use of the dye, Janus green B, as a specific stain for the filamentous cytoplasmic structure now known as mitochondria. This blue dye is a conjugate of the red diethyl safranine through an azo linkage to dimethyl aniline. The nature of the specificity of mitochondrial staining by this dye has been unraveled by Lazarow and Cooperstein (1953). The dye is specifically adsorbed by many proteins; there is no evidence for a specific mitochondrial adsorption. The dye can be dehydrogenated in the following sequence of reactions:



These conversions are effected by reduced flavoproteins, and, with the exception of the cleavage step leading to the formation of red diethyl safranine, the reactions may be reversed by the oxidation of reduced flavoprotein through cytochrome systems or by molecular oxygen. The dye is initially adsorbed on all parts of the cell. In the absence of sufficient  $\text{O}_2$ , the reductions via  $\text{FADH}_2$  to the colorless stage proceed rapidly throughout all fractions of the cytoplasm. In the mitochondria, which are able to reoxidize reduced intermediates, the reduction to colorless forms is reversed. Staining in the mitochondria does not occur anaerobically in the presence of cyanide (Showacre, 1953). Thus, the mitochondria stain blue in solutions of excess dye and red in dilute solutions, as a result of reductive cleavage to diethyl safranine, while the remainder of the cytoplasm is colorless or slightly pink.

Potter *et al.* (1951) have used this stain to analyze the extent of contamination of mitochondrial preparations. It has been possible to demonstrate that all mitochondria are uniform to the extent that each mitochondrion is able to prevent the irreversible dehydrogenation of the dye. Nevertheless, as will be noted below, other types of biochemical heterogeneity have been reported among these particles.

### 3. Mitochondria

Of the many structures present in animal cytoplasm, we shall discuss only the three major fractions defined by the centrifugal technique described on p. 38, i.e., mitochondria, microsomes, and the clear supernatant fluid remaining after deposition of microsomes at 20,000 *g* for one hour. As noted above, the first of these was first isolated by Bensley and Hoerr (1934a and b). The isolation of mitochondria was rediscovered when the large granules obtained by differential centrifugation were observed to stain with Janus green

B (Hogeboom *et al.*, 1948). When isolated by the sucrose technique, these granules often had the rod-shaped appearance of mitochondria in cells.

The particles, visible in the light microscope, have a considerable degree of variability, being filamentous, spherical, or vesicular. In the resting cell their diameter is about 0.5 and 1 $\mu$ , with 0.2 and 2 $\mu$  as extremes, and a maximum length of 7 $\mu$ . Observations with the electron microscope, notably by Palade (1953, 1955) and Sjöstrand (1953), have revealed that mitochondria have a characteristic organization, whether derived from plant or animal material.<sup>1</sup> They possess a surface membrane and internal compartments separated by lamellae, termed "cristae mitochondriales." The laminated or, occasionally, tubular cristae show degrees of branching, which vary from cell type to cell type, and it is suggested that the purpose of the infolding is to increase internal surface rather than compartmentalization. Most often the lamellae are perpendicular to the long axis of the organelle and appear to be more or less parallel to one another, as presented in Fig. 3. In sections of mitochondria one may readily see that the surface membrane consists of 2 parallel membranes, each about 50 Å thick, and that the inner membrane connects with the one end of the cristae mitochondriales, which project finger-like within the mitochondrion. Within the inner chamber of the particle is a relatively homogeneous dense material, which occasionally contains small granules of high density.

The preservation of the fine structure of mitochondria in the process of isolation is a relatively recent event, made possible by a careful control of the isolation procedure by electron microscopy (Novikoff, 1956). The isolation technique of choice has required the use of hypertonic solutions at defined pH in order to prevent the swelling of the mitochondria. In the early isolations of mitochondria by Claude (1946), it was observed that handling or storage in hypotonic solutions resulted in a rapid disintegration or lysis. Dense submicroscopic granules containing RNA could be isolated from such a lysate. In the earliest isolations of "cyclophorase" or mitochondria, studied by Green and his collaborators as an approach to the integration of the citric acid cycle, fatty acid metabolism, etc. (Green, 1951, 1957), it was soon observed that the particles developed coenzyme requirements in the course of handling or mishandling. There appears no reason to doubt that the subsequently observed release of coenzymes and the ready diffusion into the particle of exogenously supplied coenzyme were consequences of the swelling or lysis of a semipermeable mitochondrial membrane (Lindberg and Ernster, 1954). A comparable case was studied by Berthet *et al.* (1951), who observed the exposure and release of acid phosphatase by mitochondria after exposure

<sup>1</sup> A particle of similar size, organization, and enzymatic constitution has never been observed in bacteria.

to distilled water. In this medium, a mitochondrion may swell to more than 5 times its normal volume before bursting. The stretching of the membrane in itself appears to facilitate release of internal metabolites, previously nondiffusible.

The integrity of the membrane may be preserved by isolation in the presence of hypertonic sucrose. During experimentation in isotonic media, the preservation of the membrane requires an energy source, such as added ATP or UTP (uridine triphosphate) (Lehninger, 1956). The particles will concentrate  $\text{Na}^+$  and  $\text{K}^+$  without swelling under conditions in which ATP is being generated (Macfarlane and Spencer, 1953). Various aspects of the movement of water and ions in mitochondria have been discussed by Lehninger (1956), Price, C. A., *et al.* (1956), and Fonnesu and Davies (1956).

The largest number of studies in cell fractionation has been effected with animal tissues. Although particles bearing enzyme activities have been isolated from homogenates of plant tissues, in most early studies their appearance was no longer comparable with the structures visible within the cell. In recent studies (Martin and Morton, 1956; Hodge *et al.*, 1957), as a result of efforts to preserve the surface membranes, plant mitochondria have been isolated whose morphological and enzymatic properties are comparable to mitochondria observed in sections of plant cells and which resemble the isolated mitochondria of animal tissue.

As will be seen below, mitochondria fulfill the role of a powerhouse for the cell, storing the energy derived from respiration and dehydrogenations in compounds such as ATP or thioesters, which are capable of supporting the performance of work. In addition, the special requirements of differentiated cells result in the differentiation and specialization of mitochondria. For example, the striated flight muscles of certain adult insects contain large spherical intrasarcoplasmic bodies called sarcosomes (Watanabe and Williams, 1953). These are arranged in rows along the fibril and constitute about one-third of the total muscle mass. Sarcosomes are really differentiated mitochondria in which the supply of chemically stored energy is integrated with the functional demand of the muscle fiber. In *Drosophila* their size is independent of the ploidy of the cell; it is estimated that in this organism their RNA content is less than 5 % (Rudkin and Schultz, 1956).

The accumulation of foreign substances is effected very actively by the mitochondria of kidney tubules (Zollinger, 1950a). According to this worker, proteins are taken up to form droplets, which coalesce within the mitochondrial core. Eventually, the membrane degenerates and the mitochondrion becomes a drop of secretion. It is postulated that a similar process occurs in

mast cells<sup>1</sup> and pancreas to form the secretory granules<sup>2</sup> and indeed represents a general pattern of development of secreted morphological structures. Recent developments on secretory granules are summarized by Schneider and Hogeboom (1956).

In addition to this type of transformation, considerable discussion has arisen over the possible mitochondrial origin of the chloroplasts. One school holds that there are two kinds of mitochondria, one of which gives rise to plastids and fails to stain with Janus green B (Newcomer, 1951). Other students of this question believe that plastids arise only from plastids.

Some workers consider that mitochondria divide and multiply independently of cell division, as do chloroplasts and some intracellular parasites. As will be discussed below, Lindberg and Ernster (1954) have concluded that the microsomal fraction of cytoplasm is converted into mitochondria, while Ephrussi (1953) considers it likely that mitochondria demonstrate a considerable measure of genetic continuity requiring self-duplication. These opinions are not necessarily contradictory, since the latter hypothesis may be thought of as referring to the continuing multiplication of fully formed mitochondria. Nevertheless, the effects of carcinogenic azodyes and acriflavin, to be mentioned below, are usually taken to imply that the genetic continuity of mitochondria is not continually restocked from other cellular fractions. On the other hand, an instance is recorded in which egg fragments of the sea urchin, apparently deprived of mitochondria, were induced to develop into larvae whose cells were restocked with this cellular particle (Harvey, 1946). Although this appears to bolster the view that mitochondria may be synthesized *de novo*, these experiments were done at a time when electron microscopy was not being used, and it was not proved conclusively that the initial cells were devoid of all mitochondria.

Mitochondria isolated in sucrose have been observed to possess a slight constriction, which in time proceeds to an actual division *in vitro* into two fragments (Leon and Cook, 1956). Whether this process bears any relation to the *in vivo* increase of these elements is not clear. Novikoff (1956) has recorded the presence in rat liver homogenate of small mitochondria or "mitosomes," possessing a greater number of electron-dense granules; it has been suggested by Leon and Cook that such a group of mitochondria may arise in a division process. However, such a class of small mitochondria is now believed to be biochemically unique, containing the "lysosomes."

<sup>1</sup> Contrary to the views of Zollinger (1950b), heparin does not appear to be associated with the large granules of mast cells (Hedbom and Snellman, 1955); these large granules possess all the chemical characteristics of mitochondria. They also contain histamine.

<sup>2</sup> Indeed, the so-called "secretory granules" of liver appear to dissolve upon homogenization (Hogeboom *et al.*, 1948), a result entirely consistent with the above hypothesis concerning their origin.

By either mechanism of origin of mitochondria, it may be expected that the size and composition of the mitochondrial population within the cell will vary as a function of the physiological and genetic state of the cell. Such a situation bears many similarities to problems of virus multiplication. The quantitative variability of a viruslike cytoplasmic particle containing DNA has been studied in some detail in the case of the kappa factor multiplying in the cytoplasm of *Paramecium aurelia*.

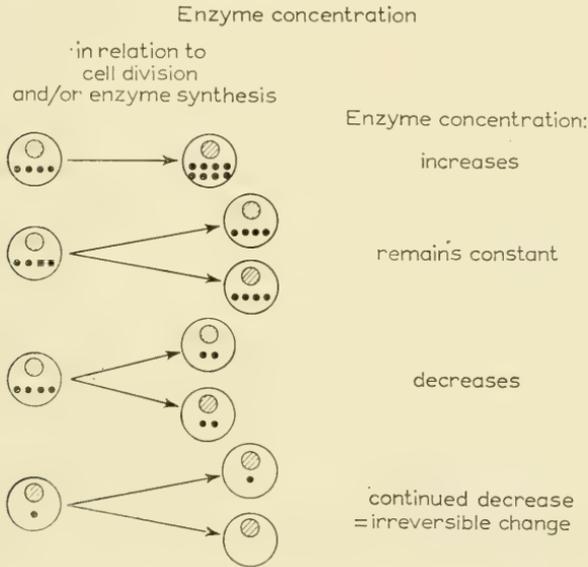


FIG. 7. Diagrams to illustrate a hypothetical mechanism of carcinogenesis in terms of the deletion of a cytoplasmic particle or an enzyme contained therein (Potter *et al.*, 1950). Each large circle represents a cell. The cross-hatched circles represent nuclei and the small black dots represent one type of cytoplasmic particle.

The problem is also of great interest from the point of view of carcinogenesis. Potter *et al.* (1950) studied the composition of rat liver as a result of feeding the carcinogenic dimethylaminoazobenzene derivatives to rats. They correlated the loss of succinoxidase, a mitochondrial enzyme, with the increasing carcinogenicity of various compounds and concluded that carcinogens might produce irreversible changes by causing the deletion of particular cytoplasmic inclusions during cell multiplication, as presented in Fig. 7.

A similar conclusion has been reached by Ephrussi and collaborators in analyzing the respiration-deficient mutants of yeast produced by the action of acriflavin (Ephrussi, 1953).

Allard *et al.* (1952a,b, 1953) have proceeded to the direct quantitation of mitochondria per cell. In Table V are presented some of their data on the

TABLE V  
MITOCHONDRIA POPULATION OF RAT LIVER CELLS<sup>a</sup>

	Mitochondria per cell	Mitochondria per gm. liver $\times 10^{-10}$
Normal	2480	33.0
Tumor	711	39.0
Regeneration (2 days)	2089	25.7
Semisynthetic diet	1940	26.7

<sup>a</sup> Allard *et al.* (1953).

number of mitochondria per liver cell of rats treated in various ways. It can be seen that liver tumors which developed as a result of 4-dimethylaminoazobenzene in the diet contained markedly fewer mitochondria per cell, although the total number of mitochondria of tumor was higher than in normal liver.<sup>1</sup>

Similar problems concerning mitochondrial synthesis arise in normal development. A diminution in mitochondrial number per cell occurs during gastrulation in the sea urchin larva (Gustafson and Lenique, 1952); this is found, in general, during growth unattended by differentiation.

The application of the principle of quantitating activity and subcellular elements per cell promises to provide much information on the nature of physiological and pathological change. Such methods have not yet been applied to virus-infected cells.

#### 4. Biochemical Heterogeneity of Mitochondria

Although isolated mitochondria exhibit similar staining and optical properties, they are highly pleomorphic. It is conceivable that differences in size and shape may reflect biochemical differences among the particles. Density gradient techniques have been successfully used to effect separations of classes of mitochondria on the basis of density differences (Ottesen and Weber, 1955; Thompson and Mikuta, 1954). In the latter study, there also appeared to be a separation of uricase from other mitochondrial enzymes, a result noted by other groups using the more conventional differential centrifugation techniques (Kuff and Schneider, 1954).

In a more recent study, however, with fractions collected by centrifugation in a density gradient (Kuff *et al.*, 1956), electron micrographs appeared to

<sup>1</sup> However, several other laboratories (Shelton *et al.*, 1953; Fiala, 1953) report, following mitochondrial counts and isolations, that even based on the weight of hepatoma and normal liver, the latter contains about 2.5 times as many mitochondria as does hepatoma.

indicate that uricase and acid phosphatase were associated with vesicles derived from the submicroscopic lamellar structures of the cytoplasm. Their fractions of liver cytoplasm and correlation of different substances with particle size are given in Table VI. Data of this type on host cells used in

TABLE VI  
THE PROPERTIES OF FRACTIONS OF RAT LIVER CYTOPLASM <sup>a</sup>

Biochemical component	Particle size, m $\mu$				Total recovery
	220-250	120	25-55	10	
Succinic dehydrogenase	91	0	0	0	91
Uricase	0	94	0	0	94
Acid phosphatase	16	58	18	9	101
RNA-P	11	26	54	11	102
DPN <sup>b</sup> -cytochrome c reductase	29	0	53	8	90

<sup>a</sup> Kuff *et al.* (1956).

<sup>b</sup> Diphosphopyridine nucleotide.

virus infections could be of considerable value in analyzing the degree of purification of a derived virus preparation.

Using polyvinyl pyrrolidone-sucrose to stabilize mitochondria and differential centrifugation as a method of separation, evidence has been obtained to show that there is indeed a class of small mitochondria, called lysosomes, containing a high concentration of the hydrolytic enzymes, acid phosphatase, ribonuclease, deoxyribonuclease, cathepsin, etc. (de Duve, 1957; Novikoff, 1957). However, these workers have concluded that the true extent of biochemical heterogeneity of mitochondria will require a great deal of additional study before a satisfactory clarification is attained. It may be noted that, although it had been claimed that there is a class of small mitochondria rich in RNA, the presence of this nucleic acid appears to stem largely, if not completely, from microsomal contamination.

### 5. Chemical Composition of Mitochondria

The mitochondria isolated from many animal tissues contain protein, phospholipids, other fatty acid derivatives, and RNA. Their coenzyme content is summarized in Table VII.

In normal rat liver the mitochondrial fraction comprises 23 to 26 % of the total nitrogen and 30 to 33 % of the total protein, figures which may increase slightly after correction for mitochondria (succinoxidase activity) present in the nuclear fraction. In many other tissues these values are considerably less. On disruption of mitochondria by sonic vibration 60 % of the mitochondrial

TABLE VII  
COENZYME CONTENT OF ISOLATED LIVER MITOCHONDRIA <sup>a</sup>

Compound	Concentration		Specific activity	Source of tissue
	Recovery (whole tissue = 100)	(whole tissue = 1)		
Vitamin B <sub>6</sub>	33	1.3	0.060 $\mu\text{g./mg. protein}$	Rat
Vitamin B <sub>12</sub>	56	2.2	0.034 $\mu\text{g./gm. nitrogen}$	Mouse
Riboflavin (total)	53	1.8	0.30 $\mu\text{g./mg. protein}$	Rat
FAD <sup>b</sup>	65	2.3	9.1 $\mu\text{g./mg. nitrogen}$	Mouse
Folic acid	37	1.8	0.089 $\mu\text{g./mg. nitrogen}$	Mouse
Pantothenic acid	43	—	—	Rat
Coenzyme A	52	—	—	Rat
Citric acid	67	4.4	55 $\mu\text{g./mg. nitrogen}$	Rat
Phospholipid	27	1.5	7.4 $\mu\text{g.P/mg. nitrogen}$	Rat

<sup>b</sup> Flavin adenine nucleotide.

<sup>a</sup> Hogeboom and Schneider (1955).

mass appeared in the form of soluble proteins. It is reported that, as analyzed in the ultracentrifuge, the proteins derived from hepatoma mitochondria lack a component found in normal liver mitochondria (Hogeboom and Schneider, 1951).

The RNA concentration of the mitochondria is far less than that of the microsomal fraction. The proportion of the total RNA contained in the mitochondria has been reported as 5 to 7 % (see Table VIII, Hogeboom and Schneider, 1955). Claude (1944) lysed the mitochondria of rat liver in distilled water and showed that the RNA was associated with small microsome-like particulates which were still sedimentable, although the ribose-containing coenzymes were now in a low molecular dialyzable state. Lowe and Lehninger (1955) have reported the presence of high molecular weight RNA in the most carefully fractionated mitochondria of rat liver, although finding that the mitochondria derived from cortisone-treated rats lack such nucleic acid. Significantly, the latter particles are still capable of normal oxidative phosphorylation.

The lipids of rat liver mitochondria have been analyzed by Swanson and Artom (1950). About half the phosphorus is present in phospholipid, comprising 80 % of the total lipid, the remainder consisting of neutral fat, cholesterol, and other substances. Lecithin, cephalin, and sphingomyelin have been demonstrated, as have relatively large amounts of inositol (0.5 %).

### 6. *The Microsomal Fraction*

The term "microsomes" was used by F. R. Lillie as early as 1906 in describing the smallest visible cytoplasmic particles in the developing polychaete embryo. The term is now widely used in the sense introduced by Claude in 1940 to describe the submicroscopic basophilic particles present in the ground substance of cell cytoplasm. Some workers are also using the term "chromidia" to apply to this cytoplasmic fraction (Fiala *et al.*, 1956). Most recently, it has been shown that in highly differentiated cells the dense basophilic particles are usually organized on membranes and are oriented internally within the cytoplasmic system of vesicles and tubules, which is given the name of endoplasmic reticulum (Palade and Porter, 1954; Palade and Siekevitz, 1956). This reticulum can be compared to an intracellular vascular system. The extended form of these particle-studded membranes does not readily survive homogenization, and, for the most part, then, isolated microsomal fractions represent much abused artifacts of preparation. In cell types characterized by rapid proliferation, the dense particles may be more or less freely distributed in the cytoplasm (Palade, 1955). A microsomal fraction has been isolated from all cells studied.

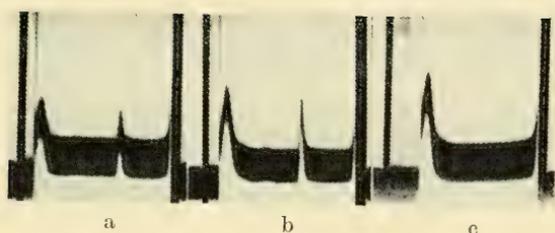
Claude believed that microsomes possess comparable chemical properties, although the isolated particles range from 50 to 300  $m\mu$  in diameter, with the

majority at 80 to 150  $m\mu$ . Chantrenne (1947) considered that Claude had fractionated the microsomes in the course of differential centrifugation, thereby limiting the observable range of particle sizes. This worker first described the heterogeneity of size, composition, and enzymatic activity of the microsomal fraction. However, electrolytes rather than sucrose had been employed in this work, and the results of the fractionation were therefore more suspect than most. More recent studies with the sucrose technique appeared to have confirmed the existence of marked chemical and enzymatic heterogeneity within this fraction (Novikoff, 1957; Hogeboom and Schneider, 1955). However, the recognition of the existence of a class of labile small mitochondria or lysosomes, which readily liberate their enzymes and thereby contribute to the apparent heterogeneity obtained in the usual fractionation, makes it difficult to be certain of the significance of numerous earlier reports.

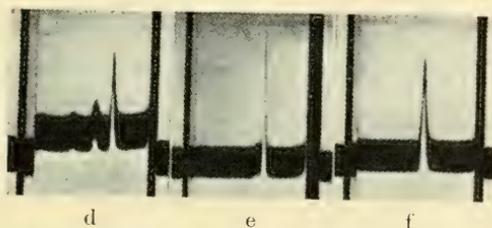
The work of Jeener (1952a,b) on the particles of the colorless flagellate, *Polytomella coeca*, has provided strong evidence of compositional and functional heterogeneity. Petermann and Hamilton (1957) have recently described the ultracentrifugal heterogeneity of the particulate fraction of rat liver after removal of nuclei and mitochondria and have isolated a major ribonucleoprotein constituent,  $S = 77.5$ .

Nevertheless, some studies on bacterial organization have revealed a high order of uniformity among particles whose size would place them within the isolated microsome fraction of animal cells. For instance, electron microscopy of lysing *E. coli* infected with bacteriophage revealed great numbers of apparently uniform particles of about 150  $m\mu$  in diameter (Luria *et al.*, 1943). Uniform spherical particles of this size ( $S_{20} = 40$ ,  $MW = 10^6$ ), have been isolated and characterized by Schachman *et al.* (1952) who found them to contain most of the RNA of all bacteria examined (e.g., *Pseudomonas fluorescens*, *E. coli*). In yeast, a similar, somewhat larger structure ( $S_{20} = 80$ ) can be found (Chao and Schachman, 1956). The relatively large quantity of such particles in a yeast extract and their apparent homogeneity with respect to sedimentation may be discerned in Fig. 8. The particles are present in the organisms in each stage of development of a synchronized culture of *E. coli* (Cohen and Barner, unpublished data). However, the  $S = 40$  particles disappear under conditions in which *E. coli* is starved (Dagley and Sykes, 1957).

Ultracentrifugal analysis of extracts of some plant tissues, e.g., pea epicotyl, similarly has revealed a high concentration (ca. 25 % of the acid-precipitable protein) of a major class of ribonucleoprotein particles of  $S_{20} = 74$  and apparent molecular weight of 4 to  $6 \times 10^6$  (Tso *et al.*, 1956). Extracts also contain about 10 % as much of another particle of 110S. On isolation these particles are found to have about 35 % RNA and far less lipid than the microsomal fraction of animal tissues. This chemical evidence



YEAST EXTRACTS



ISOLATED 80 S PARTICLES

FIG. 8. Ultracentrifuge patterns of yeast extracts and isolated 80 *S* particles.

(a) Yeast extract in water, 12 minutes after reaching speed of 42,040 r.p.m.

(b) Yeast extract stored in water for 1 week, 12 minutes after reaching speed of 42,040 r.p.m.

(c) Yeast extract in 0.15 *M* NaCl, 12 minutes after reaching speed of 42,040 r.p.m.

(d) 80 *S* particles isolated by three cycles of centrifugation in 0.0015 *M* KH<sub>2</sub>PO<sub>4</sub>, 12 minutes after reaching speed of 42,040 r.p.m.

(e) 80 *S* particles after three cycles of centrifugation and then dialysis in 0.0025 *M* K<sub>2</sub>HPO<sub>4</sub>, 0.0025 *M* KH<sub>2</sub>PO<sub>4</sub>, and 7.5 × 10<sup>-4</sup> *M* CaCl<sub>2</sub>, 56 minutes after reaching speed of 20,410 r.p.m.

(f) 80 *S* particles after three cycles of centrifugation in 0.0025 *M* K<sub>2</sub>HPO<sub>4</sub>, 0.0025 *M* KH<sub>2</sub>PO<sub>4</sub>, 0.001 *M* MgSO<sub>4</sub>, and 7.5 × 10<sup>-4</sup> *M* CaCl<sub>2</sub>, 48 minutes after reaching speed of 20,410 r.p.m. (Chao and Schachman, 1956).



suggests the absence of the lipid-rich membranes of an endoplasmic reticulum in these tissues. However, the reticulum does appear to exist in the petioles of silver beet and the roots of germinating wheat (Hodge *et al.*, 1957). The cytoplasmic fractions of various leaves have also been studied in the ultracentrifuge (Singer *et al.*, 1952; Eggman *et al.*, 1953) and have revealed a number of major particulate components possessing relatively low sedimentation coefficients.

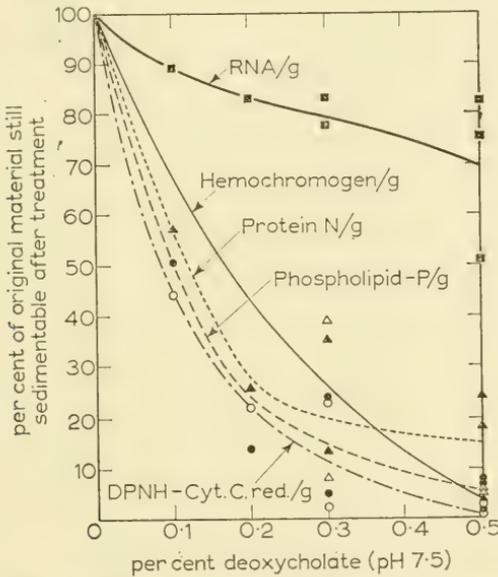


FIG. 9. Effect of deoxycholate on the biochemical composition of liver microsomes. The microsomes were obtained from rat liver homogenized in 0.88 *M* sucrose (centrifugation: 60 minutes at 105,000 *g*). They were resuspended in 0.88 *M* sucrose containing the amounts of Na deoxycholate (pH = 7.5) indicated. The treated suspensions were recentrifuged immediately for 120 minutes at 105,000 *g* and the pellets thus obtained analyzed chemically. The results, given per gram tissue equivalent, indicate what per cent of the original material is still sedimentable after deoxycholate treatment. (Palade and Siekevitz, 1956.)

KEY: ■—■, RNA; ▲-----▲, protein N; ●-----●, phospholipide P; Δ—Δ, hemochromogen; ○- - -○, DPNH-cytochrome *c* reductase activity.

The microsomal fraction contains 16% of the total solid in mammalian liver and about 25% of the total nitrogen in bacteria. This fraction from guinea pig liver contains 9.6% N and 1.5% P, being rich in RNA. It has 40 to 45% lipid, of which about two-thirds is a complex mixture of phospholipids. Among the fractionated liver and *Polytomella* microsomes, the smallest particles are richest in RNA. The more homogeneous bacterial particles may contain up to 60% RNA; the yeast particles are reported to contain 50% RNA.

Treatment of the microsomal fraction from liver with sodium desoxycholate, as in Fig. 9, solubilizes protein, lipid, hemochromogen, and DPN-cytochrome c reductase, components which appear to have been centered in the reticular membranes. Dense RNA-protein particles, which are similar in size and shape to the membrane-embedded particles, may now be isolated by sedimentation (Palade, 1956; Palade and Siekevitz, 1956).

As observed earlier, the amount of the entire microsomal fraction in rat liver decreases under conditions of protein depletion (Muntwyler *et al.*, 1950). The synthesis of this fraction and of protein in general in the adrenal gland has been shown to be stimulated by ACTH (Fiala *et al.*, 1956). The fractionation of this material into membrane and particles makes possible a more precise dissection of the changes occurring under various physiological conditions.

Hultin (1957) has begun in this way to analyze the composition of microsomal subfractions as a function of the nutritive state of the animal. After removal of RNA with bicarbonate buffer at pH 8.2, he has made desoxycholate extracts. The precipitability of these by ethanol is affected by the lipid content and it has been possible to observe apparent changes in lipid content in these extracts after starvation. One might ask whether, in the synthesis of an RNA virus, e.g., poliomyelitis, the synthesis of the components of the microsomal fraction has been affected and, if so, if this has been particularly pronounced in the lipoprotein membranes or in the dense RNA particles.

### 7. Cell Sap or Soluble Supernatant Fraction

This fraction includes the material failing to sediment in a gravitational field which deposits the microsomal fraction. It also contains substances of low density, such as lipid droplets, which would migrate centripetally in a gravitational field. About 30 to 50 % of the nitrogen of liver, kidney, and tumor is present in this soluble fraction. In the earliest analysis of leukemic cells by Claude (1944), it appeared that all of the RNA was associated with the sedimentable fraction. However, in rat liver the cell sap contains 43 % of the total nitrogen of the homogenate, 10 % of the RNA, and 66 % of the acid soluble nucleotides (Anderson, 1956c). In general, large amounts of nonsedimentable ribonucleoprotein are present in most rapidly growing cells. The systematic analysis of this fraction has barely begun, several electrophoretic studies revealing numerous lipid- and RNA-containing protein components (Gjessing *et al.*, 1951; Anderson, 1957).

As noted earlier, metachromatically staining mast cells have been fractionated in order to determine the distribution of heparin. This substance is not present on mitochondria and the soluble fraction contained 82 % of the heparin bound to very small particles, less than 10  $\mu$  in diameter (Julén *et al.*, 1950). Of course, the distinction between particulate and soluble

fractions is arbitrary and merely reflects the forces used in the separation; all molecules in both fractions may be considered to be particulate.

### 8. *On the Origin of the Mitochondria and Microsomes*

The finding of Claude (1944) that microsomal-like particles rich in RNA could be isolated from lysed mitochondria led to two suggestions: (a) that the microsomes isolated from homogenates are artifacts of mitochondrial disintegration, and (b) that the microsomes are normally produced in this way. Hypothesis (a) was clearly disproved in the ensuing years, which also revealed that isotopic amino acids and nucleotides are incorporated into microsomal proteins and RNA, respectively, at a greater rate than their appearance in mitochondria. Accordingly, as noted earlier, the hypothesis has been proposed that mitochondria are produced by the aggregation of the smaller particulates. This is not contradicted by the metabolic evidence and indeed tends to be supported, as noted earlier by the cytological evidence of Harvey (1946) and of Lillie, who, in 1906, followed the development of microscopically visible particulates. It may be added further that Lillie believed that microsomes originated in the nucleus, a hypothesis which has also been suggested on the basis of observations of nucleolar function, of the continuity of the external nuclear membrane with the endoplasmic reticulum, and the relative rates of RNA synthesis in various parts of the cell. On the other hand, it has also been suggested that these particles are self-duplicating, as are viruses, and may possess a measure of autonomy with respect to their multiplication within the cell.

### 9. *Properties of Chloroplasts*

Intensified investigation of the detailed mechanism of photosynthesis and of the role of intracellular structures in cellular activity has resulted in many studies on the isolation and properties of chloroplasts and smaller fragments of these organelles. Chloroplasts may be defined as cytoplasmic bodies containing the light-absorbing pigments, chlorophyll and carotenoids, essential to photosynthesis. It is supposed that various plant viruses arise in the variegation of plastids and are multiplied within plastids or chloroplasts. In many instances the pathology of virus infections in plants is readily revealed in aberrations of pigment and chloroplast production. Efforts to demonstrate this as a primary consequence of the viral multiplication are not yet convincing, although electron microscopy of sectioned chloroplasts of virus-infected leaves has revealed virus particles in close proximity to the chloroplast structures (Leyon, 1953).

Within a given plant there appears to be a relative constancy of the number of plastids per cell. They divide to increase their number if the cell has too few; some may degenerate if many are present. Cells possessing large

chloroplasts tend to have fewer of these units than cells possessing smaller plastids. It has been reported that division occurs by construction of an elongated plastid, culminating in a pinching-off process in the center.

The reproduction of the chloroplast and inheritance of its structures are controlled in part by nuclear genes in a Mendelian fashion. Nuclear mutations may lead to disturbances in chlorophyll synthesis and to the production of colorless plastids, as in the iojap maize character of chlorophyll striping (Rhoades, 1946). The reproduction of these mutant plastids, once formed, may now proceed and, despite the reintroduction of the normal nuclear gene, the cell may continue to produce colorless plastids. Thus, certain genetic characters determining chloroplast structure may be transmitted by the plastid relatively independently of the nucleus.

The relation of the chloroplast to mitochondria has been mentioned earlier. It has been noted that once the cell loses all its plastids, following growth in the dark or growth in the presence of streptomycin (Provasoli *et al.*, 1948), they are never regained. Chlorophyll may disappear in some instances as a result of growth in the dark, but if the plastid remains, it can generate the pigments again in the presence of light. This process has recently been studied in *Euglena* by means of electron microscopy (Wolken and Palade, 1953).

A poor supply of N or Fe can also reduce the rate of chloroplast production. On the other hand, a supply of RNA and amino acids selectively promotes chloroplast growth without affecting the rate of cellular multiplication (Deken-Grenson, 1957).

Chloroplasts may also orient themselves within the cell in response to light. It is evident that their synthesis, turnover, movement, and reproduction present many important unsolved problems, other than those related directly to photosynthesis.

It has proved to be rather difficult to obtain homogeneous preparations of chloroplasts. Careful studies of preparations of these particles have revealed that homogenization of plant tissue tended to yield materials heavily contaminated with chromatin threads, mitochondria, and other cell fragments. The botanists have been somewhat provoked by the ease with which enzymologists and other biochemists have designated their preparations as "chloroplasts" and it is felt that many studies on the distribution of enzymes in the plant cell are not to be taken seriously in the absence of careful cytology, side by side with the chemical studies.

During isolation, structures known as "grana" frequently become visible within the chloroplasts. These optically dense, wafer-shaped bodies are embedded in a clear protein-containing matrix or "stroma." The grana are about 6000 Å in diameter and 800 Å thick, and seem to be rather uniform in size. It is probable that they contain the concentrated pigments and are

therefore the center of the primary light absorption. Since these structures within the chloroplast appear most often during the process of isolation, it has been argued that grana are artifacts. This is not generally held, some workers believing that isolation and imbibition of water merely lower the refractive index of the stroma so that the similarly refractive grana become visible.

Perhaps the most interesting evidence for the functional importance of bodies of the size of grana is derived from the isolation, by means of differential centrifugation, of the cytoplasmic chromatophores of the blue-green algae, *Synechococcus cedorum*, and the photosynthetic bacterium, *Rhodospirillum rubrum*. Although most algae and higher plants contain chloroplasts, blue-green algae and photosynthetic bacteria lack these bodies. It had been believed that their pigments were dispersed throughout the cell, presumably in soluble form, but it is now known that the colored components, including the phycobilins, exist within the chromatophores and chloroplasts. The studies of Schachman *et al.* (1952) on light-grown photosynthetic bacteria revealed that these pigments are contained exclusively in large particles, (about 50  $m\mu$  in diameter), about 5000 per cell.

*Rhodospirillum rubrum* can grow aerobically in the dark and under these circumstances is relatively unpigmented. Neither chromatophores nor unpigmented particles of comparable size are present in these cells. In the light there is an extensive synthesis of pigment and particles, which then amount to about 15 % of the nitrogen of the cell. The particles lack DNA and have a small amount of RNA, although less than the small particle fraction of the same cells. Such a system provides obvious advantages for the study of pigment and particle synthesis. Deken-Grenson (1954) has also observed the synthesis of grana in leucoplasts of etiolated leaves following the exposure to light.

In addition to the presence of protein, chlorophyll, carotenoids, and RNA, chloroplasts contain many lipids, including neutral fat, sterols, waxes, and phosphatides. Phosphatides can comprise one-third of the phosphorus of the plastid and these may amount to 35 to 65 % of the lipid phosphorus of the leaf. The fat-soluble vitamins, K and E, have also been found in chloroplasts. Studies on the details of organization of various types of chloroplasts have been described (Wolken and Schwartz, 1953; Wolken, 1956).

### III. THE DISTRIBUTION OF METABOLIC FUNCTION

#### A. Methodological Notes

Several approaches to enzyme localization are widely used at the present time. Some of these shall be considered in a later section and involve the use of more or less intact cells. For example, function may be contrasted between

the cell fragments, nucleated and enucleate, into which a cell is cut. Or the synthetic capacities of a cell may be studied under the influence of metabolic blocks, imposed genetically, nutritionally, or by means of more or less specific inhibitors. However, in the immediate sections to follow we may consider the result of apparently more direct techniques, namely, the histochemical method and the method of isolation via differential centrifugation of cell components from homogenates.

Little will be said concerning the first of these; at the present time it appears strongly desirable to check results obtained by this approach by centrifugal fractionation or by other techniques. For example, it has been claimed on histochemical grounds that alkaline phosphatase is concentrated in the nucleus. Data on isolated fractions have failed to support this contention (Novikoff, 1951). A similar discrepancy has been observed in the data on the localization of acid phosphatase (Palade, 1951). At first, then, we shall concentrate on our knowledge of the distribution of enzymes obtained with isolated organelles.

An investigation into intracellular enzyme content and distribution should attempt to correlate actual cellular activity and potential cellular activity. The observed activity of a cell is not merely equal to the activity of the component parts. For example, particular activities of liver may either be lower or greater than the activities detectable in a homogenate. In addition, the activities of intact cells must be understood in terms of the external natural milieu of the cell, which would include hormones, substrates, etc., as well as in the interactions of the internal enzymatic equipment which functions in many different states of organization and complexity.

The enzyme activity of a homogenate is not always the sum of its component parts. The mere preparation of a homogenate, assuming enzymes are not being damaged in the process, may dilute or affect previously optimal concentrations of coenzymes and various ions. Furthermore, the individual activities of the components will not necessarily add up to the activity of the homogenates. Thus, the interactions of the separated cell components may be essential to the demonstration of maximal reactivities. For example, the rate of oxidation of various substrates by mitochondria can be increased several fold by adding the glucose-hexokinase system to the medium in order to trap high energy phosphate being generated at the organized enzyme sites (Lardy and Wellman, 1952). The rate-limiting factors affecting oxidation by mitochondria include not only the enzymes of this organelle but also its supply of phosphate acceptor. Hexokinase is normally in the fluid surrounding the mitochondria.

There are many systems in which fractionation reveals the dependence of maximal metabolic activity upon a considerable order of complexity. In a study of the choline oxidase of rat liver homogenate, most of the activity

was present in the mitochondria (Williams, 1952). However, in this instance the supernatant fluid was reported to contain a heat-labile inhibitor which severely depressed this activity.

Finally, we must note that any study attempting to determine the quantitative and qualitative metabolic relations of structural units must employ methods in which damage to the components does not occur or is minimized. Recent fractionation methods stress the controlling use of microscopic examination, paying particular attention to the size, shape, internal structure, and stainability of the organelle. It is no exaggeration to say that many laboratories have wasted years of effort on materials subsequently shown to be unsatisfactory from the point of view of homogeneity and structural resemblance to the native state.

When due attention is paid to these elementary orienting considerations, it is found that there are numerous enzymatic activities in homogenates that are relatively unaffected by careful fractionation. Thus, the cytochrome oxidase and succinoxidase of a homogenate are entirely limited to cytoplasm and may be quantitatively recovered in mitochondria. These structures may then be disrupted for purposes of further purification of the component enzymes.

The  $Mg^{++}$ -activated ability to dephosphorylate ATP is also concentrated in the mitochondria, but these bodies only contain about 65 % of the total activity, the remainder being distributed among all other fractions. Systems such as these, in which the total activity of a homogenate can be accounted for, are useful standards in control of the fractionation procedure. However, systems in which activators and inhibitors have been detected and in which interactions of the components of the homogenate occur, can be expected to tell us more about problems of cell regulation.

### *B. Tables of Enzyme Distribution*

A number of important reviews have appeared on the biochemical properties of the cellular components. These include general discussions (Bradfield, 1950), surveys of plant material (Van Fleet, 1952; Goddard and Stafford, 1954), of bacteria (Alexander, 1956), and of animal tissues (Hogeboom *et al.*, 1953; Dounce, 1955; Allfrey *et al.*, 1955b; Hogeboom and Schneider, 1955). It is not the purpose of this discussion to summarize these contributions in detail, but rather to extract data which can reveal the patterns directly relevant to the problems of polymer synthesis. The following tables (Tables VIII, IX, and X) of distribution of enzymes in rat liver components, taken from Hogeboom *et al.* (1953) are useful examples for these purposes.

A glance at these tables reveals that of the known enzymes assayed in rat liver only a few are concentrated in the nucleus and microsomal fractions,

TABLE VIII  
 BIOCHEMICAL PROPERTIES OF LIVER CELL NUCLEI <sup>a</sup>

Enzyme	Recovery (whole tissue = 100)	Remarks
Arginase	36	—
Adenosine triphosphatase	31	—
Adenosine-5-phosphatase	40	—
Alkaline phosphatase	16	—
DPN nucleosidase	34	over-all recovery = 130-140 %
Synthesis of DPN	19-92	—

<sup>a</sup> Hogeboom *et al.* (1953).

TABLE IX

BIOCHEMICAL PROPERTIES OF LIVER MITOCHONDRIA <sup>a</sup>

Enzyme	Recovery (whole tissue = 100)	Remarks
Cytochrome oxidase	79	—
Cytochrome c	51	Most of remainder in soluble fraction
DPN-cytochrome c reductase	28	Most of remainder in microsomes
TPN-cytochrome c reductase	49	Concentrated in microsomes
Succinic dehydrogenase	72	—
$\alpha$ -Ketoglutaric oxidase	20	Enhanced by other fractions
Oxalacetic oxidase	45	Enhanced by other fractions
Octanoic oxidase	80	—
Glutamic dehydrogenase	83	—
Synthesis of ATP (oxidative phosphorylation)	94	—
Adenylate kinase	72	—
Adenosine triphosphatase	50	—
Choline oxidase	78	—
Betaine aldehyde oxidase	50	—
Tyramine oxidase	67	—
$\alpha$ -Glycerophosphate dehydrogenase	60	—
Catalase	45	Remainder in soluble fraction
Uricase	74	Particles containing uricase may not be mitochondria
Cathepsin	46	—
Glutaminase I	78	—
Acid phosphatase	61	—
Adenosine-5-phosphatase	15	—
Ribonuclease	58	—
Deoxyribonuclease	73	—
Glutamic-phenylpyruvic transaminase	100	—
Synthesis of <i>p</i> -amino hippuric acid	90	—

<sup>a</sup> Hogeboom *et al.* (1953).

TABLE X  
BIOCHEMICAL PROPERTIES OF MICROSOME FRACTION <sup>a</sup>

Enzyme	Recovery (whole tissue = 100)	Remarks
Esterase	47	Diffusely distributed in all other fractions
DPN-cytochrome c reductase	59	Also in mitochondria
TPN-cytochrome c reductase	36	Also in mitochondria
Glucose-6-phosphatase	88	
Lactase (kidney)	71	
DPN nucleosidase	50	
Uricase	32	Over-all recovery 130-140 %

<sup>a</sup> Hogeboom *et al.* (1953).

while a great many are concentrated in mitochondria. Some enzymes, such as ATPase or nucleoside phosphorylase, appear to be distributed in many fractions. However, the apparently wide distribution of uricase arises in the difficulties of cleanly separating the lysosomes within the mitochondrial fraction. As will be noted below, many enzymes are also found in the cell sap, e.g., glycolytic system, various dehydrogenases.

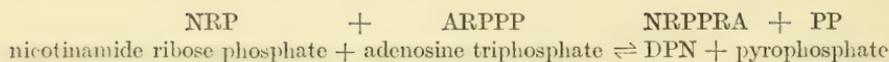
It is important to stress that these distributions are given for rat liver and that other distributions may be found for other tissues of the same animals or for other cells. For example, hippurate synthesis, centered in mitochondria of rat liver, is not found in mitochondria of heart (Green, 1954). The oxidative hexosemonophosphate pathway, found in large part in cell sap, nevertheless appears to be organized in rather tiny particulates in rabbit liver (Newburgh and Cheldelin, 1956). Also, Green (1957) refers to work of Gale to the effect that the entire glycolytic system of *Staphylococcus* is associated with the protoplast membrane of the bacterium rather than in the soluble state found in liver nuclei or cell sap.

Knowledge of these distributions should be of great value in following the course of virus purification. It should be known if a virus such as influenza virus carries some of these enzymes in addition to antigens characteristic of the host. Sharp *et al.* (1954) have described the association of ATPase with the virus of avian myeloblastic leukemia. Does this ATPase normally exist in a cellular component of comparable size? Do other enzymes exist on such a component? Are they also associated with the virus? Studies of this type have not yet appeared. Such information would also be of considerable relevance to the problems of viral origin.

### C. Enzymatic Systems of Nuclei

Table VIII A reveals that known enzymes concentrated in isolated nuclei include a group of systems capable of degrading intermediates of nucleic acid metabolism, and arginase, which converts arginine to ornithine and urea. These substrates are known to be present in nuclei. Although it has been suggested (Allfrey *et al.*, 1955b) that the relatively high concentration of nucleoside phosphorylase (as well as adenosine deaminase and guanase) reflect an accumulation of enzymes concerned with nucleic acid synthesis, the nature of these enzymes are not such as to suggest a synthetic role rather than a degradative one. Some workers have found these enzymes in a soluble fraction rather than in nuclei (Schneider and Hogeboom, 1956).

The extraordinary concentration of the system synthesizing DPN (DPN pyrophosphorylase) in this organelle should be noted; according to Hogeboom and Schneider (1952) the enzyme is present only in nuclei. The reaction catalyzed by this enzyme is:



Thus, unless another mechanism is found for the biosynthesis of DPN, it can be assumed that the coenzyme is transferred to the cytoplasm from its primary site of synthesis. Furthermore, since the sucrose method used usually results in a marked loss of protein, it would appear that this enzyme is confined to some site from which it does not leach. It has been reported that nucleoli isolated from the starfish contain a very high concentration of this enzyme (Baltus, 1954). However, it has been observed that in amoebae, enucleation does not produce a decrease in DPN content after 168 hours (Cohen, A., 1956). It is possible that cytoplasmic DPN may be highly stable in this organism since no turnover data are available for this system. Jacobson and Kaplan (1957b) have demonstrated a DPNH pyrophosphatase in the soluble fraction of mammalian liver, and DPN pyrophosphatase and DPNase in the microsomal fraction.

DPN is an essential component of the Embden-Meyerhof glycolytic system and it has been found that wheat germ nuclei are capable of metabolizing hexose diphosphate to pyruvate anaerobically with an activity comparable to that of cytoplasm (Stern and Mirsky, 1952, 1953). Indeed, enolase and pyruvate kinase are highly concentrated in these nuclei, and in calf liver nuclei, as well. In addition to these enzymes of carbohydrate metabolism, the first step in the phosphogluconate pathway, glucose-6-phosphate dehydrogenase, is also highly active in plant and animal nuclei. The first steps of this system require triphosphopyridine nucleotide (TPN) in most cells and the presence of this coenzyme in rat liver nuclei has also been recorded recently (Jacobson and Kaplan, 1957a). The distribution of the oxidized and reduced forms of these coenzymes in rat liver nuclei and other cell components have also been recorded by these authors. These coenzymes do not appear to be present in considerable amounts in nuclei despite their apparent origin in this organelle. However, it is not certain to what extent these substances were destroyed in isolation, nor is it known whether these substances may not have been leached from the nuclei during isolation, leading to their apparently high concentration in the cell sap.

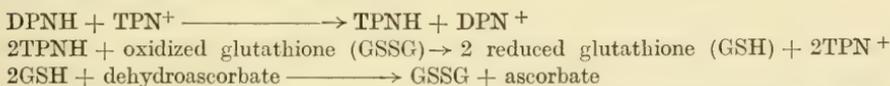
Numerous reports have indicated that neoplastic tissues have a relatively low content of DPN. The synthesis of TPN from DPN also appears to be curtailed in tumor cells (Glock and McLean, 1957). It may be asked, therefore, if tumor cells are not deficient in the capacity to synthesize these coenzymes, since the diphosphopyridine nucleotidase activity of a variety of tumors is within the range of normal tissue activities. Alternatively, the nucleotide substrates for DPN and TPN synthesis may have been shunted to the other syntheses occurring in such cells. This question is currently of considerable interest to virologists, in view of the observed dedifferentiation

of many kinds of normal cells in tissue culture and their apparent transformation to new types. Furthermore, it would be important to know the effects of tumor viruses on these metabolic systems.

Despite the presence of typical glucose-utilizing systems in nuclei with accompanying oxidation-reductions involving DPN and TPN, it is a mystery how hydrogen and electron transport continue in this cellular structure. Liver nuclei do not contain flavoproteins, cytochrome c, and cytochrome oxidase, and no mechanism is known in this organelle for the transfer of hydrogen to molecular oxygen. According to Schneider and Hogeboom (1956), FAD pyrophosphorylase does not exist in nuclei despite the concentration of DPN pyrophosphorylase and UDPG pyrophosphorylase (Smith and Mills, 1954). Stern and Timonen (1954) detected very small amounts of DPN-cytochrome c reductase and of flavin in calf thymus nuclei, amounts which they attributed to contamination. They have suggested that coenzyme reoxidation requires the participation of cytoplasm, since calf thymus nuclei contain 60 % of the total cell mass and enzymes capable of half of the glucose-6-phosphate metabolism of this cell.

In studies of nuclear glutathione, the concentration of which is relatively high, they found glutathione reductase in the amount of 16 to 20 % of that in cytoplasm. Ascorbic acid was also found in nuclei, but these investigators considered it unlikely that these substances actually participate in a major pathway of hydrogen transport.

Actually one could conceive that these latter systems are precisely those involved in the chain of electron transport required in this organelle, as in the following sequence of reactions:



Reduced glutathione generated during metabolism of glucose-6-phosphate might participate in the generation of sulfhydryl groups essential to the development of a mitotic spindle and nuclear division, as described by Rapkine (1931), Mazia (1956), and Stern (1956). The generation of soluble GSH prior to mitosis in *Lilium* is presented in Fig. 10. The cyclic production of acid in dividing sea urchin eggs has implicated glycolysis in division (Brachet, 1950), and a classic inhibitor of glycolysis, sodium fluoride, blocks prophase in mitosis (Hughes, 1952). Furthermore, ascorbate concentration also rises during mitosis of microspores in *Lilium* (Stern and Timonen, 1954), whereas O<sub>2</sub> consumption falls (Erickson, 1947).

It is premature to make much of this apparent concatenation of events. One may note, for example, that Holter and Zeuthen (1957) have reported that, in sea urchin development, O<sub>2</sub> consumption is maximal at prophase and falls thereafter. It will be necessary to have many of these measurements

of different metabolic activities on the same material at the same time to permit conclusions concerning their interrelations and significance for nuclear metabolism. One may wonder whether the large requirement for vitamin C is not related to the existence of a special mechanism necessary for disposing of this possible receptacle of nuclear hydrogen transport. It is conceivable that, in addition to an oxidative disposal of this compound to oxalate, reduced ascorbate may be implicated both in the solubilization of the mitotic apparatus by converting disulfide bonds to—SH groups and subsequently in the reductive generation of deoxyribonucleotides from ribonucleotides during interphase.

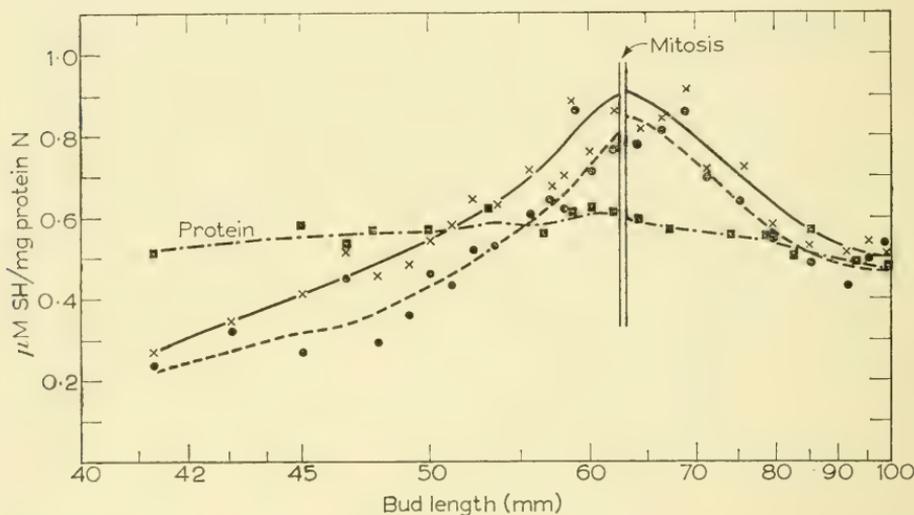


FIG. 10. Concentration of thiols in relation to nuclear division in *Lilium longiflorum* (Stern, 1956). Solid line represents total soluble thiols; the broken line, reduced soluble thiols. Bud length has been shown to be an index of cell development.

That glutathione and ascorbate may fulfill a critical role in nuclear metabolism might also be inferred from the fact that nutritional requirements for glutathione and ascorbate among microorganisms are quite rare, suggesting that mutation to auxotrophy for these substances might be lethal. Unfulfilled glutathione requirements in *Glomerella* are reported to lead to chromosome abnormalities (Markert, 1952) and ascorbate deficiencies lead to blocks in DNA synthesis (Sokoloff *et al.*, 1955).

As noted earlier, isolated thymus nuclei can phosphorylate adenylic acid to ATP (Osawa *et al.*, 1957). This reaction is blocked by cyanide, azide, and dinitrophenol, compounds which do not affect glycolysis but do typically affect respiration and oxidative phosphorylation in mitochondria. Nevertheless, a number of reagents, e.g., dicumarol, and methylene blue, which block

oxidative phosphorylation of mitochondria, do not inhibit phosphorylation in the nucleus. Osawa *et al.* (1957) have suggested that phosphorylation in the nucleus is oxidative and involves a cytochrome, as yet undetected. Since one must postulate an as yet undetected cytochrome, it seems equally plausible to consider the possible existence of other as yet undetected oxidation-reduction systems involved in oxidative phosphorylation, and one may wonder if the glutathione-ascorbate systems are not of interest in this regard.

Protein synthesis in isolated nuclei has been studied by Allfrey *et al.* (1957a,b). Thymocyte nuclei, isolated in sucrose, have been the major objects of study; these have been incubated with radioactive amino acids in the presence of 0.07 M Na<sup>+</sup> and the incorporation of the amino acids into isolated nuclear proteins were followed, as in Fig. 11. L-amino acids are

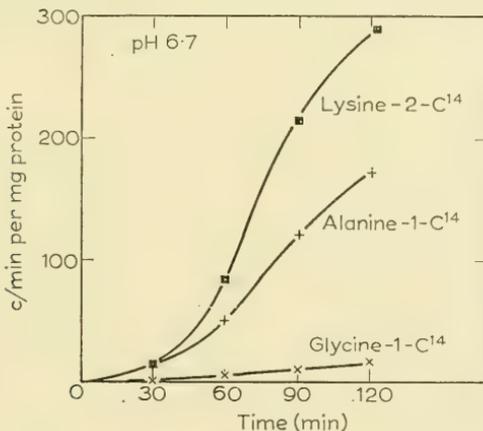


FIG. 11. The time course of incorporation of isotopic amino acids into the isolated thymus nuclei (Allfrey *et al.*, 1957a and b).

incorporated specifically in a mechanism which does not involve exchange. Under the best conditions of *in vitro* synthesis obtained, it has been estimated that each nucleus synthesizes 22 molecules of protein of average molecular weight 50,000 per second; this is a very small amount of synthesis.

The synthesis is inhibited by cyanide, azide, dinitrophenol, dicumarol, etc. However, methylene blue, which inhibits oxidative phosphorylation in mitochondria, and fluoride, which inhibits glycolysis, are relatively without effect. Chloroamphenicol and ethionine, which block protein synthesis very considerably in intact cells, had little effect on amino acid incorporation in nuclei. Small amounts of cortisone reduced uptake of C<sup>14</sup>-alanine by more

than 50 %. In addition, a structural analog of a purine nucleoside, 5, 6-dichloro- $\beta$ -D-ribofuranosyl benzimidazole, which is reported to inhibit influenza virus multiplication (Tamm *et al.*, 1954), is also an effective inhibitor of uptake. To be maximally inhibitory, a  $\beta$ -D-ribofuranoside is required (Allfrey *et al.*, 1957b).

These workers have also shown that amino acid incorporation is greatly inhibited by degradation and removal of DNA after treatment with DNAase.<sup>1</sup> Readdition of large fragments of DNA to such DNAase-treated nuclei restores activity. Bases and nucleotides were ineffective in restoring activity. Although many degradation products of RNA were ineffective in this regard, dialyzable split products of RNA digestion by RNAase had some restoring activity. The synthetic polynucleotide, polyadenylic acid, possessed restoring activity. The possible role of RNA metabolism in incorporation in this system is unclear at present; on the other hand, DNA is implicated in protein synthesis in nuclei. It may be noted that of the proteins containing incorporated amino acid the histones had the lowest specific activity, whereas the proteins associated with RNA in "residual chromosomes" had the highest specific activity.

The synthesis of nucleic acid in these nuclei has been studied by estimating the incorporation of glycine-1-C<sup>14</sup> and orotic acid-6-C<sup>14</sup> into purines and pyrimidines, respectively (Allfrey *et al.*, 1957a).<sup>2</sup> The incorporation of the latter was observed far more readily. It enters nuclear RNA, from which it can be released by RNAase. Nuclei degraded by DNAase are far less effective in such incorporation. The incorporation of thymidine into the DNA of isolated nuclei had been demonstrated by Friedkin and Wood (1956), who also observed that DNAase lowers incorporation.

The benzimidazole riboside was shown by Allfrey *et al.* (1957a) to block synthesis of nuclear RNA, using orotic acid-C<sup>14</sup> as a label. This compound was capable of blocking protein synthesis when added with the amino acid. However, a 30-minute incubation and activation of the nuclei before addition of the analog protected the nuclei from inhibition of amino acid incorporation in the presence of the analog. One might imagine that a prior synthesis of nuclear ribonucleotides or RNA was essential to subsequent amino acid incorporation which, having had its precondition fulfilled, could no longer be blocked by the analog riboside.

<sup>1</sup> According to a number of workers (Brown *et al.*, 1952; Lamirande *et al.*, 1954), the nucleases, DNAase and RNAase, can be found in nuclei. However, most of the nuclease activity of the cell is associated with cytoplasmic structures, e.g., lysosomes. The liver nuclei of animals fed *p*-dimethylaminoazobenzene markedly increase their DNAase activity (Lamirande *et al.*, 1954). These workers have suggested a possible correlation of such activity with the mitotic activity of the nuclei.

<sup>2</sup> It has been shown that there are at least two fractions of RNA of differing metabolic activity in nuclei (Allfrey and Mirsky, 1957; Vincent, 1957).

### D. Enzymatic Systems of Cytoplasm

#### 1. Mitochondria

Tables VII and IX reveal the large number of enzymes present in the mitochondria of rat liver, as well as the relatively high concentration of many of these and a number of vitamins, coenzymes, and metabolites

A number of these enzymes, e.g., acid phosphatase, ATPase, glutamic dehydrogenase, etc., are "latent" or inactive when the mitochondria are isolated in an undamaged state. Disruption of the membrane permits demonstration of these enzymatic activities. Further disruption by mechanical means reveals two major classes of mitochondrial enzymes, an insoluble phosphorylating carrier of respiratory systems and ATPase, and a number of soluble enzymes which include glutamic dehydrogenase, adenylate kinase, fumarase, etc. Treatment of the insoluble fragments with sodium cholate destroys the phosphorylating ability without affecting respiratory activity (Gamble and Lehninger, 1956).

A soluble heme protein, termed mitochrome, which inhibits the aerobic phosphorylation of fresh mitochondria, has recently been isolated after release by aged or otherwise degraded mitochondria (Polis and Shmukler, 1957). Mitochrome appears to be involved in the action of the hidden ATPase of intact mitochondria, and it has been suggested that these structures are integrated in some way to effect a binding of inorganic phosphate and transfer of this moiety to ATP during oxidative phosphorylation.

From these tables it is clear that in animals mitochondria are the major cell centers of respiration, i.e., of hydrogen and electron transport terminating in reaction with molecular  $O_2$ , since this particle contains all the cytochrome oxidase of the cell (Hogeboom *et al.*, 1946; Martin and Morton, 1956). At particular stages in the life cycle of some plants, polyphenol oxidase and ascorbic acid oxidase may make the most quantitatively significant contributions to  $O_2$  consumption (James, 1953). Although in plants both cytochrome oxidase and polyphenol oxidase are associated with mitochondria, ascorbic acid oxidase is reported to be associated with the cell surfaces (Goddard and Stafford, 1954). In bacteria, the work of Weibull (1953a,b) showed that the cytochromes of *B. megatherium* existed on the membrane of the protoplast, i.e., the cytoplasmic membrane (Murray, 1957) remaining after the cell wall of the bacterium was stripped off by lysozyme in the presence of hypertonic sucrose. In addition, bacteria react with Janus green at the cell periphery, leading to the suggestion that in bacteria these oxidative sites exist in particles, which may form a continuous layer immediately within the cell wall (Alexander, 1956). Many workers have described the isolation of cytochrome-containing particles from bacteria; however, these particles are now thought to result from the disruption of the fragile protoplast membrane.

In 1912, Batelli and Stern and Warburg recognized the insoluble nature of key oxidative systems. Keilin began to study the correlation between respiration and cytoplasmic structure in the late 1920's and, in examining the activity of the cytochromes, observed that certain substrates, e.g., cysteine, capable of interacting with cytochrome c in solution, were incapable of interacting with particle-bound cytochrome c. That the oxidative enzymes are not randomly aggregated in mitochondria and that they are spatially organized and integrated in these structures was a concept developed further by Green (1957) and others in work on the citric acid cycle, fatty acid oxidation, the organization of terminal electron transport, etc.

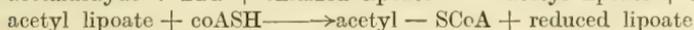
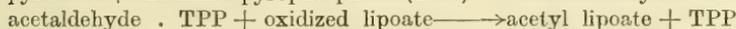
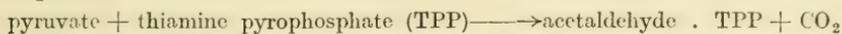
Hogeboom *et al.* (1946) were the first to show that cytochrome oxidase was entirely organized within mitochondria. As noted above, Lardy and Wellman (1952) showed that the rate of respiration of these bodies was tied to oxidative phosphorylation and that the removal of newly formed high energy phosphate by means of glucose and hexokinase was important for the maximal efficiency of the electron chain. Pressman and Lardy (1952) have also observed an enhancement of mitochondrial respiratory rate by a microsomal component.<sup>1</sup> Thus, maximal energy production in the cell requires optimal conditions of interaction of mitochondrion and its surrounding milieu.

In the operation of the citric acid cycle, which also funnels electrons to the cytochrome systems, the existence of extramitochondrial enzymes of the cycle, e.g., isocitric dehydrogenase, has been thought to argue against the concept of integration of cycle operations within the organelle. However, the mitochondrion does catalyze all the reactions of the cycle and the largest part of the citrate of the cell is found within this particle. Nevertheless, recent reviewers (Schneider and Hogeboom, 1956) are not convinced of the preponderant role of an integrated organelle in the operation of the cycle, pointing out that cytoplasm as a whole is greatly superior to mitochondria in catalyzing cycle oxidations. To this reviewer, the argument seems mainly one of terminology, since those who consider that mitochondria do embody an integrated array of enzymes do not insist that this system operate in a vacuum and, indeed, will agree that the exigencies of optimal operation will require a host of factors and reactions supplied by an enveloping cytoplasm.

Energy production and conservation in mitochondria are not entirely a function of the chemical tapping via oxidative phosphorylation of the main electron chain from the reduced pyridine nucleotides through flavoproteins and the cytochromes. The oxidation of  $\alpha$ -ketoacids, such as pyruvate and  $\alpha$ -ketoglutarate, lead to the formation of thioesters, which represent an additional mechanism for the conservation of the energy derived from

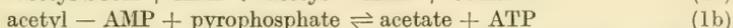
<sup>1</sup> A heat-stable component of the supernatant fluid after removal of mitochondria, which by themselves possess a small ability to glycolyse anaerobically, will markedly stimulate such glycolysis by separated mitochondria (Hochstein, 1957).

oxidative decarboxylation. Thus, pyruvate is converted via the pyruvic oxidase of mitochondria to the thioester, acetyl coA, in the following sequence:

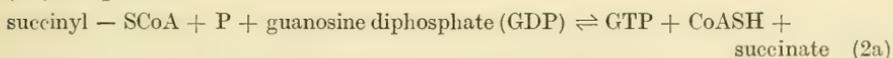


A lipoate dehydrogenase requiring DPN and linked to the cytochromes regenerates oxidized lipoate. In a comparable series of reactions,  $\alpha$ -keto-glutarate forms succinyl-SCoA plus  $\text{CO}_2$ .

These thioesters may participate in the following synthetic reactions to form ATP:



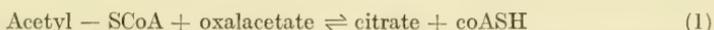
In this group of reactions, the energy conserved in the thioester linkage is stored in the carboxy-phosphoanhydride of acetyl-AMP prior to formation of ATP. The apparent role of such reactions in protein synthesis will be discussed in a later section. Reaction (2a) takes place in heart muscle and (2b) in spinach.



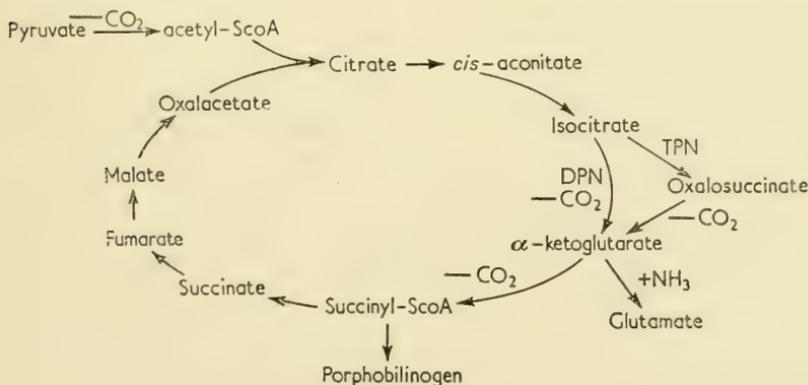
Myokinase is also present in mitochondria and catalyzes the reaction



In addition to generating ATP, acetyl coA can participate in the following reactions:

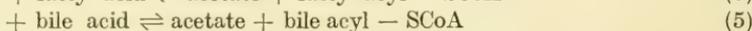
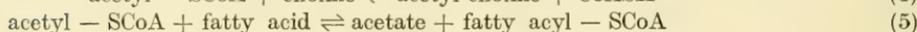
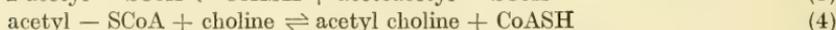
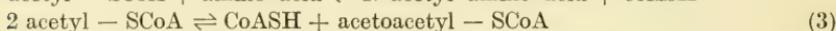
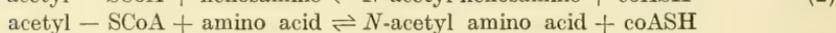
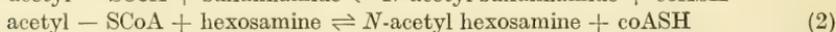
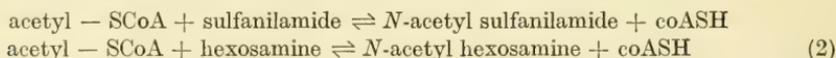


starting the turn of a tricarboxylic acid cycle as seen in formula (I) which can degrade two carbons of acetate to  $\text{CO}_2$ :

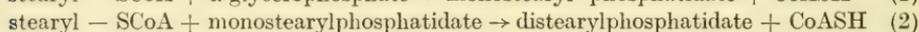
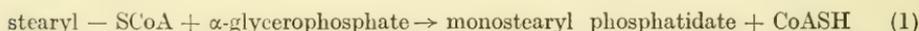


(I)

It will be seen that this cycle can not only degrade a two-carbon fragment to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  but may provide moieties essential for the synthesis of glutamate and many derived amino acids, the porphyrins, etc. In the life of a cell, it is not possible to ascribe either a catabolic or anabolic role to this system, since it may fulfill both. Additional reactions of acetyl—SCoA follow:



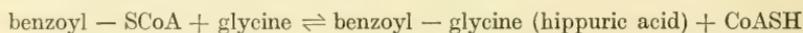
In reaction 5, it can be seen that the acetyl moiety can be exchanged for other acyl substituents. Mechanisms are then available for the synthesis of esters, as in the synthesis of phosphatides, which occurs in mitochondria (Kornberg and Pricer, 1952).



Kennedy (1953) has shown that incorporation of  $\text{P}^{32}$  into phosphatide in isolated mitochondria requires oxidative phosphorylation. Free glycerol was stimulatory and the formation of the intermediate,  $\alpha$ -glycerophosphate, was strongly indicated. Again, enzyme fractions from the soluble supernatant fraction were markedly stimulatory in this process. The synthesis of complete phosphatides will be discussed in a later section.

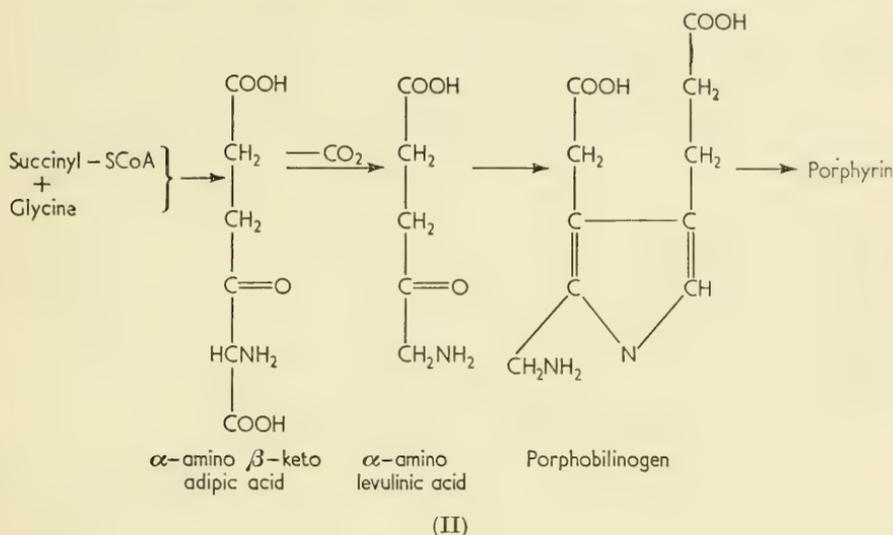
Although the conversion of acetate to cholesterol occurs to a slight degree, if at all, in liver mitochondria (Bucher and McGarrahan, 1956), the oxidation of cholesterol to acidic steroids such as cholic acid proceeds very well in these organelles (Frederickson, 1956). The accumulation of cholesterol esters of fatty acids was also noted in these isolated oxidative systems.

The formation of acyl — SCoA derivatives also facilitates the synthesis of some peptide bonds by mitochondria (Chantrenne, 1951). Thus,



The possible significance of this reaction in the syntheses of peptides in general will be considered in a later section.

As shown in formula (II), succinyl — SCoA condenses with glycine to form  $\alpha$ -amino  $\beta$ -keto adipic acid, which decarboxylates to  $\alpha$ -amino levulinic acid. Two moles of the latter condense to porphobilinogen, the immediate precursor to porphyrins.



The synthesis of the key pyrimidine, orotic acid, from  $\text{NH}_3$ ,  $\text{CO}_2$ , aspartate, and ATP is also effected by mitochondrial enzymes, whereas the further conversion of orotic acid to the nucleotide, uridylic acid (UMP), occurs in a soluble fraction (Hurlbert and Reichard, 1954; Reichard, 1954).

Other reactions of amino acid biosynthesis also occur in mitochondria. These include the amination of  $\alpha$ -ketoglutarate to glutamate, transamination from amino acid to another keto acid, and a number of synthetic reactions of the citrulline  $\longrightarrow$  arginine  $\longrightarrow$  ornithine cycle.

It will be noted that this brief and incomplete list of synthetic reactions localized in mitochondria does no more than skirt the areas of polymer synthesis with which we shall be mainly concerned. The major reason for this is the fact that the biosynthesis of nucleic acid and protein has not yet been found to be very active in these particles. At least this is so in terms of such crude measurements as the incorporation of  $\text{P}^{32}$  and  $\text{C}^{14}$ -amino acids into total mitochondrial nucleic acid and protein. One might ask if this were true with respect to the synthesis of a mitochondrial enzyme or RNA. Are mitochondrial nucleic acids and proteins made in mitochondria? Will not the syntheses of mitochondrial-specific entities occur at a maximal rate in these organelles? These questions have not yet been posed experimentally. With respect to the general problem of polymer syntheses, existing methods of measurement and data have compelled us to shift our attention away from these critical organelles.

However, it can be seen in our examination of reactions occurring in mitochondria, that, in addition to the generation of a utilizable chemical

currency, ATP, and other high energy phosphates, a number of different biochemical mechanisms are available for the conservation of the energy derived from energy-yielding reactions and for the formation of ester and peptide linkages, as well as carbon to carbon bonds. Reactions associated with mitochondria, leading to the esterification of sulfate, fixation of  $\text{CO}_2$ , and the formation of glutathione will be discussed in later sections.

### *E. Enzymatic Systems in the Microsomal Fraction*

It was not until 1951 that an enzyme was found to be concentrated in this fraction. Hers and collaborators (1951) reported on the distribution of glucose-6-phosphatase and, as presented in Table X, it appeared that essentially all of this enzyme in liver was present in the microsomal fraction. A similar situation exists for kidney lactonase (Meister, 1952).

Glucose-6-phosphatase is not inactivated by RNAase and may be solubilized in an active nonsedimentable form by deoxycholate. Its inactivation by trypsin and a lecithinase suggests a close relationship with lipoprotein, presumably on the membranes of the endoplasmic reticulum (Beaufay and de Duve, 1954).

In the 48-hour fasting rat, under conditions in which liver glycogen is reduced to 5 % of the normal value, the glucose-6-phosphatase activity per unit weight of liver is increased 60 % (Weber and Cantero, 1954). It is possible that there has been a synthesis of enzyme under conditions in which glycogen is being mobilized, and it is of interest that this apparently adaptive phenomenon appears to be associated with the membrane component of the reticulum.

Some oxidation-reduction systems have been found concentrated in the microsomal fraction.<sup>1</sup> These include DPN- and TPN-cytochrome c reductases. However, although the DPNH and TPNH-cytochrome c reductase of mitochondria are inhibited by antimycin, this antibiotic has no effect on the reductases of the microsomal fraction. Cytochrome c is not present in the microsomal fraction nor is it certain that cytochrome c really exists outside of mitochondria. It therefore seems likely that these reductases transfer electrons from DPNH to other acceptors. The liver microsomal fraction has a reddish hue, and a slowly autoxidizable protoporphyrin hemochromogen, now termed cytochrome m, distinct from cytochromes a, b, and c, was demonstrated in this fraction by Strittmatter and Ball (1952). More recent work of Strittmatter and Velick (1956) has shown that cytochrome m is the true microsomal electron acceptor for the DPNH reductase of this fraction.

<sup>1</sup> Thiers and Vallee (1957) have recently analyzed subcellular fractions for S cations. Microsomes are low in Cu and Mn and relatively rich in Fe, suggesting the presence of Fe hemochromogens (see Fig. 5).

Strittmatter and Ball (1952) have suggested that this system plays an important role in the reductive syntheses of lipids. The microsomal fraction of various plants has also been found to contain a hemeprotein, called cytochrome  $b_3$ , which is localized exclusively in this fraction (Martin and Morton, 1957). This cytochrome is reduced enzymatically by the addition of reduced DPN.

Many of the key steps of cholesterol synthesis, as well as the hemochromogen, do appear to be centered in the vesicular structures of the microsomal fraction (Bucher and McGarrahan, 1956). In short term experiments with injected acetate- $C^{14}$ , over 90 % of the formed cholesterol is formed in the microsomal fraction. However, in *in vitro* systems, the synthesis of cholesterol requires the participation of glycolytic systems and oxygen as well as other systems derived from the soluble fraction. Squalene accumulates in the absence of air and this hydrocarbon is converted to cholesterol only in the presence of oxygen.

Mueller and Miller (1949) and Brodie and his collaborators (1955; Brodie, 1956; Gillette *et al.*, 1957) have studied the detoxication of drugs by liver microsomes. Only nonpolar compounds are degraded by these metabolic systems and it may be imagined that this has provided a mechanism for the survival of "normal" metabolites, which are predominantly polar in character. However, these systems have not been found in microsomes of other cells. These reactions involve TPNH and oxygen and the TPNH oxidase system involved is not cyanide-sensitive. The end product of the TPNH oxidase activity is hydrogen peroxide. The system is also inhibited by detergents.

Of outstanding interest to this discussion is the fact that the microsomal fraction is a major site of protein and RNA synthesis.<sup>1</sup> Various aspects of these polymer syntheses will be discussed in detail below.

#### *F. Enzymatic Activities of Cell Sap*

This fraction is rich in protein and it may be suspected of containing many enzymatic activities. Many enzymes have indeed been found. Phosphorylase is mainly contained in this fraction, as is the largest part of cell glycogen (Sacks *et al.*, 1957). Glycolytic enzymes are found largely in this fraction, although certain enzymes, such as hexokinase, may be bound in considerable degree to sedimentable material in particular organs. Enzymes of the oxidative hexose monophosphate shunt are also present in this fraction, as are some enzymes of the tricarboxylic acid cycle, e.g., isocitric dehydrogenase,

<sup>1</sup> It has been discovered that the heavier components of a homogenate of hen oviduct appear most active in protein synthesis (Hendler, 1956). On closer investigation, it appears that the endoplasmic reticulum of this tissue is better preserved after homogenization, in contrast to rat liver, and is therefore sedimented rather readily (Hendler *et al.*, 1957).

aconitase. Other systems of the fraction are briefly summarized by Hogeboom and Schneider (1955). Considering the state in which it is obtained, it is difficult at this time to correlate the biological relations of this fraction with its chemical properties. At present, the cell sap constitutes a splendid fishing pool for the elementary stages in the training of an enzymologist.

### *G. Polymer Synthesis in Cytoplasm and Nucleus*

#### *1. Methodology*

Before examining data on the synthesis of proteins and nucleic acid at the enzymatic level within specific cell fractions, it is desirable to consider the variety of approaches used to localize these functions within more or less intact cells and to summarize the relatively large body of information arising from such studies. These studies are of particular interest for the design of experiments by virologists. The approaches used include the following:

1. Incorporation of a labeled precursor into the test system. Cell fractionation and autoradiography have been employed to estimate the extent of incorporation into various cell parts. Numerous modifications of this type of experiment have been made in order to obtain data on the possible kinetic interrelations of cell fractions and components.

2. Separation of intact cells into enucleate and nucleate fragments. The metabolic capabilities of such organized fragments have then been explored by a variety of techniques, including the isotopic approach indicated above. In recent years these experiments have been performed for the most part on two selected types of differentiated giant cells, the alga, *Acetabularia*, and the protozoan, *Amoeba*. It may be noted that the induction of giantism in virus-susceptible mammalian cells in X-irradiated tissue cultures (Cieciura *et al.*, 1957) may now permit the performance of many of these kinds of experiments with this infectable material.

3. Specific enzymatic elimination of cell components without cell destruction. The use of enzymes, such as ribonuclease to degrade basophilic components in a variety of living cells and lysozyme to eliminate the cell wall and produce viable bacterial protoplasts, permits a comparison of the metabolic capabilities of normal and partially degraded cells.

4. Nutritional deficiencies and genetic lesions have been exploited in bacteria to produce specific deficiencies of cell parts.

5. Inhibitors have been similarly employed to induce specific deficiencies. These have included chemical substances, such as chloramphenicol and penicillin, or physical agents, such as ultraviolet irradiation.

#### *2. Protein Synthesis*

- a. Relations to Problems of Protein Structure.* We wish to know if protein synthesis can occur throughout all parts of a cell, i.e., at all the sites of

deposition in which protein is found, or if such synthesis occurs predominantly at a particular organelle from which protein or polypeptide may be transported to its particular site of deposition, or if some combination of these more accurately describes the cellular biosynthesis of particular proteins. Present data relates for the most part to two extremes of protein synthesis, i.e., to the site of incorporation of amino acids into the peptide linkages of protein and to the origin of enzymatic activity. Most current experimentation has not yet dissected the problem in terms of the various levels of protein structure. These levels include:

(1) Primary structure—the specific order of amino acids in a polypeptide chain.

(2) Secondary structure—the folding of a specific polypeptide chain into a helix or other form such that hydrogen bonding between amino acid residues on the same chain confers stability on this configuration of the chain and produces a specific three dimensional geometry to a single polypeptide chain.

(3) Tertiary structure—the side to side arrangement of folded polypeptide chains, fixed by a variety of bonds, polar, covalent, etc., between functional groups, e.g.,  $-\text{SH}$ ,  $-\text{NH}_2$ ,  $-\text{COOH}$ , etc., between amino acid side chains. These arrays of peptide chains determine the size and shape of specific proteins and the linkages between chains confer stability to the protein as a whole. If the problems of protein synthesis are posed in terms of these three different levels of structure, it can be seen, for example, that the incorporation of an amino acid into protein-bound peptide in one or another part of the cell may relate only to an early step in the total process of producing functional protein. Thus, this functional unit may really be assembled at a very different site from that indicated by incorporation data.

*b. Sites of Protein Synthesis. i. Incorporation Data.* Caspersson (1941) proposed that the nucleus is the main center of protein synthesis. He considered that the genetic material of the chromosomes controlled the quality of protein synthesis. More particularly, heterochromatin and nucleoli were believed to be responsible for the synthesis of basic proteins, which were thought to accumulate in the latter organelle. From the nucleolus, basic proteins diffused to and through the nuclear membrane and induced the synthesis of cytoplasmic RNA, which subsequently induced the synthesis of cytoplasmic proteins. At approximately the same time Brachet (1941, 1942) presented the hypothesis that RNA was somehow concerned with protein synthesis, which he did not yet relate to nuclear activity.

As described earlier, modern data on the composition of nuclear components do not fit well with the details of Caspersson's hypotheses. However, that the nucleolus can indeed be an important center of RNA and protein synthesis is strongly supported by the autoradiographic studies of Ficq (1955,

1956), who showed that the nucleolus of the starfish oocyte incorporates adenine-8-C<sup>14</sup> and phenylalanine-2-C<sup>14</sup> at far greater rates than do other parts of the nucleus and cytoplasm (Fig. 12). The use of the specific labels for

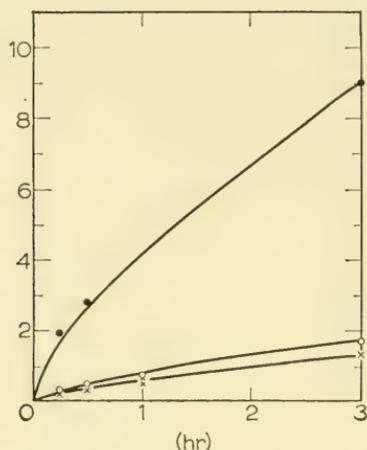


FIG. 12. Incorporation of adenine-8-C<sup>14</sup> into the oocytes of *Asterias* (Ficq, 1956) —●— nucleus, —○— cytoplasm (radioactivity:  $\times 70$ ), —x— nuclear sap (radioactivity:  $\times 2$ ). Ordinate = number of tracks  $\times 10^4$  per  $\mu^2$  after 3-day exposure to photographic emulsion.

nucleic acid and protein, respectively, followed earlier work showing similar results with a relatively nonspecific label, glycine-1-C<sup>14</sup> (Ficq, 1953). In this study it was shown that glycine was incorporated into the nucleolus of the starfish oocyte at a rate 100 times greater than its incorporation into cytoplasm.<sup>1</sup> These results and others of Ficq and Brachet (1956) have also served to support the hypothesis that organelles relatively rich in RNA are active in protein synthesis.

Experimentation on the incorporation of amino acids into cell protein has not yet provided data on the specific activity of the nucleoli, but only of the nuclear fraction and various cytoplasmic fractions. The possibility that very high nucleolar activity is a general phenomenon in all cells has been obscured by the dilution of incorporated isotopic amino acid by the excess of inactive nuclear protein. Most data on incorporation into protein have been obtained in just this way and in general appear to show that in animal tissue the microsomal fraction (Borsook, 1956) and occasionally a supernatant fraction have a higher rate of incorporation of labeled amino acid than do mitochondria and nuclei.

<sup>1</sup> However, it has been reported that, in most mammalian tissues, the nucleus is not appreciably more active than cytoplasm at early stages of amino acid incorporation (Brachet, 1957).

In the experiments of Littlefield *et al.* (1955; Littlefield and Keller, 1957), it was observed that, when a  $C^{14}$ -amino acid is injected intravenously into a rat, the initial incorporation into the microsomal fraction of the liver is several times greater than into other cell fractions. Furthermore, as shown in Fig. 13, when this fraction was separated into the deoxycholate-insoluble

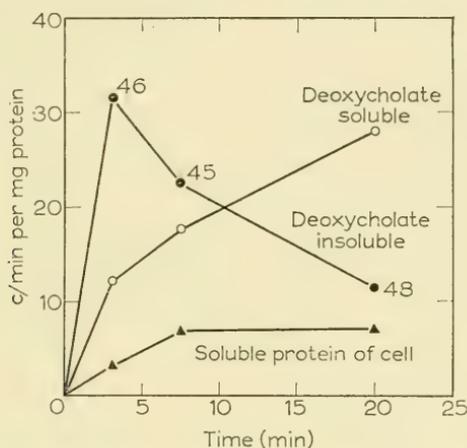


FIG. 13. Incorporation *in vivo* of a small dose of leucine- $C^{14}$  into the two components of the microsomes and into the soluble protein of the cell (Littlefield *et al.*, 1955). The per cent RNA by weight of each deoxycholate-insoluble sample is indicated.

ribonucleoproteins and deoxycholate-soluble lipoproteins, the former possessed a markedly greater activity. In addition, under the conditions of the experiment presented in Fig. 13, i.e., using a small dose of labeled leucine- $C^{14}$ , it appeared that after initial fixation, the newly formed protein left this fraction at about the rate the specific activity of the deoxycholate-soluble fraction increased. That amino acid incorporation in these systems involves peptide bond formation has been demonstrated by Zamecnik and Keller (1954).

It may be asked if such an apparent localization does not arise from primary peptide synthesis in the nucleolus, transport to the cytoplasm, and selective fixation on the microsomal components. However, appearance of isotope in the microsomal fraction is immediate and continues linearly (Littlefield and Keller, 1957). Furthermore, we shall see that enucleate cell fragments are capable of protein synthesis. Finally, as will be discussed in greater detail below, the isolated microsomal fraction is capable of incorporating amino acids *in vitro*, and a transport mechanism need not be invoked under these experimental conditions.

A number of interesting variants of the results of this basic incorporation experiment may be mentioned. The incorporation of methionine- $S^{35}$  into the

proteins of rat pituitary was most rapid in the soluble fraction and nuclear incorporation occurred at less than half this rate (Ziegler and Melchior, 1956). Incorporation of leucine- $C^{14}$  into TMV-infected leaf disks over a two-hour period revealed that TMV had a lower specific activity than any of the isolated fractions, including chloroplasts; the microsomal fraction was most active, more than twice that of the isolated TMV (Stephenson *et al.*, 1956). In this study it was also found that isolated chloroplasts synthesize protein quite actively, and that oxygen and light stimulated this process.

In studies of Allfrey *et al.* (1955a) on the incorporation of glycine- $N^{15}$  into various mouse tissues, special attention was paid to the activity of nuclear components. In general, cytoplasmic ribonucleoprotein was most active in these tissues and the residual protein of the chromosomes approached the over-all rate of cytoplasm. However, glycine- $N^{15}$  uptake into histones was least rapid of any protein fraction, including the lipoproteins and globulins of nuclei (Rotherham *et al.*, 1957). Although protein synthesis has been described in isolated nuclei, the quantitative role of the nucleus in the over-all protein economy of the cell has not been greatly clarified by the experiments indicated above.

*ii. Enucleate and Nucleate Cell Fragments.* Brachet and his collaborators have studied fragments of the unicellular alga, *Acetabularia mediterranea*, and the protozoan, *Amoeba proteus*. Mazia and his group have concentrated on the latter organism. The experiments of these groups have attempted to clarify the nature of the interactions between nucleus and cytoplasm that occur in the metabolizing cell. The procedure adopted is essentially one of study of differences in metabolic behavior when the possibility of these interactions is eliminated in one of the fragments. Studies of these systems have yielded considerable fundamental information.

Although virologists are familiar with amoebae, probably few are acquainted with *Acetabularia*, an organism studied in detail by Hammerling (1953). The fully developed giant cell consists of a rhizoid, stalk, and cap. The stalk may reach 4 to 6 cm., the cap diameter being up to 1 cm. The single large nucleus lies within the rhizoid and removal of the rhizoid produces an enucleate part devoid of DNA. The cap may also be cut from this enucleate, chloroplast-containing fragment. The cap may be regenerated in the light, photosynthesis permitting the fixation of  $C^{14}O_2$  into cell components. Even after 5 weeks, the synthetic activity of enucleate parts may be as much as 70 % that of nucleate fragments. It may be mentioned that the nucleolus of the intact organism is rich in RNA, as indeed is the cytoplasm of the rhizoid in the vicinity of the nucleus.

According to Brachet *et al.* (1955), removal of the nucleus has no effect on  $O_2$  consumption or photosynthetic activity for two more weeks, and little on the incorporation of  $P^{32}$  for shorter intervals, e.g., hours. However,  $P^{32}$

uptake has been found to be most rapid in the rhizoid and in the nucleolus. Enucleate fragments also maintain their ATP content aerobically and anaerobically. Active protein and RNA synthesis occur simultaneously in enucleate fragments; indeed, over a period of three weeks protein synthesis is much more active in these than in the nucleate fragments, although it then stops in the former while the latter continues to synthesize protein. With the exception of this point, no evidence has been obtained in this organism to indicate that protein synthesis by the nucleus is quantitatively significant.

With *Amoeba proteus*, somewhat different results are obtained (Brachet, 1955a). Enucleation results in little change of O<sub>2</sub> consumption but ATP accumulates in air. Anaerobically the ATP content of the enucleate fragment falls quickly, suggesting that in this organism the nucleus may play a quantitatively significant role in the anaerobic generation of ATP. Unlike *Acetabularia*, enucleate amoebae shrink and their glycogen, RNA, and protein contents fall markedly after a very few days. In the nucleate half, P<sup>32</sup> uptake is maintained at a normal rate, whereas this falls sharply in the enucleate fragment.<sup>1</sup>

Enzyme studies have proven more difficult in *Amoeba*. Many enzymes have not yet been found in this organism, e.g., hexokinase, DPN pyrophosphorylase, nucleoside phosphorylase. Enolase, believed to be concentrated in the nuclei of other cells, is not so concentrated in *Amoeba* and may be maintained unchanged for many days in an enucleate fragment. On the other hand, dipeptidase, phosphatase, and esterase fall markedly in enucleate fragments; the last two are known to be associated with the microsomal fraction. The results on RNA and enzyme disappearance suggest that, in this organism, the nucleus appears to control the maintenance of the microsomal fraction in the cytoplasm, a fact which parallels the absence of such a fraction in enucleate mammalian erythrocytes. Such control apparently does not apply to other enzymes, such as enolase, protease, amylase, and ATPase, which may be associated with other cell fractions.

More direct data on protein synthesis in amoebae stem from the work of Mazia and Prescott (1955). Enucleation reduces immediate total methionine-S<sup>35</sup> uptake by a factor of 2.4; indeed, the rate of incorporation into protein in enucleate halves was only one-sixth that in nucleate halves. The latter effect accounts for the observed effect on uptake, i.e., the nucleus does not control penetration into the cell, but only incorporation into protein. In this system, nuclear synthesis is estimated to account for 40 % of the methionine incorporation into the whole cell. On the other hand, intact

<sup>1</sup> The marked decrease of anaerobically-generated ATP has also been observed in two other systems in which nuclei have been damaged, i.e., ultraviolet-irradiated *E. coli* (Kanazir and Errera, 1955) and strongly lethal interspecific frog hybrids, as well as in frog eggs fertilized with sperm treated with nitrogen mustard (Brachet, 1954).

amoebae incorporate twice the amount of methionine used by the nucleate half. Mazia and Prescott suggest that the amino acid is activated by the rate-limiting amount of cytoplasm, which then transfers the active precursor to the nucleus.

It should be noted that protein synthesis in the cytoplasm in *Amoeba* is quite significant, and, in agreement with the work on *Acetabularia*, the studies on *Amoeba* have shown that the cytoplasm is capable of independent protein synthesis. Such a conclusion is in agreement with the conclusions derived from work on reticulocytes (basophilic nonnucleated erythrocytes), which also appear capable of synthesis of globin and hemin (London *et al.*, 1950; Borsook *et al.*, 1952).

Functional enucleation may be accomplished, at least in part, in a variety of biological materials, of which the reticulocyte is one. In many adult tissues, mitosis may be a rare event among many metabolizing cells actively synthesizing protein. In such systems, e.g., nerve, liver, etc., general protein synthesis is at least separable from the duplication of DNA and histones. Similar results may be obtained with thymine-requiring bacteria deprived of thymine (Barner and Cohen, 1954) or with ultraviolet-irradiated bacteria (Kelner, 1953; Kanazir and Errera, 1954), in both of which protein and RNA are synthesized in the absence of DNA synthesis.

In many respects a cell is functionally enucleate during division (Brachet, 1957). In amoebae, growth and protein synthesis stop at this time (Prescott, 1955) and  $P^{32}$  uptake also decreases 50 % in this organism (Mazia and Prescott, 1954). In early development of the frog (until gastrulation), RNA synthesis stops during the rapid series of mitoses. This function and related protein synthesis approach a normal rate only when the mitotic rate slows down.

*iii. Enzymatically Degraded Cells. (a) Bacterial Protoplasts.* Lester (1953) showed that *Micrococcus lysodeikticus*, treated with lysozyme in a medium containing concentrated sucrose, was able to incorporate leucine- $C^{14}$  into protein. The incorporation was enhanced in the presence of DNAase and abolished by RNAase. It seems probably that these results were obtained by protoplasts, i.e., bacteria lacking their cell walls; these results have been reproduced and extended with carefully prepared protoplasts (Bridoux and Hanotier, 1956).

McQuillen (1955a) has shown that intact but not lysed protoplasts of *B. megatherium* are capable of actively synthesizing protein and nucleic acid. Despite their lack of cell walls, protoplasts are capable of bacteriophage multiplication, if infection is accomplished prior to the removal of the cell wall (Salton and McQuillen, 1955; Brenner and Stent, 1955). They are also capable of growth and division (McQuillen, 1955b) and the induced biosynthesis of enzymes, including permeases (Landman and Spigleman, 1955;

Rickenberg, 1957). Indeed, a recent report indicates the most rapid incorporation of labeled glycine occurs in the protein of the protoplast membrane (Hunter *et al.*, 1957).

Thus, in these forms at least, the activation of amino acids prior to later assembly stages does not occur at the cell wall. As will be noted below, a considerable degree of synthesis of cell wall peptides is accomplished within the cell prior to final assembly into cell wall.

(b) *Effect of Ribonuclease*. If fertilized amphibian eggs are injected with pancreatic ribonuclease, mitosis is inhibited (Thomas *et al.*, 1946; Ledoux *et al.*, 1954). This inhibition is not obtained with DNAase, trypsin, or pepsin. Ribonuclease has also been observed to inhibit amino acid incorporation by protoplasts (Lester, 1953), bacteria degraded by ultrasonic waves (Gale and Folkes, 1954), and by microsomal preparations (Allfrey *et al.*, 1953; Zamecnik and Keller, 1954; Hultin *et al.*, 1957).

When this enzyme is added to intact plant and animal cells, various effects have been noted on basophilia, cell permeability, and mitosis (Lansing and Rosenthal, 1952; Kaufmann and Das, 1954), suggesting that the enzyme of molecular weight 13,000 can penetrate the cell membrane of many kinds of cells quite readily. These results were readily confirmed and extended (Ledoux *et al.*, 1954), and the effects were shown not to be produced by basic proteins in general. However, Brachet (1956) considers that the enzyme works in two ways on onion root tips; a ribonuclease complex is formed first with intracellular RNA and the RNA is subsequently degraded. Relatively enormous amounts of enzyme can be taken into treated cells, perhaps by pinocytosis.

As summarized by Brachet (1955b), onion roots, frog eggs, starfish oocytes are moderately sensitive to the enzyme; amoebae and ascites tumor cells are very readily attacked. A number of cell types, e.g., *Acetabularia*, fungi, yeasts, and ciliates, which possess tough cell walls, seem impermeable. Although bacteria such as *E. coli* at first seemed resistant to the enzyme, RNAase can penetrate at sufficiently low ionic strength (Jerne and Maaløe, 1957). In the hands of these workers, it could be shown that the application of RNAase to *E. coli* reduces the RNA content, stops protein synthesis, and kills the bacterium. If the bacteria had been infected with virus, e.g., T4, the plaque-forming abilities of the complex were also destroyed after a 4-minute exposure to the enzyme.

Treatment of onion roots with RNAase produces a marked inhibition of incorporation of labeled amino acids and of growth, which is relieved by the addition of RNA. The enzyme affects ATP concentration, producing an initial rise and then a fall, but does not affect O<sub>2</sub> consumption.

Ledoux and his collaborators (Ledoux and Revell, 1955; Ledoux, 1956; Easty *et al.*, 1956) have studied the effect of the enzyme on neoplastic growth.

In intact animals the enzyme produces a fall of 50 % in the content of RNA in Landschutz ascites tumor and effects a general slowing down of tumor growth. On isolated tumor cells RNAase frequently produces an initial apparent increase of RNA and protein, which is subsequently followed by cell degradation.

*iv. Inhibition of Protein Synthesis.* Protein metabolism is influenced by energy production; indeed, the nature of the coupling of these activities is at the core of our problem. When the rat is fed a high protein diet, there is a linear relationship between change in body weight and the amount of energy source, i.e., carbohydrate or fat in the diet (Munro and Naismith, 1953). When the synthesis and secretion of specific proteins, e.g., lipase and ribonuclease, are studied in pigeon pancreas slices in physiological saline, it is found that the formation of these enzymes is abolished by anaerobiosis, or by a compound uncoupling oxidative phosphorylation, e.g., 2, 4-dinitrophenol (Schucher and Hokin, 1954). Secretion of the enzymes may be stimulated without affecting synthesis.

As might be expected, an exogenous source of amino acids will stimulate protein production in a synthesizing tissue. A mixture of all the amino acids is usually more stimulatory than are single amino acids or limited mixtures of these. In certain systems one may study the removal of single exogenous amino acids from the environment and their concentration within the cells. As studied in Ehrlich mouse ascites cells, the concentration of glycine is also strongly inhibited by anoxia, cyanide, and dinitrophenol (Christensen and Riggs, 1952). Comparable phenomena have been observed in bacteria by Gale (1953).

A deficiency of a single essential amino acid will inhibit protein synthesis. In the presence of the required amino acid, amino acid analogs will serve to prevent protein synthesis. Thus, 5-methyl tryptophan will block tryptophan utilization in animals (Gordon and Jackson, 1935), plants (Kessler, 1956), and bacteria (Fildes and Rydon, 1947; Cohen and Fowler, 1947) and inhibit protein synthesis in general.

Three classes of amino acid analogs may be recognized. These are:

1. Analogs, such as 5-methyl tryptophan, which do not replace tryptophan and block protein synthesis.
2. Compounds, such as *p*-fluorophenylalanine, which are incorporated into protein instead of phenylalanine and tyrosine and severely affect protein specificity and function (Munier and Cohen, 1956).
3. Compounds, such as selenomethionine, which replace methionine in proteins, e.g.,  $\beta$ -galactosidase, but have only a relatively slight effect on protein specificity and function (Cohen, G. N., and Cowie, 1957).

The inhibitory effects of some antibiotics on protein synthesis will be discussed in later sections.

As indicated above, in discussing the effect of dichlorobenzimidazole riboside on protein synthesis in nuclei or the inhibitory effect of ribonuclease on protein synthesis in amoebae, the integrity, synthesis, or metabolism of RNA and the component nucleotides is intimately tied to protein synthesis. However, a precise analysis of the mechanism of various compounds on protein synthesis is difficult to obtain with intact cells. Since it is known that all of the ribose nucleotides found in RNA may act as coenzymes in various phases of intermediary metabolism, it is difficult to be certain that inhibitory analogs of RNA components are carrying out their primary effect on RNA synthesis. For example, high energy phosphate is presumably essential to protein synthesis. Does dichlorobenzimidazole riboside inhibit synthesis of ATP, RNA synthesis, or both? Uracil-deficient organisms are usually unable to synthesize protein. However, in at least one instance uracil deficiency in bacteria markedly inhibited respiration (Cohen and Barner, 1955). In this system the significance of a concomitant inhibition of RNA and protein synthesis is obscured by the fact that the synthesis of these polymers is tied to energy production.

An analysis of the precise mode of action of analogs of nucleic acid components presents problems similar to those seen with amino acid analogs. As will be discussed below, analogs of purines and pyrimidines may or may not enter intermediates in nucleic acid metabolism or into the nucleic acids themselves, resulting in the production of more or less damaged nucleic acids or the inhibition of nucleic acid biosynthesis.

*v. Protein Synthesis and the Nucleic Acids.* It may be asked whether existing data on intact cells indicate that protein synthesis is tied either to the presence of RNA or to its synthesis. An excellent recent survey of these data is that of Borsook (1956). It has been reported that mature rabbit erythrocytes contain about 4 % RNA and are unable to synthesize protein (Holloway and Ripley, 1952), thereby indicating the existence of other controlling factors, as indicated in the discussion of enucleate cells.

In the course of erythropoiesis, hemoglobin appears most rapidly when cytoplasmic nucleic acid has almost disappeared (Hammarsten *et al.*, 1953). However, it can be suggested that this very decrease in RNA content is linked in some way to the synthesis of protein. An alternative hypothesis suggests that a small fraction of the RNA is undergoing a rapid turnover, thereby permitting a concomitant RNA synthesis and protein synthesis (Kruh and Borsook, 1955). Some unequivocal evidences of such RNA turnover are now known. For example, although some years ago it appeared that protein synthesis in T2-infected bacteria occurred without RNA synthesis (Cohen, 1947), it has been found that a rapid RNA synthesis and turnover occurs in a small fraction (1 to 3 %) of the total RNA of the infected cell (Volkin and Astrachan, 1957).

Most recently, it has been possible to study extensive protein synthesis in a particular strain of *E. coli* requiring both thymine and uracil for growth (Barner and Cohen, 1958). In the absence of thymine, DNA is not made; net synthesis of RNA does not occur in the absence of exogenous uracil. In a medium devoid of both pyrimidines a net synthesis of nucleic acid does not occur, but such organisms make protein at a normal rate for considerable periods (as in Fig. 14) and may even be induced to make a new enzyme,

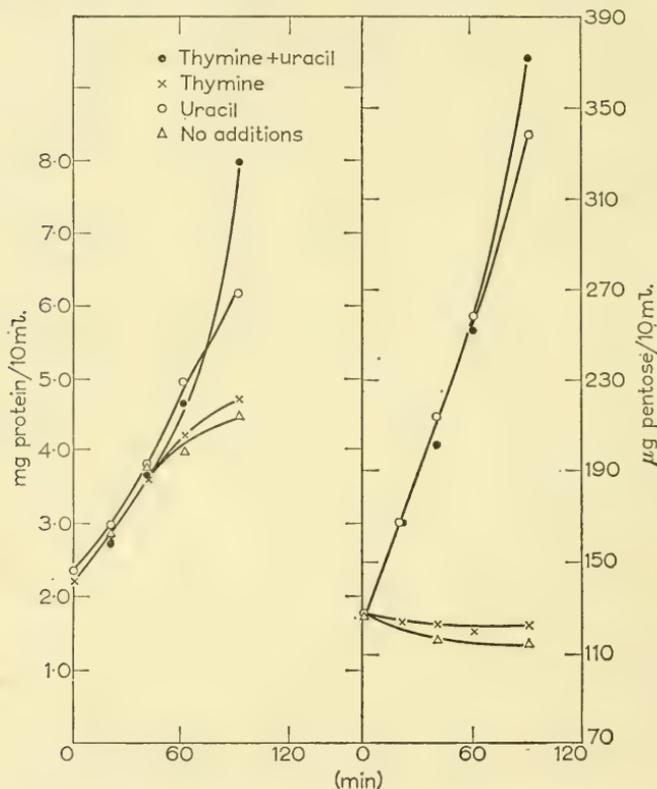


FIG. 14. The synthesis of protein in a thymineless, uracilless strain of *E. coli* under different conditions of pyrimidine deficiency (Barner and Cohen, 1958, in press).

xylose isomerase. On analysis of these cells synthesizing protein in glucose- $C^{14}$  in the absence of exogenous pyrimidines, it was observed that a fraction of the RNA (up to 15% of the total) was turning over, since RNA could be isolated containing uridine in which preformed uracil was now linked to ribose formed from the isotopic glucose.

In summary, we may say that, at the present time, no case (with the exceptions noted in footnote 1, p. 54) is known in which protein synthesis has

been proved to occur in the absence of RNA or within cells in the absence of RNA synthesis. On the other hand, the phenomenon of protein synthesis in enucleate cells and in cell-free preparations devoid of nuclei indicates unequivocally that protein synthesis in the cytoplasm is not tied to the presence of DNA or its synthesis.

### 3. RNA Synthesis

*a. Incorporation Data.* RNA is found among all the subcellular fractions which have been so far discussed. Using the colorless flagellate, *Polytomella coeca*, grown successfully in P<sup>32</sup> and P<sup>31</sup>, Jeener (1952a,b) isolated five different fractions of sedimentable particles containing RNA and analyzed their relative rates of RNA synthesis. He observed that the rates of RNA synthesis in a given fraction were proportional to the amounts of RNA present. He concluded, in addition, that the smallest particles did not behave as precursors to the larger particles.

TABLE XI  
BASE COMPOSITIONS OF RNA'S OF RAT LIVER<sup>a, b</sup>

	Adenine	Guanine	Cytosine	Uracil	purine	A + U
					pyrimidine	G + C
Nuclei	20.20	26.00	29.85	23.95	0.860	.790
Cytoplasm						
All fractions	17.99	31.64	30.04	20.30	0.989	.624
Small particles	17.56	32.26	29.97	20.17	0.996	.609
Supernatant fluid	18.42	31.03	30.12	20.43	0.983	.639

<sup>a</sup> Elson *et al.* (1955).

<sup>b</sup> Moles per 100 moles of nucleotide.

Although most of the RNA is in the cytoplasm, a significant amount is found in the nucleus. As in the case of DNA, the base composition of the cytoplasmic RNA is the same in all cytoplasmic fractions in various tissues of the same animal (Elson *et al.*, 1955); indeed, the base compositions of the RNA of the various cytoplasmic fractions remain the same whether one is dealing with resting or regenerating rat liver (Cox, 1957). However, these and other workers are agreed that the base composition of nuclear RNA is substantially different from that of cytoplasmic RNA (Crosbie *et al.*, 1953), as indicated in Table XI. It may be supposed that the nuclear and cytoplasmic fractions have independent origins, or that RNA is made first in one site and then disassembled for reassembly at another site.

The first indication of an unusual role of the nucleus in nucleic acid metabolism stems from the work of Marshak (1941), who showed that when  $P^{32}$  is injected into an animal, the rate of uptake by the nucleus is far more rapid than that by cytoplasm. He showed that the rate of  $P^{32}$  uptake in nuclei was sufficient to account for the doubling of nuclear P in one division time, and that, in a lymphoma nucleus of division time 27 hours, this amounted to  $2.8 \times 10^5$  nucleic acid nucleotides per second.

In subsequent studies, it was demonstrated that when an animal was administered glycine- $N^{15}$  (Bergstrand *et al.*, 1948),  $P^{32}$  (Marshak, 1948), or the pyrimidine orotic acid- $C^{14}$  (Potter *et al.*, 1951), the early isotope content of nuclear RNA in liver was far greater than that of any cytoplasmic fraction. A typical experiment of this type is given in Fig. 15. It can be seen that the

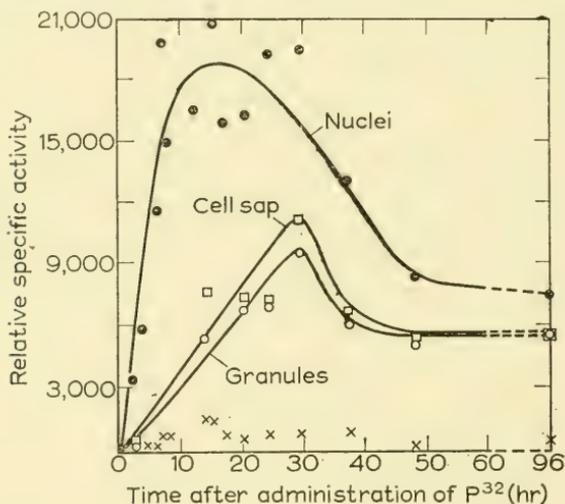


FIG. 15. Relative specific activities of the cytidylic acids of nuclear RNA, mitochondrial RNA, and microsomal RNA separated from rabbit liver at various times after the administration of  $P^{32}$ . The points for mitochondrial and microsomal RNA were coincident and the resultant curve has been termed "granules." (Smellie, 1955.)

activity of a nucleotide (cytidylic acid) of nuclear RNA rose to a maximum rather early and fell precipitously, while the same moiety in cytoplasmic RNA reached a maximum somewhat later and fell more slowly. The activity of this substance in cell sap was slightly greater than that of various granules, which in this experiment had comparable activities. As summarized by Smellie (1955), many workers have confirmed these results, although a few have considered that one or the other of the cytoplasmic particles had slightly greater rates of incorporation than the other.

In any case, the nucleus is evidently an important site of RNA synthesis,

as shown independently in isolated nuclei. It is, of course, evident that these data are consistent with the concept of the nucleolus, as a particularly active center in the synthesis of RNA and protein, as described in Fig. 12. The close association of heterochromatin with the nucleolus and the relation of the latter to RNA metabolism (Ficq, 1956) have also led to the studies of quantitative relations of heterochromatin to RNA metabolism. Although additional heterochromatin does not increase the RNA content of *Drosophila* eggs, to which a Y chromosome was added, it is reported to alter the base composition of the RNA that is made (Schultz, 1956).

It must now be asked if this nuclear RNA or component nucleotides are now made available for the synthesis of cytoplasmic RNA, a result consistent with the kinetics presented in Fig. 15.

*b. Enucleate Cells.* Brachet and Szafarz (1953) studied the incorporation of orotic acid- $C^{14}$  into fragments of *Acetabularia*. It was found that both nucleate and enucleate fragments of the alga were able to make radioactive nucleic acid.

Plaut and Rustad (1956) have examined the uptake of adenine- $C^{14}$  into fragments of *Amoeba proteus*. In this organism, the enucleate fragments also take up adenine, but at a rate of about 50 % that of the nucleate half cell. Acid fractionation of the fragments was not undertaken to distinguish nucleotide and RNA-adenine. However, as shown most recently by Prescott (1957), the uptake of uracil- $C^{14}$  in enucleate fragments levels off after 72 hours and apparently none of the radioactivity contained in the fragments is bound in acid-insoluble RNA.

*c. Nuclear Transfer.* A direct demonstration of the transfer of nuclear RNA to cytoplasm has been made by Goldstein and Plaut (1955). These workers labeled amoebae by feeding an organism labeled with  $P^{32}$ . After 2 to 3 days of feeding, essentially all nuclear  $P^{32}$  was present in the nuclear RNA and was not in DNA. These  $P^{32}$ -labeled nuclei were transferred by micromanipulation to unlabeled enucleated amoebae or to normal amoebae. The isotope remained in the transplanted nucleus for 5 hours, but appreciable radioactivity appeared in the cytoplasm after 12 hours and continued to increase in this fraction with time. All of the radioactivity in nucleus and cytoplasm was removable by treatment with ribonuclease, suggesting that the radioactive material leaving the nucleus was not inorganic  $P^{32}$  and was at least at the ribonucleotide level of organization. In experiments of transfer to normal cells, label derived from one nucleus did not pass significantly to the second unlabeled normal nucleus. Thus, the transfer may be effected from nucleus to cytoplasm but not in reverse. The nature of the  $P^{32}$ -containing fragments involved in this transfer are as yet unknown.

*d. Dissociation of RNA Synthesis from Protein Synthesis.* In all instances discussed until now, RNA synthesis has been associated with a concomitant

protein synthesis. However, not all data support the concept of a compulsory coupling of the two functions. Hotchkiss (1956) has shown that, in resting staphylococci, the uptake of  $P^{32}$  into nucleic acid was inversely related to the rate of protein synthesis. In an unusual auxotrophic mutant of *E. coli*, requiring methionine, RNA accumulated under conditions of methionine starvation (Borek *et al.*, 1956). No other amino acid-requiring mutant has been observed to react in this way; in all other auxotrophs the cessation of protein synthesis as a result of amino acid deficiency also prevents RNA synthesis.

Of particular interest in this regard is the action of the antibiotic, chloramphenicol or chloromycetin, which at suitable concentrations inhibits protein synthesis in bacteria up to 2 to 5 % of the normal value (Gale and Folkes, 1953; Wisseman *et al.*, 1954). Despite this marked inhibition, nucleic acid synthesis proceeds actively. A similar result has been effected with cobalt (Levy *et al.*, 1949). Chloramphenicol has been a powerful tool in the study of nucleic acid metabolism in phage-infected bacteria, as described by Tomizawa and Sunakawa (1956) and by Hershey and Melechen (1957), who showed that, by the judicious use of the antibiotic at an early stage of infection, one may block the synthesis of a protein essential to the production of phage DNA. This result had also been obtained by other techniques, such as the use of amino acid-deficient mutants (Burton, 1955) or amino acid analogs (Cohen and Fowler, 1947). By blocking protein synthesis at a later stage of infection, it was possible to accumulate viral nucleic acid (DNA) under conditions in which the production of viral protein was either negligible or at least very markedly reduced. In addition, the presence of chloramphenicol exaggerates RNA synthesis in virus-infected bacteria.

Bacteria were permitted to accumulate RNA in the presence of chloramphenicol and their extracts have been compared electrophoretically with extracts of normal organisms (Pardee *et al.*, 1957). A new nucleoprotein component, which was relatively nonsedimentable in contrast to the S40 component, was detected by electrophoresis in the extract of chloramphenicol-treated bacteria, i.e., the newly accumulated RNA did not exist in the free state. On sonic vibration this nucleic acid was released in the free state from the new nucleoprotein fraction, unlike normal RNA which was not thus readily released from the S40 component.

Despite these results, Gros and Gros (1956), and Pardee and Prestidge (1956) have asked whether RNA synthesis does not still at least require the presence of amino acids. Both groups have shown that chloramphenicol did not permit RNA synthesis in amino acid-deficient bacterial mutants nor did bases accumulate in the organism. However, the addition of traces of the required amino acids now permitted extensive RNA synthesis. Gros and Gros (1956) have observed that if the amino acid is added after the antibiotic, RNA synthesis starts but not that of DNA. The RNA's made in the presence

or absence of antibiotic were found to possess identical base compositions (Pardee and Prestidge, 1956).

#### IV. PATTERNS OF POLYMER SYNTHESIS

##### A. Exponential Growth

From the point of view of the virologist, it is most useful to have suspensions of separate and viable cells which, when mixed with virus, will act as discrete separate units subject to the relatively simple statistics of a Poisson distribution. Much of the important progress in the study of the bacteriophages was possible because these conditions were met in the mixing of

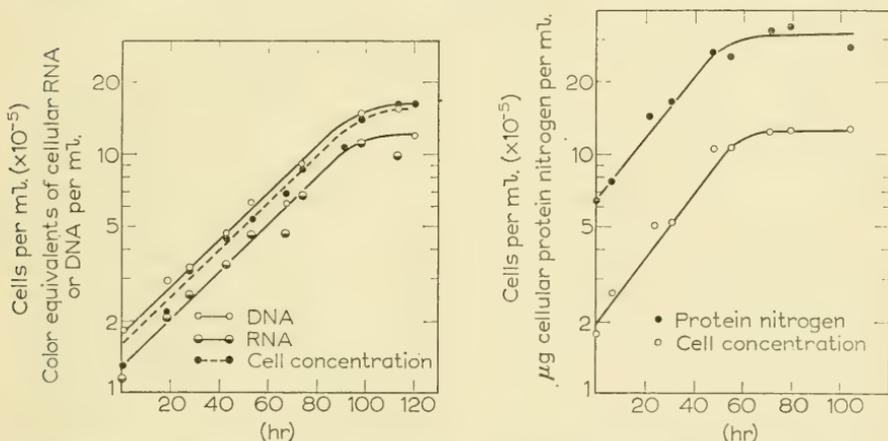


FIG. 16. Increase in population, RNA, and DNA of L cells propagated in suspension. Increase in population and cellular protein nitrogen of a similar culture (Siminovitch *et al.*, 1957).

bacteria and virus, and the recognition that progress with animal viruses might similarly be facilitated has in recent years evoked considerable effort to prepare animal cells in a manner comparable to bacterial suspensions. An example of outstanding success in this direction has been provided by Siminovitch *et al.* (1957), who have grown various animal cells derived from the progeny of a single cell, e.g., mouse L cell, monkey kidney cell, in suspension in tissue culture.

The kinetics of cell multiplication and of the parallel increments of protein, DNA, and RNA are fully comparable to those of exponential growth of a bacterial culture, e.g., *E. coli* in a glucose-mineral medium, as given in Fig. 16. Furthermore, the L cells will support the multiplication of various animal viruses, e.g., vaccinia, herpes simplex, and be lysed by them, in a manner analogous to a virulent phage-bacterium system.

As noted earlier, a growth pattern of this type suggests an integrated interdependence of polymer syntheses in cell development, since concentration of the investigator upon the substance of the entire culture instead of upon individual cells tends to obscure the discontinuities of growth and division. Such an appearance of apparent integration and interdependence of the parts has even been considered to be a general law, as in the analysis of the induced biosynthesis of enzymes in bacteria which produce the new protein under conditions of gratuity. Thus, in a culture containing bacteria randomly distributed in various phases of growth and division and growing in a substrate which neither induces nor otherwise affects the enzyme under consideration, enzyme production is proportional to the increase of mass of the culture. One might ask if such a result would be obtained in all phases of a synchronized culture. It will be noted below that this result is not obtained for the light-stimulated pigment synthesis that occurs in photosynthetic bacteria.

A similar conclusion concerning the integration of polymer synthesizing mechanisms is often drawn from the observation that bacteria in exponential growth may tend to excrete few of their metabolites, i.e., there is little waste, since the enzymes which govern later reactions are produced and operate in such a way as to handle earlier metabolites most efficiently. This type of result only suggests that the system under investigation is selected automatically for just these properties and prunes off the more wasteful and less efficient members of the culture by growing and multiplying at a maximal rate.

This apparent balance of cell syntheses during exponential growth may often be changed by changing the nutritional conditions. For example, growth of *E. coli* in lactate instead of glucose will produce curves of exponential growth of cell number and cell mass which are not parallel. The bacteria are found to be decreasing in size at each division. With such cultures a lag in cell division, which occurs before the period of exponential increment begins, represents a period of cell enlargement to an apparently critical size before division can start.

In vegetative growth in diatoms, unequal division is a common phenomenon. The cell wall consists of two rigid half-walls, which in most of these forms fits one within the other. At division, the halves separate and a new half-wall is secreted within each. Thus, a smaller half-cell must be produced at each division and cell size becomes progressively smaller. A stage is reached in which a relatively labile large protoplast is produced, which leaves both parts of the cell wall behind and eventually deposits new, large half-cell walls around itself.

### *B. Differentiated Cells*

As remarked earlier, it is difficult to handle differentiated cells for purposes of virology, although the possibilities of effecting an experimental separation

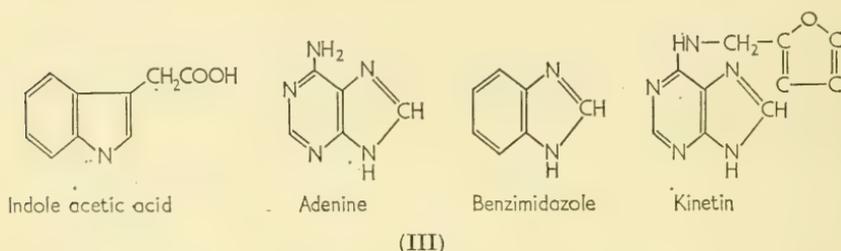
of phenomena of growth and division are maximal with such materials. Indeed, one might suppose that the obvious separation of function within cell parts is precisely what is meant by differentiation, since the development of a division of labor in a multicellular animal requires the exaggeration of one or another of the potentialities and functions of cells possessing a common genome, and hence results in the exaggeration of some structures and activities among the cell parts. In unicellular organisms, such as bacteria, the whole cell participates in the survival of the species and differentiation cannot confer the selective advantage gained by organisms which possess various kinds of tissues and which obtain improved habitats at the expense of gamete number.

In nervous tissue, the exaggeration of cytoplasmic function is effected to the point of essentially eliminating mitosis and cell division in the adult nerve cell. One could imagine that the problem of information transfer in nerve and brain would be quite difficult to manage if new synapses were continually being created as a result of cell division. On the other hand, although mitosis in adult liver slows to 1 to 2 % per day, concomitant with a massive cytoplasmic development, the enlarged differentiated cell has not lost its capacity for division since in regenerating liver a division may occur at the rate of about once per day. Therefore, the separation of growth and division observed in differentiated cells may be further subdivided according to the irreversibility of the loss of mitotic activity. In such differentiated systems the upper limit of the low rate of mitosis can be described by the low rate of synthesis of DNA and of chromosome sets.

In addition, many differentiated cells, characterized by active cytoplasmic function and particularly that related to protein synthesis, display a very active RNA synthesis. In the differentiated cells of multicellular forms, therefore, it is frequently possible to dissociate RNA and DNA synthesis completely, even as this is possible in *Amoeba*, *Acetabularia*, and their various derived fragments.

In some plant tissues, nuclear divisions may be separated readily from cell divisions. In some tissues, as in the elongation and differentiation of cells behind root tips, DNA may increase without cell division to produce polyploid cells (Deeley *et al.*, 1957). Tobacco pith tissue cultured on a nutrient agar medium will fail to grow in the absence of indole acetic acid (IAA); however, in the presence of this hormone, a 10-fold increase in cell size is realized after nuclear divisions, which begin at 40 hours and reach a maximum by 70 hours. Throughout this process, cell divisions do not occur (Jablonski and Skoog, 1954; Naylor *et al.*, 1954). When another substance, kinetin or 6-furfuryl aminopurine, which is formed in the chemical decomposition of DNA (Miller *et al.*, 1956), is added together with indole acetic acid, mitoses are accompanied by cell division (Das *et al.*, 1956). Cell enlargement induced

by indole acetic acid also appears to be mediated through effects on nucleotide metabolism, since benzimidazole, an analog of adenine, inhibits the IAA-induced elongation. Since benzimidazole also stimulates water intake, the cells swell in the transverse direction and become barrel-shaped (Galston *et al.*, 1953). The structures of these compounds are presented in formula (III).



In addition to these effects of plant hormones, some hormones in differentiated animal systems have also been implicated in the control of polymer synthesis. For example, some steroid hormones, such as estradiol, induce a marked synthesis of RNA and protein in the uterus, followed only somewhat later by an increase in DNA synthesis (Mueller, 1957). The first 6 hours are characterized by an imbibition of water. As presented in Fig. 17, there is a

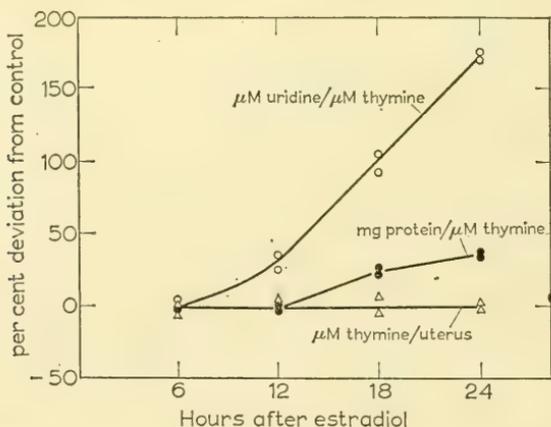


FIG. 17. Alteration in uterine composition following single dose of estradiol. Ten  $\mu\text{g}$ . estradiol injected intravenously at zero time; DNA measured as  $\mu\text{moles thymine}$ . RNA measured as  $\mu\text{moles uridine}$  and expressed as ratio,  $\mu\text{moles uridine per } \mu\text{moles thymine}$  during the first 24 hours following hormonal treatment. (Mueller, 1957.)

rapid synthesis and accumulation of RNA in the interval from 6 to 24 hours after administration of the hormone; thus RNA synthesis is then followed

by protein synthesis. DNA synthesis occurs between 40 and 72 hours. The mechanisms of these effects are not known, although it has been observed that syntheses localized in the microsomal fraction are also stimulated. Thus, these experiments show the possibility of exaggerating further one or another facet of metabolism, even in differentiated systems.

### C. Synchronous Cultures

In the past few years, many studies have appeared on the synchronization of division in microbial cultures. These efforts have been sufficiently rewarding to presage the possibility of comparable results with animal cell cultures. Zeuthen (1953) started with single *Tetrahymena* cells and studied synchronized growth through four division cycles. During growth, respiration increased linearly rather than exponentially. Linear growth and protein synthesis have also been observed in synchronous cultures of amoebae (Prescott, 1955).

Scherbaum and Zeuthen (1954) synchronized division in mass cultures of the same organism by raising the temperature 5 degrees above the optimum (29°C.) for one-half hour. All division in progress continued to completion, but growth and the initiation of division stopped. On return to the optimum for 8 hours the cells grew very considerably without division. When the temperature was then lowered to 24°C., 85 % of the cells divided after a lag of 90 minutes. Two further cycles followed at less than 2-hour intervals.

Hotchkiss (1954) applied the technique of shifting temperatures to pneumococci. Bacteria grown at 35°C. were cooled to 25°C. for 15 minutes and returned to 37°C., after which most of the bacteria divided. When a similar method is applied to *B. megatherium*, DNA synthesis is far less inhibited by chilling than is RNA synthesis, thereby favoring nuclear synthesis in the cold and permitting the apparent phasing of the bacteria (Falcone and Szybalski, 1956; Hunter-Szybalska *et al.*, 1956).

Lark and Maaløe (1954, 1956) have also used temperature shifts between 37 and 25°C. and back to produce synchronous division in *Salmonella typhimurium*. They found that DNA synthesis slows down selectively at the lower temperature and that elevation to 37°C. selectively speeds up DNA synthesis, leading almost immediately to nuclear division. Throughout these events RNA synthesis remains essentially unchanged. However, following the period of DNA synthesis in each cycle, a period of enhanced RNA synthesis was observed.

Using the thymine-requiring strain of *E. coli*, strain 15<sub>T</sub><sup>-</sup>, Barner and Cohen (1956) were able to synchronize the first division by withholding thymine for a carefully controlled period and then resupplying this pyrimidine, essential for DNA synthesis. DNA synthesis began promptly and, after slightly more than a doubling of DNA, synthesis stopped and was followed by bacterial division. Throughout these events, as seen in Fig. 18, RNA

synthesis continued substantially unchanged, as did the increment in mass, measured turbidimetrically. Repeated temperature shifts with *Salmonella* have synchronized cultures with respect to turbidimetric (mass) changes, as well as to changes of DNA (Bruce *et al.*, 1955).

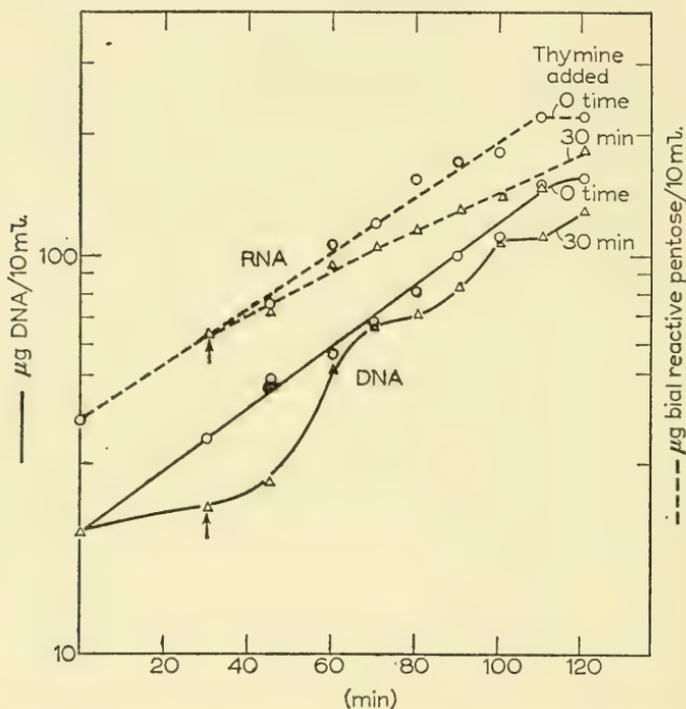


FIG. 18. Synchronization of DNA synthesis by addition of thymine to a thymine-starved strain of *E. coli* (Barner and Cohen, 1956).

Thus, as in normal mitosis in most higher cells, DNA synthesis in synchronized cultures of bacteria immediately preceded division. Furthermore, the synthesis of DNA was apparently unrelated to the bulk of RNA synthesis. Synchronized cultures of *Salmonella* have been used to explore lysogeny (Lark and Maaløe, 1956), and similar cultures of *E. coli* were used to study the effect of different parts of the division cycle on T2 multiplication (Barner and Cohen, 1956).

A number of other methods of obtaining synchronous cultures of microorganisms for purposes of following compositional changes have been described (Ogur *et al.*, 1953; Maruyama and Yanagita, 1956; Maruyama, 1956). We may note that still other techniques have been employed in rather special animal systems. Thus, fertilization of ova by sperm permits the

development of synchronized embryos, at least for early divisions. Such systems were used for the isolation of mitotic figures in different stages of mitosis (Mazia and Dan, 1952). In many such systems, e.g., fertilized sea urchin eggs, etc., DNA synthesis proceeds without net RNA synthesis, since chromosome sets are increased within the segmenting ova, which do not significantly change their mass. However, as in the case of phage multiplication, we must ask if RNA synthesis and turnover of a small fraction of the total RNA are not involved in this developmental process.

In regenerating rat liver, Hecht and Potter (1956a) were able to demonstrate an apparent synchrony of DNA synthesis, as measured by the incorporation of orotic acid- $C^{14}$  into DNA (cf. Fig. 19). There is essentially no DNA

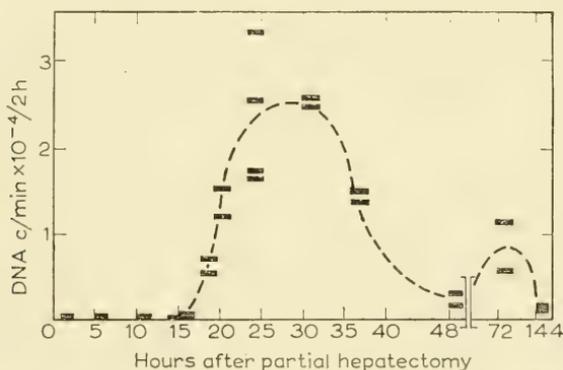


FIG. 19. Synchronization of DNA synthesis in regenerating rat liver (Hecht and Potter, 1956a).

synthesis for about 18 hours, after which this function is suddenly activated, rising to a maximum between 24 and 30 hours. In this system, incorporation of isotope into nuclear RNA was maximal at 2 hours and into cytoplasmic RNA about 10 hours later. In an extension of the study of this system with injected orotic acid, the distribution of isotope in acid-soluble pyrimidine nucleosides and nucleotides suggested that there was a conversion of low molecular ribose derivatives to low molecular deoxyribose derivatives, which were then incorporated very rapidly into DNA.

#### D. Pathological Systems

The control of differentiation is a subtle process inasmuch as the exaggeration of one or another facet of metabolism must be effected without destroying the cell. A cytopathogenic effect in virus infection may be considered to be an instance in which a deviation and exaggeration of metabolism cannot occur without destroying the ability of the cell to maintain itself. Cellular integrity is maintained in liver and nervous tissue despite the growth and

development of cytoplasm in the absence of mitosis. However, in liver cells such cytoplasmic growth obviously cannot continue indefinitely or the cell would burst. In the case of liver, differentiation has provided mechanisms for the liberation or excretion of synthesized protein; nervous tissue has well-developed mechanisms of turnover. Evidently, an integrated system of multiplying nucleus and cytoplasm without such mechanisms cannot be blocked selectively in the development of one component alone without the development of pathology.

Ultraviolet and X-irradiation are other tools which produce such selective damage. A common effect of such treatment is the development of giant cells, as nuclear syntheses are inhibited and cytoplasmic growth continues. In many cases such inhibited cells will eventually grow and lyse or necrose. The loss of the ability of such cells to multiply is what is commonly meant by "death" as applied to irradiated cells, although much of the metabolism of such cells may appear to continue normally for many hours. Although many workers have demonstrated an inhibition of DNA synthesis following irradiation of many tissues and cell types, it has been difficult to be certain in most of these experiments that the effect on DNA synthesis is indeed the primary effect of irradiation. As pointed out by Howard (1956), a primary effect on some stage of protein development in mitosis might be expected to inhibit DNA synthesis, which should occur prior to subsequent mitotic cycles. Indeed, it has recently been reported (Deering and Setlow, 1957) that low doses of ultraviolet (UV) light prevent bacterial division without apparent effect on DNA, RNA, and protein synthesis. A similar result has just been obtained after treatment of *E. coli* with nitrogen mustard (Harold, personal communication).

In a possibly related result, it is known that ultraviolet-irradiated (or mustard-inactivated) bacteria, incapable of DNA synthesis, will synthesize DNA after infection by phage, and this suggests that the radiation-induced lesion may be somewhere other than in host DNA. However, a number of counterarguments could point to the fact that such irradiation lesions may be repaired anyway, and that perhaps infection merely hastens this process; or that if the lesion is in the DNA of the bacterial genome, thereby producing a block in the use of the template for synthesis, infection merely replaces the damaged host genome by undamaged phage DNA. Indeed, the latter hypothesis is supported by a great deal of evidence, of which the most interesting derive from the lethal effects of radiation on DNA viruses. The damaged DNA of a UV-irradiated phage may be repaired within the host bacterium by photoreactivation of the host-virus complex, or may be replaced by undamaged phage DNA in cells infected with a multiplicity of virus particles.

Although limited irradiation damage of cells produces a marked inhibitory effect on DNA synthesis to a greater extent than on RNA or protein synthesis

e.g., irradiated cells incapable of division may be induced to synthesize a new enzyme, RNA viruses and the infectious RNA from these viruses can also be inactivated by irradiation. It must be asked, therefore, whether damage of this component does not play an unsuspected role in cellular damage. Kelly (1952) has reported that in X-irradiated animals, there is a depression of  $P^{32}$  uptake into nuclear RNA, without a similar effect on cytoplasmic RNA. One would evidently wish to know the relative sensitivities of nuclear RNA and DNA and the sensitivity of DNA and RNA as a whole.

### *E. Unbalanced Growth*

In addition to the instances of radiation damage noted above, a number of other pathological situations have been provoked in experimental systems which have been instructive in revealing the extent to which cell functions can be separated. We will briefly consider four of these.

#### *1. Thymineless Death*

It was discovered initially that when a thymineless strain of *E. coli* strain 15<sub>T</sub><sup>-</sup> was grown at 37°C. in an aerated glucose-mineral salts medium in limiting amounts of thymine, it was not possible to establish a simple relation between cell number and mass of the culture (Barner and Cohen, 1954). When incubated in the presence of a carbon source and essential salts and in the absence of thymine, the cells lost the ability to produce colonies ("died") on thymine-containing medium at the rate of about 90 % per division time. The cells did not die in the absence of a carbon source, and appeared to have to metabolize and grow in order to produce this irreversible change, the pattern of which is presented in Fig. 20. Synchrony may be produced in such a culture if thymine is added just before the cells begin to lose the ability to multiply; at this moment they are maximally phased (Barner and Cohen, 1956).

Such cells made RNA and protein, but only traces of DNA, and became quite enlarged. After many hours they finally lysed. If thymine is added to a "dead," unlysed culture, the cells make considerable amounts of DNA but are not resuscitated (Barner and Cohen, 1956). Thymine-deficient cells can be induced by xylose and other substrates to produce new enzymes (Cohen and Barner, 1955), indicating that, as with irradiated cells, DNA synthesis is not essential to this type of enzyme synthesis. Although it may be suggested from this type of result that such synthetic functions are localized in the cytoplasm, this problem should be more cleanly answered by studies with enucleate *Acetabularia* or *Amoeba*.<sup>1</sup>

<sup>1</sup> Although Brachet has stated that enucleate *Acetabularia* did on occasion produce catalase in response to the presence of  $H_2O_2$  in the medium, he has more recently remarked that these results have been difficult to reproduce.

This strain of *E. coli* is unique in having this single metabolic deficiency. It appears that unless accompanied by other deficiencies which prevent growth, mutation to an unrequited thymine deficiency is lethal. Such mutants would not be recovered in most routine screening operations for nutritional mutants. Cell enlargement as a result of DNA deficiency has been observed in certain other nutritionally deficient organisms cultivated in the absence of their requirement. Filament formation has been observed with *Thermobacterium acidophilus* R26 cultivated in the absence of deoxyribosides

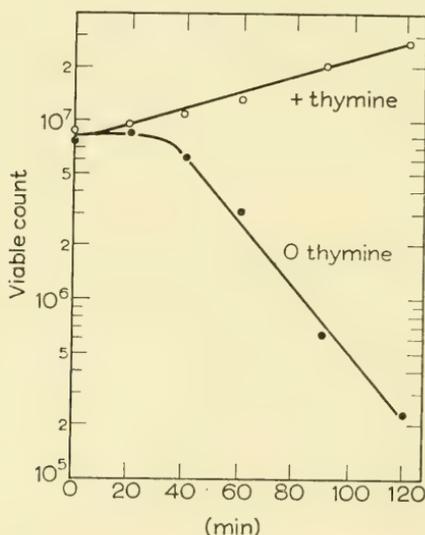


FIG. 20. The irreversible loss of the power to multiply ("thymineless death") as a consequence of withholding thymine from a thymineless strain of *E. coli* under conditions of continuing cytoplasmic growth (Cohen and Barner, 1954).

(Jeener and Jeener, 1952) and in *Lactobacillus leichmannii* or other deficient bacteria cultured in the absence of vitamin B<sub>12</sub> (Deibel *et al.*, 1956; Chaplin and Lochhead, 1956). Filament formation may be induced by a variety of antibiotics, e.g., azaserine, although it is not known that the primary effect is on DNA synthesis (Maxwell and Nickel, 1954). However, folic acid analogs, e.g., Amethopterin, also produce this effect, which in some instances is reversed by thymine<sup>1</sup> (Nickerson and Webb, 1956).

<sup>1</sup> Indeed, Nickerson and his collaborators have made many contributions to the experimental dissection of growth and division in microorganisms, particularly processes related to the control of dimorphism in the pathogenic fungi. Webb (1953), in turn, has studied similar phenomena in bacteria, arising from magnesium deficiencies of the growth media.

Strain 15<sub>T</sub><sup>-</sup> is almost but not completely blocked in the synthesis of thymine; it may make 2 to 4 % of its normal requirement (Cohen and Barner, 1954). The enzymatic site of this block appears to be in the conversion of deoxyuridylic acid to thymidylic acid (Flaks and Cohen, unpublished data), and the cells accumulate uracil in amounts comparable to the thymine deficiency. Uniquely, this block may be overcome by T2 phage infection, since infected cells show a gross net synthesis of thymine (Barner and Cohen, 1954).

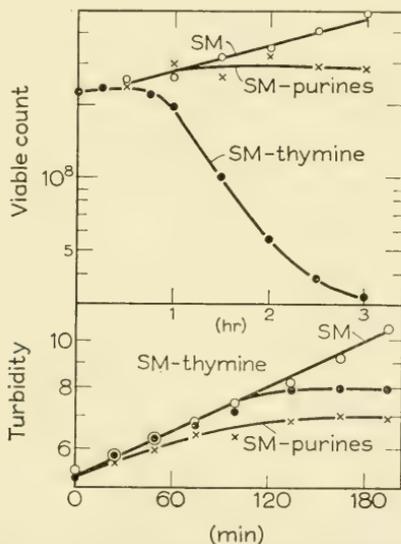


FIG. 21. The induction of thymine deficiency and "thymineless death" in *E. coli* grown in the presence of sulfanilamide (S) and metabolites (M) containing essential one carbon fragments, including purines and thymine (Cohen and Barner, 1954, 1956).

The thymineless organism is useful for the exploration of the interrelations of RNA and protein synthesis. Under conditions of thymine depletion, inhibition of RNA synthesis with 5-hydroxyuridine inhibited protein synthesis proportionately (Ben-Ishai and Volcani, 1956). In amino acid deficiencies both RNA and protein syntheses were inhibited, although a significant synthesis of DNA occurred (Cohen, 1957).

Multiple deficiencies, including those for thymine, purines, and a number of amino acids, may be induced in nonauxotrophic strains of *E. coli* with sulfanilamide (Rutten *et al.*, 1950). The production of folic acid is inhibited, thereby producing deficiencies in compounds containing the one carbon fragments handled by folic acid derivatives. Growth may be restored by the addition of these metabolites to sulfanilamide. If thymine is specifically eliminated from this medium, unbalanced growth is produced and thymineless death ensues, as presented in Fig. 21.

Why do the cells die? Two types of hypothesis may be offered: (1) Continuing cytoplasmic growth rings the nuclei in such a fashion that some stage in the division process aborts, or (2) The nuclei attempt to divide in the absence of an incomplete complement of DNA, producing incomplete and dispersed fragments of the genetic complement.

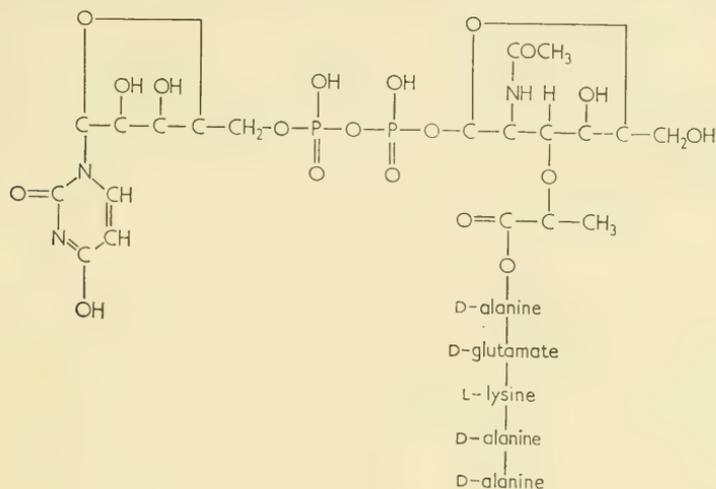
Recent data suggest that the latter may be more likely, although this is far from proved. Strains of  $15_T^-$ , carrying an additional requirement for a single amino acid will die for the first hour, as does the parent, and then stop, i.e., 90 % of the cells will die, although cytoplasmic growth is very slight (Barner and Cohen, 1957). The lag observed in Fig. 20 may be shortened by incorporation and decay of  $P^{32}$  in the DNA of strain  $15_T^-$ , suggesting that this lag describes the inactivation of multiple bacterial nuclei, of which the average cells of this strain have at least two (Fuerst and Stent, 1956). However, it may be noted that the DNA of "dead" cells does not become acid-soluble; this substance remains sufficiently viscous to produce a self-sharpening effect when extracts are examined in the analytical ultracentrifuge (Cohen and Barner, unpublished data). Thus, if breaks have occurred in this DNA, they have not led to an extensive depolymerization of this bacterial substance. Furthermore, synchrony effected by addition of thymine at the end of the presumed nuclear decay results in a doubling of DNA (Barner and Cohen, 1956). Thus, the presumably inactivated nuclei appear to be able to make a normal complement of DNA.

## 2. Mode of Action of Penicillin

It has been known for many years that the lethal action of penicillin on microorganisms is a function of microbial growth. Many workers have observed the production of spherical bodies and L forms in the presence of the antibiotic. The production of similar forms during the production of protoplasts by means of lysozyme suggested to Lederberg (1956) that the bizarre cell types observed in the presence of penicillin were intermediates in protoplast formation. When *E. coli* was grown in broth in the presence of 5 % sucrose and 0.1 %  $MgSO_4$ , the bacteria were quantitatively transformed into spherical protoplasts, which lysed readily in distilled water. In the absence of penicillin, about half the protoplasts will revert to rods and form typical bacillary colonies; thus this system can provide an excellent experimental system for the selective study of cell wall synthesis, as well as for the production of uncorseted, perhaps more permeable, bacteria.

It had been observed that staphylococci in the presence of penicillin accumulated large amounts of various peptides (Park, 1951) which were found to be associated with uridine nucleotides and amino sugars. The isolation of the 3-O-lactyl ether of N-acetylhexosamine (Strange, 1956) and of derived peptides from the cell walls of gram-positive bacteria (Strange and

Powell, 1954) permitted the clarification of the chemical structure and role of these relatively bizarre accumulation products (Park and Strominger, 1957). A typical nucleotide of this type is presented in formula (IV).



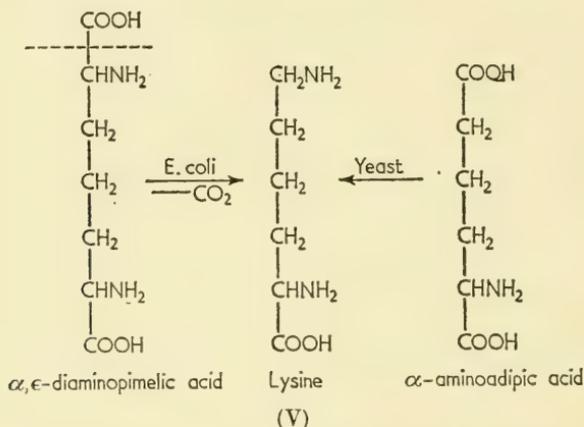
(IV)

Many variations on this structure are known, particularly in peptide composition. In *E. coli*, for example, L-lysine is replaced by diaminopimelic acid. It appears that the nucleotides are the bearer of the sugar-peptide fragments which are incorporated as units into cell wall. Penicillin in some way blocks the synthesis of these units into cell wall, permitting the cell to grow through and out of a wall which can no longer cover the enlarging surface. Outside of its restraining corset, the cell lyses in unsuitable osmotic conditions and dies. It should be noted that the lactyl ether of *N*-acetyl glucosamine has not yet been discovered in animal or plant tissues. The existence of this exclusively microbial product and associated compounds may then be considered to account for the lack of toxicity of penicillin to animal cells, since limiting membranes of animal cells are evidently constructed of somewhat different materials. Nevertheless, a unit similar to the lactyl ether, namely, the pyruvyl derivative, sialic acid (see Fig. 2), is a constituent of erythrocyte stroma and of gangliosides, which are probably organized in the surface membranes of nerve cells. One might ask if this type of structure does not represent a general feature of limiting membranes, and if the various mucoproteins which contain sialic acid are not either degradation products of such membranes or products which accumulate as a result of their inefficient incorporation into cell membranes. The increased appearance of serum mucoproteins in infections perhaps suggests the former

possibility; an increase of these materials in patients with metastasizing tumors warrants the exploration of both possibilities in connection with the origin of metastases.

### 3. Diaminopimelic Acid Deficiency

As mentioned in the previous section, and as summarized by Work (1955), diaminopimelic acid (DAP) is confined to the cell wall in *E. coli* and indeed in many microorganisms. In *E. coli*, this amino acid is the precursor to lysine, although lysine is derived from another metabolite,  $\alpha$ -aminoadipic acid, in yeast (Davis, 1956). These relationships are given in formula (v).



A number of workers have discovered independently that a DAP deficiency can lead to cell lysis (Rhuland, 1957; Meadow *et al.*, 1957; Bauman and Davis, 1957) and, if the deficiency is maintained under conditions of growth and in the presence of hypertonic sucrose, a protoplast will be formed. Meadow *et al.* (1957) have demonstrated that lysine is essential for the development of cell lysis in the absence of DAP. Under these conditions there is thus a selective interference with cell wall formation, leading to continuous cell growth without production of cell walls, as in the case of penicillin. Bauman and Davis (1957) have exploited the use of thymine-deficiency in strain 15<sub>T</sub><sup>-</sup> and of DAP-deficiency in auxotrophic mutants for the selection of other requirements in these bacteria, since the suicidal tendencies of these organisms are minimized in the presence of multiple deficiencies, a fact also used in the isolation of polyauxotrophs of the molds, *Ophiostoma* and *Aspergillus* (Fries, 1948; Pontecorvo, 1953).

### 4. Carotenoid Deficiencies in Photosynthetic Bacteria

The previous instances describe pathology causally related to the inability to produce nuclear and cell wall constituents during continuing synthesis of

other cell constituents. Lethal consequences of unbalanced growth as a result of the inability to produce cytoplasmic constituents have not yet been clearly documented, with the exception of the case described by Griffiths *et al.* (1955) and Cohen-Bazire *et al.* (1957), in which the synthesis of a cytoplasmic particulate, a chromatophore, is distorted in carotenoidless mutants of *Rhodospseudomonas spheroides*. This organism is a photosynthetic nonsulfur purple bacterium (family Athiorhodaceae), which normally may grow aerobically in the dark on a suitable substrate or anaerobically in the light. In the light, the normal pigment system of carotenoids and bacteriochlorophyll, centered in particulate chromophores (50  $m\mu$  in diameter), facilitates photosynthetic fixation of  $CO_2$ .

Mutants were isolated in which, instead of the two normal red and yellow carotenoids, the chromatophores contained the colorless  $C_{40}$  polyene, phytoene, which is considered to be a precursor of the more unsaturated carotenoids. The spectra of the mutant chromatophores are altered in the position of the chlorophyll band and it is believed that this change arose from structural inadequacies, even holes, in the particles. The mutants synthesize porphyrin at the normal rate, but unlike wild type organisms, excrete significant amounts of these substances, indicating an inability to pack them efficiently into the chromatophores. The mutant grows normally in the dark, and anaerobically in the light, although less rapidly than the wild type.

Air only inhibits pigment synthesis in the wild type in the light, respiration replacing photosynthesis. In the mutant, however, air kills the cells in the light. This result has been interpreted as a photodynamic action due to the presence of porphyrins, in which the oxidizing fragment (OH), generated by the photolytic decomposition of water, is not removed as a result of the absence of carotenoids. It is proposed that the protection of cells from photodynamic destruction by chlorophyll is a major function of carotenoids, accounting for its ubiquitous association with the photosynthetic apparatus.

In still another instance, then, the apparent integration of polymer synthesis in the production of cellular components does not reflect a compulsory interlacing of these activities at the enzymatic level, but reflects the fact that these independent syntheses must occur concomitantly or have pathological consequences detrimental to cell survival. It is of interest that the cytopathology observed in liver, in response to infection by ectromelia virus, has been ascribed to an initial inhibition of mitosis, followed by a continuing production of cytoplasm (DeBurgh and Miller, 1955). Subsequent cell damage is attributed by the authors to this abnormal form of growth.

*F. Syntheses with Abnormal Metabolites*

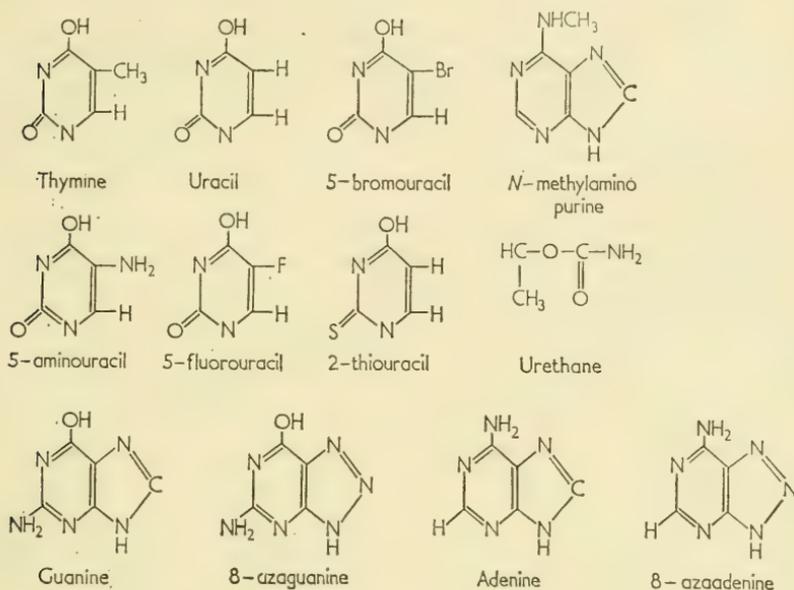
The pathological systems described above provide excellent materials for the study of the specificity of polymer syntheses. As noted in the case of the carotenoidless *Rhodospseudomonas spheroides*, the carotenoid precursor, phytoene, is accepted as a carotenoid replacement in the synthesis of chromatophores to produce an altered and pathological cell structure. In the case of the thymineless bacteria, a number of variations on this possibility have been observed.

A synthetic analog of thymine, 5-bromouracil (BrU), permits the synthesis of bacterial DNA containing BrU (Dunn and Smith, 1954; Zamenhof and Griboff, 1954a,b). This occurs in the absence of thymine and a 6-fold accumulation of DNA and RNA then may be obtained (Cohen and Barner, 1954). A single cell division is permitted in the presence of BrU, but the cells then lose the power to multiply and form giant filaments (Cohen and Barner, 1954, 1956). The DNA containing a massive dose of BrU in strain 15<sub>T-</sub> is evidently incapable of fulfilling the function of DNA containing thymine. However, if cells are grown in the presence of both thymine and BrU, the cells do not die and contain both bases in their DNA (Zamenhof *et al.*, 1956a,b).

T2 phage has been grown in the presence of BrU in *E. coli* rendered thymine-deficient in a sulfanilamide-containing medium. Phages are produced which contain BrU; for the most part these are inactive. However, Litman and Pardee (1956) have observed that a high proportion of mutants (15 %) are obtained among the surviving progeny. It was reported that in one such preparation, in which all of the thymine had been replaced by BrU, 9 % of the total phages produced were still infectious.

Chlorouracil and iodouracil are also capable of replacing thymine in DNA. Other apparent thymine analogs are not replacements, but nevertheless prevent the insertion or synthesis of thymine. These include 5-aminouracil (Duncan and Woods, 1953) and 5-fluorouracil. When applied to *E. coli*, 5-aminouracil caused the accumulation of *N*-methylamino purine in bacterial DNA, as if the methyl group originally involved in thymine synthesis were redirected to a new acceptor (Dunn and Smith, 1955). Chromosome breaks can be produced by high concentrations of uracil, which appears to act like a thymine antagonist under these conditions (Deysson, 1952). Both the production of chromosome breaks by urethane, an inhibitor of pyrimidine biosynthesis, and its carcinogenic action in mice are inhibited by thymine (Rogers, 1957). The action of urethane in the production of pulmonary adenomas is also potentiated by aminopterin, a folic acid antagonist, which inhibits thymine synthesis. Thus, it would appear that the presence of these compounds leads to the formation of thymine-deficient chromosomes. The

structures of the above-mentioned compounds are given in formula (VI).



(VI)

The folic acid antagonist, Amethopterin, is considered to inhibit thymine synthesis to a greater extent than the synthesis of purines. In the presence of this compound, Balis and Dancis (1955) have observed that leukemic animals effect an apparently greater synthesis of DNA purine than of thymine. As in the case with aminouracil in *E. coli*, it appears possible to synthesize a thymine-deficient DNA, and it seems reasonable to relate the antitumor activity of Amethopterin to its antithymine activity.

5-Fluorouracil and some derivatives have recently been described. The free base and nucleosides appear to be very potent antitumor and antibacterial agents and, in the latter, effects are inhibited by the presence of thymine or its deoxyriboside (Heidelberger *et al.*, 1957). These fluoro compounds will provoke thymine deficiency and thymineless death in nonauxotrophic strains of *E. coli* (Cohen and Barner, unpublished data). The fluorouracil deoxyriboside is converted to the deoxyribotide by *E. coli* strain 15<sub>T</sub><sup>-</sup> and this compound is a very potent inhibitor of the enzymatic conversion of deoxyuridylic acid to thymidylic acid (Cohen, Flaks, and Barner, unpublished data).

Unlike chlorouracil, fluorouracil will not enter DNA (Heidelberger *et al.*, 1957). However, like chlorouracil, which is capable of entering RNA, fluorouracil also enters this type of nucleic acid. It may be asked now whether these types of RNA are pathological.

Both purine and pyrimidine analogs are known to enter RNA. These include purines, such as 8-azaguanine and 8-azaadenine, and pyrimidines, such as 2-thiouracil. 8-Azaguanine was first shown to enter the RNA of tobacco mosaic virus (Matthews, 1953) and was subsequently observed to be incorporated into the RNA of bacteria, mouse organs, and tumors (Lasnitzki *et al.*, 1954). Particles of tobacco mosaic virus containing the unnatural base appear to be noninfectious and a comparable result has been observed for virus particles which have incorporated thiouracil in their RNA (Jeener and Roseels, 1953; Jeener, 1956). The RNA of *B. cereus* which has incorporated azaguanine (Matthews and Smith, 1956) was found to contain a number of relatively short polynucleotide chains in which the proportion of the unnatural purine was high, particularly so at the end of the chains. Similarly, the distribution of 2-thiouridylic acid in the virus RNA indicated that it tends to be concentrated at the end of polynucleotide chains, and at one end more than the other (Mandel *et al.*, 1957a). The incorporation of small amounts of azaguanine into DNA has recently been recorded for *B. cereus* (Mandel *et al.*, 1957b), but not for the DNA of *E. coli* or T2 bacteriophage (Smith and Matthews, 1957).

Mention has already been made of the incorporation of unnatural amino acids into proteins, even as unnatural substances, such as acetyl pyridine, may replace nicotinamide in an enzymatic system to generate unnatural pyridine nucleotide coenzymes (Kaplan *et al.*, 1954) or the toxic fluoroacetate can form the toxic fluorocitrate (Peters, 1957), capable of jamming the tricarboxylic acid cycle. It is possible to fool an enzyme with an analog but the resulting unnatural product is rarely capable of maintaining the deception with many enzymes and fulfilling a normal function. One apparent exception,  $\beta$ -galactosidase containing selenomethionine (Cohen, G. N., and Cowie, 1957) is produced to not quite a degree of activity as the enzyme containing normal methionine, although it is not known if this reflects an inefficiency in enzyme production or an inherently low enzymatic activity when selenium is built into the protein.

### G. Problems of Turnover

#### 1. Proteins

Until relatively recently, it was believed, as a result of the work of Borsook and Keighley (1935) and of Schoenheimer (1942), that all proteins in the mammalian organism were being constantly broken down and resynthesized at various rates. This situation was also applicable to most other chemical components of mammal tissue and was termed "the dynamic state of body constituents." Re-examination has demonstrated that most of this turnover of protein can be attributed to proteins of the viscera (Borsook, 1950).

Indeed, a recent study of Thompson and Ballou (1956) has revealed that in the rat some proteins, such as collagen, show a marked stability, not being replaced during the lifetime of the animal. Most chemical fractions isolated from this animal contain large amounts of long-lived components.

The validity of the concept of a dynamic state of any protein has been called to question in recent years by the discovery of Monod and his collaborators that many amino acid-requiring mutants of bacteria could not be induced to make a new protein in the absence of the required amino acid in the medium. Since the new protein would probably not exceed 1% of the protein of the cell, this implied that not even 1% of the bacterial protein could be degraded and release the required amino acid for the resynthesis of a new protein (Monod *et al.*, 1952). It was then shown (Hogness *et al.*, 1955; Cohn, 1954) that  $\beta$ -galactosidase, synthesized in nonisotopic medium in heavily labeled bacteria was nonradioactive and remained in this condition despite subsequent growth in isotope, as in Fig. 22, a and b. Furthermore,

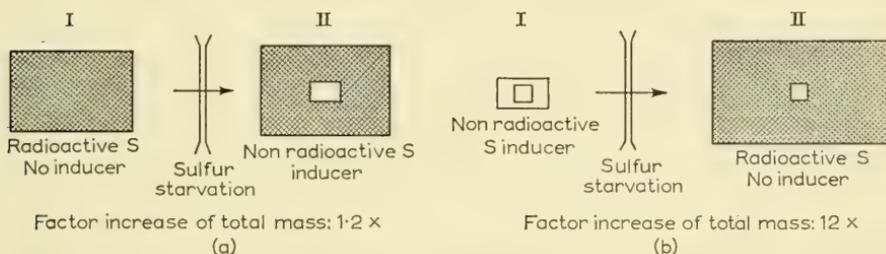


FIG. 22a. The synthesis and stability of nonradioactive  $\beta$ -galactosidase (Cohn, 1954).

Diagrammatic representation of experiment in which *Escherichia coli* were grown in the presence of radioactive S (crosshatched areas) without inducer until lack of S prevented further growth (I). Following the S starvation, nonradioactive S plus inducer were added, whereupon growth was immediately resumed. The  $\beta$ -galactosidase (small, clear rectangle in II) manufactured from nonmarked S within cells with marked proteins were then analyzed for their isotope content.

FIG. 22b. The stability of  $\beta$ -galactosidase (Cohn, 1954).

Diagrammatic representation of experiment in which *Escherichia coli* were grown in S starvation on nonradioactive S plus inducer (I). The inducer was then removed and radioactive S was added. The radioactive bacterial proteins (crosshatched area) increased 12 times, but the enzyme (clear rectangle) remained constant (II).

labeled enzyme produced in isotopic medium remained intact, as in Fig. 23, despite growth of bacteria in unlabeled media in the presence or absence of an inducer of additional enzyme synthesis. The formed proteins of *E. coli* were static in all conditions of growth and enzyme synthesis, a fact now confirmed by numerous investigators (Rickenberg *et al.*, 1953; Rotman and Spiegelman, 1954; Koch and Levy, 1955). It could be estimated that the rate

of exchange of protein-bound sulfur, for instance, was only one-thousandth as rapid as the synthesis of protein during the logarithmic phase of bacterial growth.

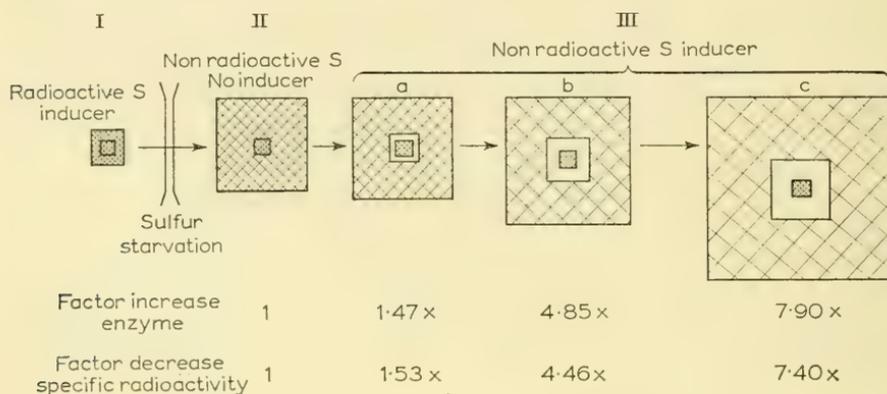


FIG. 23. The stability of  $\beta$ -galactosidase (Cohn, 1954).

Diagrammatic representation of experiment in which *Escherichia coli* was grown with radioactive S plus inducer (crosshatched areas) I. The inducer was then removed, and nonradioactive S substituted. The bacterial cells increased 11-fold, but the enzyme remained constant (II). The III inducer was added to the nonradioactive S and growth continued as shown in (a), (b) and (c). The degree of radioactivity in the cells is shown by the closeness of the crosshatching, and the amount of enzyme by the inner clear rectangles.

It was then asked whether the apparent turnover of any proteins in the mammal did not reflect the death and resynthesis of cells, rather than of components of intact cells. As of this writing, definitive experiments with animal cells have not yet been performed to prove or disprove this hypothesis. Velick (1956) studied three well-defined crystallizable enzymes, aldolase, glyceraldehyde-3-phosphate dehydrogenase, and phosphorylase in muscle, which has a long life span and a slow protein metabolism. He found that the three enzymes did not become labeled at the same rate, a result which at least suggests the independent metabolism of individual proteins in intact and functioning cells. He estimated half-life times of 20 to 100 days for different glycolytic enzymes and contractile proteins of muscle.

Related to this question have been the observations on the changes in enzyme content of various tissues as a consequence of depletion of some essential metabolite. As a typical report may be noted the study of Van Pilsum *et al.* (1957), in which it was shown that a deficiency for tryptophan or other essential amino acids did not reduce the activity in rat liver of arginase, aconitase, and D-amino acid oxidase, but did decrease the content of catalase and xanthine oxidase. Although the authors thought this was best

explained by the relative priority of certain synthetic systems for the amino acid pool, the possibility that these results arise from a selective degradation of the latter enzymes must also be considered.<sup>1</sup>

For a long time, brain proteins were considered to have a very slow turnover, a fact thought to support the concept that turnover reflected population changes rather than events within individual cells. The phenomena of chromatolysis behind severed axons and the regeneration of the axon suggests that individual cells possess the capacity for protein turnover, at least under these abnormal conditions. Using lysine-C<sup>14</sup>, an amino acid which more readily penetrates the blood-brain barrier, Lajtha *et al.* (1957) have demonstrated a rapid uptake of the amino acid into mouse brain, particularly into the microsomal fraction. Furthermore, the half-life of lysine in brain was a maximum of 20 days. Since such cells must maintain a constant size for many years, one would anticipate the existence of a renewal mechanism, even as seems to have been found.

Furthermore, studies of protein degradation in a variety of mammalian cells have revealed that, as in protein synthesis, the release of labeled amino acids requires an energy source and is inhibited by anaerobiosis and dinitrophenol (Simpson, 1953; Steinberg and Vaughan, 1956). The release is also inhibited by the amino acid analog, *p*-fluorophenylalanine. These results suggest that the release is not primarily autolytic. A similar result was obtained by Moldave (1957) on the release of labeled amino acids from structures of ascites cells.

The hypothesis of Hogness *et al.* concerning the absence of protein turnover relies exclusively on work with *E. coli*. However, in yeast, conditions of nitrogen deficiency produce enzymatic deadaptation, i.e., the apparent destruction of enzymes (Robertson and Halvorson, 1957), and an initial stage in deadaptation to  $\alpha$ -glucosidase included a release of the enzyme from a bound to the soluble state, before the latter also disappeared. In this connection, reference may be made to the disappearance of the S40 particles of *E. coli* during starvation (Dagley and Sykes, 1957). Evidently the "static state of body constituents" in microorganisms also warrants a closer examination.

## 2. Nucleic Acids

In general, the rate of P<sup>32</sup> incorporation into DNA in a given cell parallels the mitotic activity of the cell (with a few exceptions, as in the development

<sup>1</sup> We may note the existence of experiments on enzyme synthesis in amino acid-deficient rats which are superficially similar to the bacterial mutant experiments of Monod *et al.* (Cohn, 1954), but which have yielded contrary results. The livers of rats fed nonprotein diets are depleted with respect to a number of enzymes, such as xanthine oxidase, succinic oxidase, and choline oxidase. When such rats are fed diets deficient only in a single amino acid, such as histidine, there are marked restorations of these enzymes (Prignore *et al.*, 1955). It was suggested by these authors that under these conditions missing amino acids may be salvaged from the body proteins. Such experiments, however, do not take the turnover of cellular populations into account.

of polyteny, virus infection, etc.). A few years ago, several groups of workers presented evidence to suggest that in animal tissues twice as much  $P^{32}$  entered DNA as could be accounted for by the net increase in DNA content at mitosis (Stevens *et al.*, 1953; Daoust *et al.*, 1954; Barnum *et al.*, 1953). This was taken to mean that mitosis involves a synthesis of two new DNA molecules accompanied by a breakdown of one old DNA molecule. A reexamination of the methods employed, particularly that of the estimation of mitotic index, revealed that in regenerating rat liver the rate of formation of  $P^{32}$ -labeled DNA could be accounted for by the net increase of DNA (Daoust

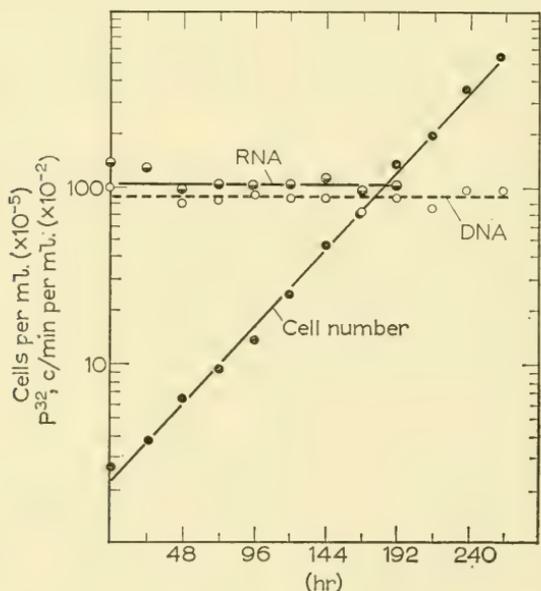


FIG. 24. Retention of  $P^{32}$  in the RNA and DNA fractions of L strain mouse cells propagated in suspension by the rotating culture technique (Siminovitch and Graham, 1956).

*et al.*, 1956). The hypothesis that pre-existing DNA is replaced at mitosis by twice its amount of DNA was not correct. The carbon, nitrogen, and phosphorus atoms of DNA appear not to be replaced in the life of normal cells.

The metabolic stability of DNA has been confirmed for fibroblast cultures (Healy *et al.*, 1956; Thompson *et al.*, 1956; Siminovitch and Graham, 1956), not only by the method of correlating incorporation of label with net synthesis, but also by demonstrating that label once incorporated into this fraction is not lost but only diluted by new synthesis in the absence of isotope. Such a result is presented for suspended cell cultures in Fig. 24 (Siminovitch and Graham, 1956). It can also be seen that in this system and in this sense RNA

is also metabolically stable. The stability of DNA and RNA for actively growing and multiplying bacterial cultures had also been established earlier by a number of laboratories (Fujisawa and Sibatani, 1954; Manson, 1953; Hershey, 1954).

Of the results presented in Fig. 24 on mammalian cells, surely that concerning the stability of RNA is the most surprising. However, we must note that these studies apply to cultures in an exponential phase of growth and that cells in which mitosis has substantially stopped have not yet been subjected to such an analysis. Siminovitch and Graham (1956) have pointed out that even in exponentially growing cultures the initial rates of incorporation of  $P^{32}$  into RNA and DNA may differ considerably, but the observed stabilities of the nucleic acid suggests the need for explanations other than that of turnover, explanations such as differences in precursor pool size and mechanism of incorporation in each instance. Thus, since most of the so-called turnover data concerning RNA in the tissues of intact animals do not take these possibilities, as well as the turnover of cell population, into account, Siminovitch and Graham (1956) conclude there are no satisfactory data on the *in vivo* turnover of the nucleic acids.

As in the case of the proteins, it seems likely that this judgment, while rigorous at the time made, may possibly be too extreme and fails to take into account a good deal of data concerning RNA metabolism under conditions of metabolic stress. Two days of starvation will produce a sharp decline in the RNA content of mammalian liver; this increases again after feeding, although the DNA content remains unchanged (Davidson, 1947). As noted above, starvation provokes the degradation of an RNA particle in *E. coli* (Dagley and Sykes, 1957) and, indeed, of RNA structures in a variety of microorganisms, followed by resynthesis on feeding. Also, the phenomenon of chromatolysis and regeneration in nervous tissues is correlated with the degradation and resynthesis of ultraviolet-absorbing basophilic structures (Caspersson, 1950). The turnover of RNA in phage-infected bacteria and in a uracil-requiring strain of *E. coli* strain 15<sub>T</sub><sup>-</sup> has also been mentioned earlier. Most recently, it has been shown that the RNA formed by *E. coli* in the presence of chloramphenicol is rapidly degraded *in vivo* when the antibiotic is removed from the medium (Neidhardt and Gros, 1957).

The existence of this data suggests that under conditions of metabolic stress the system of Siminovitch and Graham may produce another type of result. One would wonder if virus infection may constitute such a stress, as indeed it does in the case of DNA turnover in phage-infected bacteria, wherein, as summarized by Kozloff (1953), host DNA is degraded to deoxyribotides, which are then resynthesized to form viral DNA.

However, in apparent contradiction of the possibility that RNA turnover occurs primarily in cells which have stopped growth and multiplication is the

report of Brody (1957), indicating that in developing placenta the ribonuclease activity is roughly proportional to the rate of growth of the tissue. This has been taken to suggest a possibly direct relation between RNAase activity and the cellular synthesis of RNA. Ledoux *et al.* (1957) have also shown a linear relation between the RNAase and RNA contents of the normal organs of mice and rats.

The problem of demonstrating nucleic acid turnover in rat liver has recently been re-examined in great detail (Swick *et al.*, 1956; Swick and Koch, 1957). These workers have analyzed the difficulties stemming from the use of a single dose of isotope as a consequence of varying pool sizes, exchange, transfer, and reincorporation phenomena, variability of turnover rates of fractions of the substance under investigation and of its precursors. They have used isotope ( $C^{14}O_2$  and  $P^{32}$ ) continuously under conditions which eliminate transient changes in the specific activity of intermediate pools and, having developed corrections for various reutilization phenomena, have obtained equations which permit the estimation of incorporation and disappearance of isotope from the nucleic acids of the growing rat. Their results affirm the high degree of stability of DNA and the nonreplaceability of its atoms and indicate that the average life of all liver cells is about 150 days. On the other hand, the half-life of substantially all of the RNA of the same tissue was on the order of 4 to 6 days.

Recent results with unnatural purine and pyrimidine bases have provided another type of evidence pointing to exchange or RNA turnover in bacteria, and, in one instance, to the possibility of this phenomenon with DNA. In studies with 8-azaguanine in *B. cereus* by Matthews and Smith (1956), bacteria were prepared in which the RNA contained the unnatural base. The growth of such bacteria is inhibited, but may be renewed in the presence of guanine. On supplying the normal purine, a normal growth rate is restored and 8-azaguanine disappears from the RNA much more rapidly than can be accounted for by the dilution of existing RNA by newly synthesized RNA containing guanine. These results then suggest an exchange of guanine for azaguanine without breakdown of RNA or a rapid degradation and resynthesis of polynucleotides. In a more extensive study of this phenomenon, Mandel (1957) has excluded the possibility of a simple replacement of azaguanine by guanine.

Zamenhof and his collaborators have described a reversible replacement of thymine in DNA by 5-bromouracil in a thymine-requiring strain of *E. coli* (Zamenhof *et al.*, 1956b). It has been shown further that the replacement is accompanied by an incorporation of  $P^{32}$  with the new base, indicating that the new base is incorporated as a new nucleotide (Price, T. D. *et al.*, 1956). The experiments reported to date state that when organisms which are no longer growing are incubated for considerable periods, e.g., 20 hours, in the presence

of the analog of the base present in DNA, there is a very considerable replacement of the original base. Data on the shape of the replacement curve, particularly in the early periods, are not available and it is important to be sure that the phenomenon does not arise from cellular turnover within a population of labile cells.

This experiment is so important that it is certain that this question will be clarified in the near future. In addition to providing a major exception to the concept of DNA stability, it raises the possibility that the induction of thymine deficiency in some way labilizes the DNA chain. The phenomenon may assist in explaining thymineless death in bacteria and may prove to be a phenomenon of potentially great significance in approaching problems of chemotherapy. Such a labilization may also be of considerable interest in the study of mutation, as suggested by Coughlin and Adelberg (1956) and may suggest an added role for the ubiquitous RNAase and DNAase.

It may be suggested that the experiments so far constructed to test the metabolic activity of the nucleic acids are valid only if complete units, e.g., nucleotides, are added or removed. Even as proteins may possess several polypeptide chains, the nucleic acids are not known to exist in biological materials as single polynucleotide chains. In DNA, bases are bonded together so that each nucleotide is held in three ways, including the two phosphodiester bonds of the internucleotide linkage. In RNA, bases may also be bonded to other bases or possibly to protein. Thus the possibility exists that internucleotide bonds may be cleaved without disrupting polynucleotide chains. If, as we believe likely as a result of enzyme studies to be discussed in a later section, these cleavages are phosphorylytic and involve the formation of pyrophosphate bonds which may be exchanged with amino acids to give phosphocarboxyanhydrides, a mechanism is available for organizing amino acids selectively at nucleic acid templates as a preliminary step to peptide synthesis. The release of such an anhydride-bound amino acid may permit a concomitant closing of the phosphodiester bond, as in Fig. 25. In such a sequence the nucleic acids may be intensely active and would show no evidence of phosphorus, nitrogen, or carbon replacement. Indeed, it is not immediately evident that one could expect a turnover of oxygen in the system, although this can certainly be tested. Although some information is available concerning the phosphorylytic cleavage of RNA and the pyrophosphorylytic cleavage of DNA, and the attachment of an amino acid in such a fragment has recently been recorded, the nature of amino acid transfer to nucleic acid and of amino acid acceptors is entirely hypothetical and is presented only to complete a possible mechanism of nucleic acid activity which may operate without detectable turnover in the atoms so far examined.

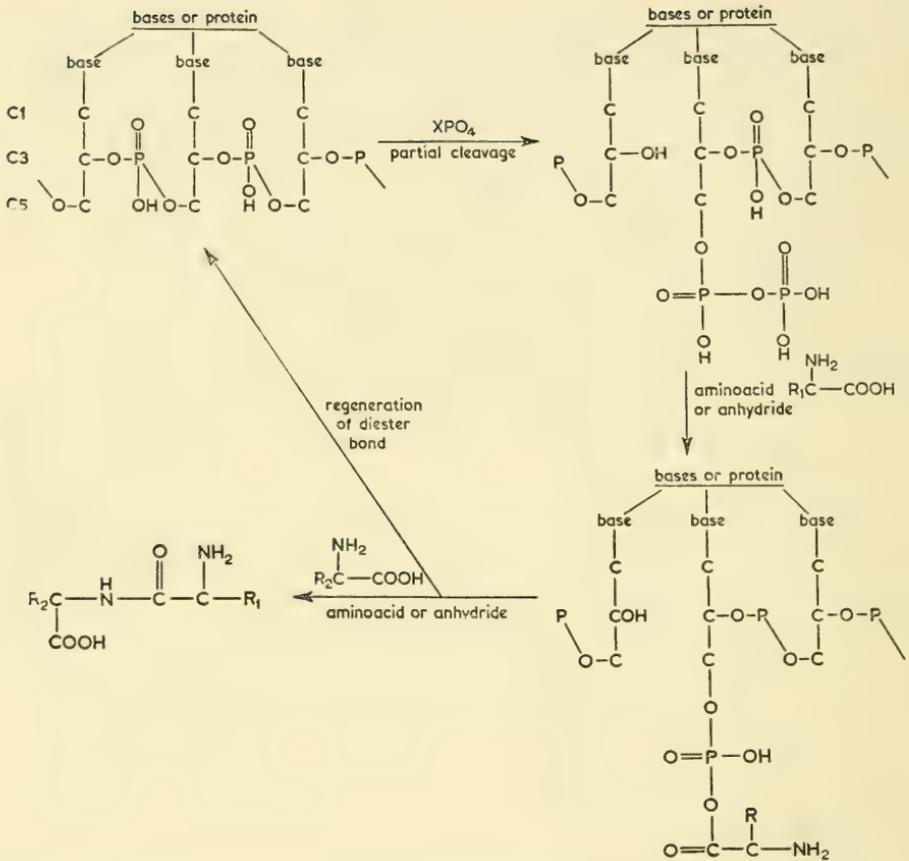


FIG. 25. A possible mechanism for the participation of DNA in metabolic activity without replacement of carbon, nitrogen, and phosphorus atoms.

## V. CONTROLLING MECHANISMS OF ENZYME AND PROTEIN SYNTHESIS

### A. Introduction

Until now this survey has been concerned for the most part with the control of polymer synthesis imposed by existing cell structure. The development of cell structure is in turn controlled by genetic and environmental factors; indeed, such factors are determinative not only in the formation of structure but also in their operation. Determining genetic factors are built into cell structure in nucleus and cytoplasm and confer the potentialities for metabolic and synthetic reactivities; the realization of these potentialities, i.e., phenotypic expression, is dependent upon the existence of a suitable internal and external milieu, i.e., pH, ionic strength, temperature, pressure, nutrients, substrates, and other substances.

Recent advances on the participation and interaction of these genetic and environmental factors in polymer syntheses have been obtained on biological materials of relatively little interest to virologists. Of the major experimental materials, *Drosophila*, yeast, *Neurospora*, *Escherichia coli*, and most recently *Salmonella typhimurium*, only the bacteria have seriously attracted the interest of virologists.<sup>1</sup> Very little of this type of information is available for the cells which are infectable by animal and plant viruses. However, the development of tissue cultures of animal cells is beginning to provide suitable biological materials for these types of analysis. Thus, papers have begun to appear on inheritance and mutation in clones of animal cells (Puck and Fisher, 1956), on the detailed nutritional requirements of such cells (Eagle, 1955), and on their metabolic patterns and their variability as a function of environmental conditions.

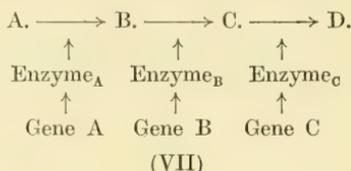
We shall briefly consider three types of controlling elements that have begun to yield information on the mechanisms of polymer synthesis and shall in the main confine our attention to these aspects of metabolic activity.

### B. Genetic Controls

This topic has been the subject of many books and reviews, of which a recent volume can serve as one guide to the literature (Wagner and Mitchell, 1955). Since the original work of Garrod on inborn metabolic errors in man, it was recognized that a close relationship existed between the genetic units of Mendelian inheritance and the control of metabolic activity. Garrod's studies of this problem appeared at the turn of the century (1902) and Cuénot probably made the first statement of a one gene-one enzyme hypothesis, in 1903, before the word "gene" was coined by Johannsen, in 1911. Many forms of such a hypothesis now exist and state in general that genes exert their physiological power through enzymes whose synthesis or activities they control. The variations on this theme extend from the early view of Haldane that a gene may manufacture particular chemical species of enzyme or an antigen to the more recent and cautious statements to the effect that there is a large class of genes, each of which controls the activity or the specificity of a single enzyme. We may note that, in the latter definition of the one gene-one enzyme hypothesis, not all genes necessarily affect enzymes, that the moderating control may be either direct or indirect, and that a single enzyme need not be controlled by a single gene. However, if the activity of a single enzyme is controlled by more than one gene, it is postulated that the contribution of the various genes must be different.

<sup>1</sup> There is the outstanding exception in the studies of the virus-like CO<sub>2</sub> sensitivity factor in *Drosophila* (L'Heritier, 1951).

We may picture the position of genetic units in a metabolic sequence in the simplest form, as shown in formula (VII).



At the level of a specific reaction, this apparently simple genetic control may be complicated by a network of other possible reactions (see Fig. 26) for all of which known examples exist.

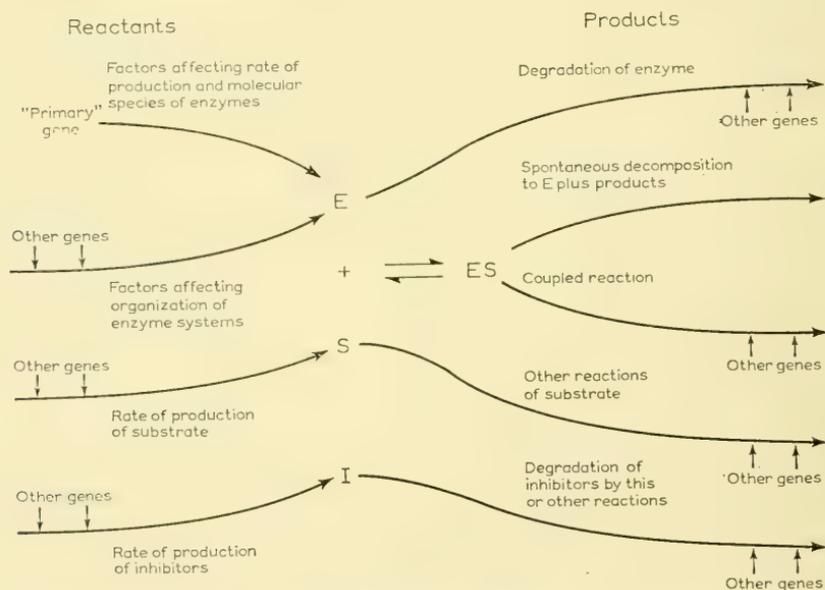


FIG. 26. A summary illustrating some factors of genetic origin that influence reaction rates (Wagner and Mitchell, 1955).

As this scheme demonstrates, the normal or distorted actions of a single gene can and do impinge on a wide variety of more or less directly related reactions and thereby can and do influence an ever-widening circle of metabolic patterns, cellular structures, and morphological units. As a result of these complicating factors, the task of determining the primary site of gene action has much in common with determining the primary effects of viral infection. We may note in addition that many genes appear to be able to

affect the activity of a single enzyme, as in the case of  $\beta$ -galactosidase production in *E. coli* (Lederberg, 1951), and it may be asked how many of these are primary genes and how many fulfill the functions of "other genes" described in Fig. 26. In the case of genes controlling the synthesis of a compound enzyme, such as tyrosine decarboxylase, for which pyridoxal phosphate is the prosthetic group, or catalase containing Fe protoporphyrin IX, auxotrophs have been obtained unable to synthesize pyridoxine or hemin, respectively. In each case, the organisms nevertheless synthesized the protein portion of the enzyme, demonstrable by exogenous addition of the prosthetic group (Gunsalus, 1952; Beljanski and Beljanski, 1957).

In the early 1940's, Beadle and Tatum and their collaborators undertook to probe the problem of the mode of gene action, and elected to use the mold, *Neurospora*, for this basic study. In this fungus the alternating sexual and vegetative phases of the life cycle permitted both classic genetic analysis and the study of enzyme production, and the simple nutritional requirements of the wild type enabled the ready detection of auxotrophy among mutant progeny. By 1950, about 1500 different auxotrophic mutants of three different fungi, *Neurospora*, *Aspergillus*, and *Ophiostoma*, possessing a single genetic change had been obtained; of these 92% were found to have a requirement for but a single growth factor (Horowitz, 1950). By 1952, only a single mutant of *Neurospora* (over 500 auxotrophic mutants were known) had been recognized in which the genetic control of metabolic capability was not centered in a nuclear Mendelian gene (Mitchell and Mitchell, 1952). A few more "cytoplasmic" mutants have been recorded since then; some of these in turn have been recognized to be controlled by nuclear genes (Mitchell and Mitchell, 1956).

Given this wealth of biological material, possessing lesions in pathways leading to the formation of essential metabolites, the work was concentrated for the most part during the 1940's on a delineation of many of the pathways themselves, so that it became possible to establish the position of the genetically controlled metabolic lesions. Many of such lesions have been analyzed in detail and results covering the major pathways have been summarized by Vogel and Bonner (1956), and by DeBusk (1956). Difficulties in establishing the precise positions of metabolic lesions have been discussed by Adelberg (1953) and Davis (1955b); these discussions record the variety of biological and chemical techniques which must be employed to define exactly the site of action of the enzyme affected by the gene mutation. In view of these difficulties, it is not surprising that, even as in determining the primary site of virus action in an intact animal, we still have not defined the nature and site of most of the inborn metabolic errors of man described by Garrod over 50 years ago (Wagner and Mitchell, 1955).

However, in the microbial systems, it has not only been possible in many instances to define the site of the metabolic block but also to describe an enzymatic reaction with known substrates and products that exists in the parent organism and is modified in the mutant. Several types of such modification have been found. In most auxotrophic mutants, the enzyme is missing or at least the reactivity characteristic of the enzyme cannot be detected. Obviously, this distinction is of fundamental importance for the problem of the nature of gene action; in these instances it must be determined whether the enzyme has not been made or if it is inhibited. In the case of the system synthesizing pantothenate in *Neurospora*, it has been shown that the enzyme is present in an inhibited state in cell-free extracts of certain mutants but not in extracts of the parental strain. The enzyme may be purified away from the inhibitor in this instance. It is evident that if this separation were not possible, it would be a very difficult matter to determine if the enzyme were really not made or were merely hidden from detection.<sup>1</sup> Several approaches to this difficult problem are available. For example, one might prepare highly purified enzyme and antisera to this protein, and test extracts of mutant organisms for cross-reacting proteins. Many possible complications could obscure the results of such an analysis. In a more biological approach, used to analyze the apparent absence of glutamic dehydrogenase in *Neurospora* mutants, Fincham (1951, 1954) prepared heterocaryons of varying numbers of dehydrogenase-deficient nuclei. The dehydrogenase activities of the various heterocaryotic mycelia were related to the numbers of nondeficient nuclei and indicated that the mutant nuclei did not facilitate the production of inhibitors. It was concluded as a result of this and other analyses that in these systems the mutation of a single gene did indeed prevent the formation of a single enzyme.

That mutant genes did not always result in complete metabolic blocks was clearly demonstrated by Bonner *et al.* (1952) in the case of tryptophan-requiring mutants of *Neurospora*. When N<sup>15</sup>-labeled precursors of tryptophan were fed to these mutants, in addition to an unlabeled carbon and nitrogen source, and various accumulation products of tryptophan metabolism were isolated, these compounds were found to contain only a portion of their nitrogen in the form provided by the fed precursor. This indicated that a portion of the tryptophan could be synthesized through the apparent block. Such mutants have been said to possess "leaky" genes and have been

<sup>1</sup> It will be evident to most readers that the problem of detecting the presence in uninfected bacteria of the enzyme for generating hydroxymethyl cytosine is of this type. A similar question may be raised concerning the origin of neuraminidase in influenza-infected animal cells. If such enzymes should prove to be inhibited in normal cells, as is deoxyribonuclease in many, the problem may then be posed as to the mechanism by which a virus releases such an inhibition.

observed in many other types of mutation (Abrams, 1952; Cohen and Barner, 1954).

The analysis of steps in tryptophan metabolism has also been most instructive concerning the nature of the gene itself. Two apparently identical mutants of *Neurospora* were found incapable of synthesizing tryptophan desmolase, the enzyme condensing indole and serine to form tryptophan. These mutations had occurred apparently at the same loci and were allelic, since the strains were functionally identical in all respects and crosses did not result in the production of wild type strains. However a mutation at another locus (a suppressor gene), which in itself did not restore the desmolase, did so when present with one of the mutant genes but not the other. Thus, the original apparently allelic mutants were structurally and functionally distinct and two genes at different loci, each incapable of desmolase synthesis, could effect synthesis when operating together (Yanofsky, 1952). Indeed, of five independent suppressor mutations that have been studied for the ability to restore desmolase synthesis, all have been different (Yanofsky and Bonner, 1955).

This possibility of the dissection of structure within a gene locus has been greatly extended in recent years by crossing-over techniques (Benzer, 1955; Pontecorvo and Roper, 1956). Such techniques have revealed the existence of units of recombination in *Aspergillus*, *Drosophila*, and phage, representing fractions of  $2 \times 10^{-7}$ ,  $2.8 \times 10^{-6}$ , and  $6 \times 10^{-5}$ , respectively, of the total genetic maps of these organisms. In chemical terms, if these genetic units consist exclusively of DNA in the form of a double-threaded helix, if crossing over involves nucleotide units, and if the probability of crossing over is the same at all nucleotides, the number of nucleotide pairs in the smallest recombination fractions consist of as few as 8 for *Aspergillus*, 216 for *Drosophila* and 12 for phage, whereas the site of mutation is sufficiently large to contain on the order of 1000 to 8000 nucleotide pairs. The possibility of the existence of subunits of DNA molecules had been realized earlier in the discovery of an apparent crossing over between molecules of transforming agents (DNA) in *Pneumococcus* (Taylor, 1949) and by evidence indicating that units of transformation are sometimes linked within a single DNA molecule (Hotchkiss and Marmur, 1954).

McIlwaine (1947) had observed that some coenzymes were present in bacteria in such small amounts that it would be most efficient to have their synthesis occur on a belt line comprised of single and unique enzyme molecules. In such cases one might imagine that this belt line of enzymes was comprised of the linear array of genes of a chromosome, and that indeed the genes might be the enzymes themselves. The demonstration of genetic subunits by Pontecorvo and Roper began with the dissection of the genetic control of vitamin synthesis in *Aspergillus* on the hypothesis that a close

linkage was required between genes involved in consecutive steps in a series of millimicromolar reaction systems (Pontecorvo, 1952). Although Mellwaine's hypothesis has not yet been proved or disproved, a remarkable result has recently been obtained by Demerec *et al.* (1955) and Hartman (1957), who have shown that in *Salmonella typhimurium* the linear order of many genes controlling the enzymes involved in tryptophan and histidine biosynthesis corresponds to the sequence of reactions of intermediary metabolism. The significance of these apparent evidences of a genetic order corresponding to metabolic order is obscure at present.

How may a gene control the enzyme itself? No instance is known wherein specificity of an enzyme has been altered by a mutant gene. However, several instances are known in which an altered protein may be produced. In the case of the hereditary disease resulting in the production of sickle cell hemoglobin, it has been reported (Ingram, 1957) that the mutant hemoglobin differs from normal hemoglobin by the replacement of one amino acid (glutamic acid) by another (valine) in a single peptide sequence per half-molecule of hemoglobin.

Maas and Davis (1952) have studied a temperature-sensitive mutant of *E. coli* that required pantothenate above 30°C. It was found that the pantothenate-synthesizing enzyme produced by the mutant was extraordinarily heat-labile, as compared to the enzyme produced by the wild type organism. Thus, an altered protein had been produced. In *Neurospora crassa*, a pair of allelic genes has been found governing the thermostability of tyrosinase; the enzymes produced in the mutant organisms also appear to be structurally different (Horowitz and Fling, 1953). In neither instance has evidence been obtained of the formation of inhibitors.

In neither of these two cases has it been possible to distinguish between the possibilities that there has been an alteration in the template leading to differences in primary structure, as appears to be the case for the production of sickle cell hemoglobin, or that the genes affect the organization of secondary or tertiary structure and that the primary polypeptide chains are invariant. In a series of allelic tryptophan-requiring mutants lacking tryptophan synthetase, in all but one a protein was detected which cross-reacted with antibody to tryptophan synthetase and possessed other structural features in common with the enzyme (Suskind *et al.*, 1955). This suggests that in these instances the gene does not affect primary protein structure related to serological specificity, but perhaps controls that aspect of organized tertiary structure related to specific catalytic activity (also see Lerner and Yanofsky, 1957).

It is evident from the above that genetic investigation has so far provided little direct information of the contribution of the gene to the synthesis of polymers. In an unusual approach to this type of problem, Horowitz and

Leupold (1951) have concluded, from an analysis of temperature-sensitive mutants, that a majority of all genes (not only those of isolable mutants) affecting biosyntheses are not involved in more than one metabolic process. Since only about one-quarter of all mutants (if they are all similar to the temperature-sensitive group) have requirements which cannot be supplied exogenously by relatively simple substances, it has been concluded that proteins, for example, do not involve the formation of all the possible intermediate peptides via genetically controlled, distinguishable enzymatic steps, but are somehow assembled from the low molecular weight building blocks in a very few steps (perhaps one) involving very few catalysts (perhaps a single template). Atwood and Mukai (1953), using a technique permitting the preservation of mutant lethal genes in heterocaryons, consider that only a small percentage of all mutants have been isolated and analyzed and that in the majority of mutants we are unable to supply the required metabolite whose synthesis has been blocked. Such a result, of course, casts doubt on the validity of Horowitz's conclusion.

It is possible that a more direct approach to this problem can be made, since properties transformable by DNA are beginning to be known that can be analyzed at the enzymatic level. Thus, Marmur and Hotchkiss (1955) have described the transformability in *Pneumococcus* of the ability to metabolize mannitol. The transformed cells are able to form the new enzyme, mannitol-6-phosphate dehydrogenase, which catalyzes the conversion to fructose-6-phosphate. Thus, one may now hope to explore the events between the addition of DNA to the bacterium and the induced biosynthesis of the new enzyme, an experimental advance obviously important in analyzing the mode of action of genetic material. As noted earlier, the viruses have also provided two perhaps comparable experimental materials, in which the production of new enzymes occurs after incorporation of new genetic units. For the T-even phages, there are the cases of the evocation of the deoxyuridylic acid hydroxymethylase by infection of *E. coli* strain 15<sub>T</sub><sup>-</sup>, as well as the apparent induction of deoxycytidylic acid hydroxymethylase by infection. These events are also possibly controlled by the addition of new DNA. The evocation of neuraminidase by influenza virus which contains only RNA is possibly of particular interest in dissecting the mode of action of this genetic material.

### C. Some Nutritional Phenomena

As pointed out earlier, organisms unable to make the prosthetic group of key enzymes as a result of genetic blocks are nevertheless able to make the protein portion of the enzyme, a point of some theoretical interest. In a recent study of Burch *et al.* (1956) on the development of riboflavin deficiency and the effect of realimentation of the vitamin in rat tissues, it was observed that the concentration of the various apoenzymes in riboflavin-deficient

tissues had fallen to 10 %, or less, of the normal content of these proteins in the tissues. This would signify that the protein apoenzymes are less stable in the absence of the prosthetic groups or that their rate of synthesis is diminished under these conditions.

However, growth and polymer synthesis in general may continue in the absence of certain essential metabolites, as has been discussed above in sections of pathological growth and elsewhere. In most of these instances, the control of the synthesis of particular polymers has been related to particular metabolites which they contain, e.g., diaminopimelic acid in cell wall synthesis, or to the presence or absence of their controlling mechanism, e.g., the maintenance of microsomal enzymes in the presence of the nucleus in *Amoeba*. In some organisms, nutritional deficiencies with respect to a particular metabolite have led to quite unusual growth patterns.

When *Proteus vulgaris* is grown in a medium with limiting concentrations of nicotinic acid, essential for pyridine coenzyme production, and all other requirements are supplied in excess, growth does not cease immediately after the vitamin disappears from the medium. There is a linear phase of protoplasmic synthesis and O<sub>2</sub> consumption, apparently determined by the temporarily fixed complement of pyridine coenzyme (Jackson and Copping, 1952). A comparable growth pattern has been observed for *Mycobacterium tuberculosis* var. *hominis* in *in vitro* cultivation, indicating a deficiency in the medium with respect to some essential metabolite stored earlier in a previous phase of growth (Fisher *et al.*, 1951).

When certain strains of *Staphylococcus aureus* are grown in fresh broth, the hyaluronidase produced is proportional to the increment in mass of the bacteria. However, the use of media partially deficient in thiamine or nicotinic acid results in a lower ratio of this enzyme to the mass of the organism (Rogers, 1957). Thus, the synthesis of specific polymers may be more sensitive to vitamin deficiencies than the synthesis of all the bacterial polymers in general. The presence of  $\alpha$ -aminobutyric acid in cultures also decreases the proportion of cell protein which appears as hyaluronidase.

A number of workers have studied effects of nutritional deficiencies in plants on protein and enzymatic deficiencies. A sulfur deficiency in alfalfa, for example, results in the decrease of methionine and cystine in plant protein. However, the plant protein is now enriched with respect to asparagine and arginine, of which the former accumulates earliest (Mertz *et al.*, 1952). Thus, the protein composition of an intact organism appears far more plastic than experiments with microorganisms would imply, when growth stops in the absence of a required amino acid.

Even more bizarre results were observed by Nason and collaborators, who studied the effect of metal deficiencies on the growth of tomato plants. Fe and Cu deficiencies produced marked drops in enzymes containing the

respective atoms in catalytic centers, e.g., Fe in peroxidase, Cu in polyphenol oxidase and ascorbic acid oxidase (Nason *et al.*, 1952). However, a Zn deficiency doubled the ascorbic acid oxidase content of the plant and copper-deficient tomato leaves showed 10- to 30-fold increases in isocitric dehydrogenase per unit weight of protein, as contrasted to normal leaves (Nason, 1952). These increases did not arise from effects on inhibitors or activators concentrations but appeared to represent increases of the enzymes involved. Thus, the apparent plasticity could be expressed in determining the relative proportions of cellular enzymes.

It may be remarked that the causal relations of these phenomena are quite obscure at the present time. However, it is well known that unusual metabolites may accumulate in many plant virus diseases and indeed be useful in the diagnosis of such infection. Although the relation of these metabolic disturbances to virus multiplication is not known, there seems no reason to doubt that such interconnections might be traced. The problem of the origin of compounds appearing in infected plants that are not found in comparable amounts in the normal plant is relevant to many problems of normal plant metabolism. In analyzing the problem of the distribution, function, and synthesis of the alkaloids, for example, it has been suggested that these compounds cannot all be thought of as useful in plant metabolism, and that the metabolic pathways involved are probably not all maximally efficient. It seems more reasonable to imagine that random mutation may increase the production of some metabolites and, as a result of the accumulation of these reactive substances, open routes to new and possibly useless end products. It appears possible that this type of metabolic disorder may also exist at the level of protein synthesis in plants, and indeed account for the production of normal and pathological proteins in many virus diseases, e.g., masked strain of TMV, as long as the foreign unit is relatively inert and does not deplete metabolites essential to normal synthesis.

#### *D. The Induced Biosynthesis of Enzymes*

##### *1. General Remarks*

From the point of view of the control of enzyme synthesis and indeed with respect to the origin of enzymes, the phenomenon of the induced biosynthesis of enzymes has been explored and analyzed to a greater extent than any other (Monod, 1947; Monod and Cohn, 1952; Spiegelman, 1951; Cohn, 1957). Until several years ago, the study of this phenomenon provided the most complete body of information concerning the mechanism of synthesis of specific proteins in intact cells; most recently, it has been possible to press this analysis to protoplasts and to cell fragments, bypassing barriers presented by cell walls and membranes. It is

with this subject that we almost bridge the gap between biology and enzymology in the field of polymer synthesis. Studies in this area have provided little information concerning the manner in which the genetic apparatus establishes a potentiality for protein synthesis or the nature of the link between the gene and the protein-synthesizing apparatus; however, given the existence of the gene and the potentiality which is in some way conferred upon the cell, it has been possible to analyze many of the steps in bringing the protein-synthesizing apparatus into play to produce a given enzyme.

The modern history of work in this area is only 15 years old and is mainly confined to the study of microorganisms. It begins with the dissertation of Monod (1942) on the growth of *E. coli* in various carbon sources and the studies of Spiegelman and his collaborators on enzyme production in yeast. Although the earliest work described the elaboration of poorly defined catalytic systems for the metabolism of one or another carbon source, the discovery of an inducible sucrose phosphorylase in *Pseudomonas saccharophila* (Doudoroff, 1940) pointed to the possibility of studying the production of a single specific protein. This lead has been pursued in most serious investigations.

Karstrom (1937) made the distinction between "constitutive" and "adaptive" or inducible enzymes,<sup>1</sup> i.e., constitutive enzymes are present in

<sup>1</sup> The terminology to be used in this discussion is one which was adopted relatively recently by a number of groups working in this field (Cohn *et al.*, 1953b). Until this time, it had been customary to use the term "enzymatic adaptation" to describe the elaboration of an enzyme by a cell in response to the presence of a specific exogenous substance. These workers preferred to avoid the usual biological connotations of "adaptation," i.e. structural or functional changes of an organism to increase fitness by a process which is usually associated with genetic variation and selection, particularly since the induced biosynthesis of enzymes was being studied under conditions of constant genetic background. Stanier (1953) has discussed the varieties of evolutionary and physiological adaptations in microorganisms and their relation to the more limited phenomena of the inducible production of enzymes.

It is relatively simple to distinguish the two classes of adaptation which appear in a culture in response to a new carbon source. If in a culture the development of the ability to metabolize a substrate as carbon source is of genetic origin, the acquisition of potentiality is found originally in only a few cells of the entire culture, i.e., in mutants, and these may be detected by plating large numbers of cells on the substrate as sole carbon source. Only a small percentage of the total number of plated cells will form colonies. These mutants, of course, have a selective advantage in the presence of the new substrate and their progeny will soon replace the original cells in this medium. On the other hand, if all the cells do possess the potentiality but lack the enzyme because the culture has been grown under conditions in which inducer is absent, plating on the substrate, nevertheless, will reveal as many inducible colonies as cells plated.

When a culture of bacteria possessing the potentiality for induced biosynthesis is exposed to a high concentration of a new inducer, e.g., a  $\beta$ -galactoside, such as lactose,

the cell regardless of the presence of an exogenous inducer. The validity of this qualitative distinction between classes of enzymes is still obscure for the following reasons:

a. It is possible that the inducers for "constitutive" systems are generated endogenously.

b. No inducible enzyme systems are known which are entirely missing when cells are grown in the absence of an exogenous inducer.

c. By a process of selection, organisms may be obtained in which relatively high levels of inducible enzymes are formed in the absence of exogenous inducer, i.e., inducible enzymes may become constitutive.

d. Many constitutive enzymes may be synthesized in greater amounts if an inducer is present under appropriate conditions.

Nevertheless, the distinction is useful in describing quantitative relations among enzymes in the presence or absence of specific inducers. Thus, *E. coli* will be able to metabolize glucose without delay and contains constitutive hexokinase whether grown on glucose or D-xylose. On the other hand, if grown in glucose, *E. coli* will contain the merest trace of xylose isomerase and subsequent systems for metabolizing xylulose, the first reaction product of xylose utilization. Such cells will not be able to handle xylose at a significant rate unless a far greater biosynthesis of these enzymes is induced. It may be suggested as one hypothesis that organisms do indeed have a more or less fixed battery of metabolic systems ("constitutive" enzymes) and that the inducible systems represent more or less disposable baggage whose main function is to insert substrates into constitutive systems when such substrates become available. It would be inefficient to have to make such enzymes even when these substrates are not present. On the other hand, it will be seen below that enzyme levels for the synthesis of even essential metabolites may vary considerably as a result of the action of various controlling mechanisms.

In one instance different constitutive and inducible enzymes are known for the cleavage of the same substrate, maltose, in the same bacterium, *E. coli* strain K12 (Pontieri, 1955). Thus, the constitutive maltase is an  $\alpha$ -glucosidase, converting maltose to 2 moles of glucose. The adaptive maltase is an

under suitable conditions of gratuity, it has been observed that the synthesis of the new enzyme,  $\beta$ -galactosidase, does indeed occur at similar rate in all the cells (Benzer, 1953). This study was done by taking advantage of the fact that bacteria infected by certain phages are no longer inducible (Monod and Wollman, 1947). Infection at different times after induction thus fixes the enzyme content of the cell and this in turn governs the rate of utilization of lactose, the rate of production of virus, and the development of cell lysis. The extent of lysis may be quantitated by estimating the amount of enzyme liberated into the medium.

A different result has been obtained at low inducer concentrations in which only some cells are induced (Novick and Wiener, 1957).

amylomaltase, converting maltose to 1 mole of glucose and polymerizing the residual mole of glucose to a straight chain glucose polymer, amylose.

As in the case of xylose metabolism, there may be a number of metabolic steps for which a number of enzymes may be required before insertion into a constitutive path is possible. In this case, we may expect stepwise production of substrates and sequential induction of each of these enzymes for these substrates in turn, until a metabolite is generated which the constitutive path is equipped to handle. Sequential induction, or "simultaneous adaptation" has been widely used in the analysis of the paths of degradation of numerous carbon compounds in organisms capable of deriving most of its carbon from these substances as sole carbon sources (Stanier, 1947; Karlsson and Barker, 1948; Cohen, 1949; Suda *et al.*, 1949). The most detailed investigations have been those of Stanier and his collaborators (Stanier, 1955), who have not only explored the utilization of tryptophan and many other aromatic compounds in *Pseudomonas* by the method of sequential induction but also confirmed the existence of many of the metabolic steps by demonstration of the suspected enzymes in cell-free extracts and by studying the fate of isotopically labeled substrates. The method of sequential induction applied to microorganisms is perhaps the simplest available for outlining a suspected pathway involving penetrable intermediates and will probably prove of interest in the analysis of certain pathways in animal cells as well. In particular instances it has been possible to prepare inducible microorganisms as specific analytical reagents (Cohen and Raff, 1951).

## 2. Problems of Protein Synthesis in Inducible Systems

That newly induced functional units were actually proteins and that induced biosynthesis did not involve (a) the synthesis of a coenzyme, (b) the formation of special intermediates, or (c) the removal of an inhibitor were clearly demonstrated by studies of extracts of galactose-induced and non-induced yeast. It was shown that (a) the catalytic system for galactose fermentation was not present in extracts of uninduced cells, although the coenzymes required for galactose fermentation were present in such extracts, and (b) the system for galactose fermentation, present in extracts of induced cells, was nondialyzable and heat-sensitive, while the essential coenzymes were dialyzable and heat-stable.

The well-known case of the conversion of inactive, preformed trypsinogen to enzymatically active trypsin raised the possibility of a similar occurrence in all biological systems characterized by the appearance of new enzymatic activities. That a comparable phenomenon, i.e., the uncovering of an inactive protein or the slight remodeling of an existing protein precursor, does not occur in inducible systems rested for some years on indirect evidence. In addition, the situation was obscured at first by the fact that yeasts could perform induced biosynthesis in the absence of an exogenous nitrogen source.

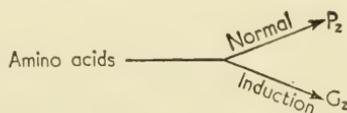
It was discovered relatively recently that yeasts contain significant amounts of free amino acids and that the depletion of this amino acid pool then limited enzyme synthesis (Halvorson and Spiegelman, 1953).

On the other hand, it was known quite early that inducible enzyme synthesis required an energy source and was blocked by inhibitors of oxidative phosphorylation, such as dinitrophenol, and other inhibitors, azide, arsenate, etc. In gram-negative bacteria, subsequently discovered to contain relatively small internal amino acid pools, exogenous nitrogen and carbon sources were also found to be essential to enzyme synthesis.

In yeast, the induced synthesis of  $\alpha$ -glucosidase is inhibited by a wide variety of amino acid analogs, and in most instances a parallelism exists between the inhibition of enzyme formation and growth (Halvorson and Spiegelman, 1952). As noted earlier, the unfulfilled requirement for a single amino acid in a series of auxotrophic mutants of *E. coli* is sufficient to prevent formation of  $\beta$ -galactosidase (Monod *et al.*, 1952). Thus, an active metabolism and *de novo* protein synthesis seemed required in the production of new catalytic units.

In the case of  $\beta$ -galactosidase of *E. coli*, it was shown by Cohn and Torriani (1952) that extracts of  $\beta$ -galactoside-induced bacteria contained a new antigenic activity,  $G_z$ , associated with the enzyme. The new antigen was capable of stimulating the production of precipitating antibody in rabbits. However, noninduced cells contained a cross-reacting protein,  $P_z$ , which is nonenzymatic and nonantigenic in rabbits, and precipitates poorly with antisera to  $G_z$  in the presence of  $G_z$ . An immunochemical analysis of the formation of  $G_z$  and  $P_z$  in extracts of induced bacteria revealed a fall in  $P_z$  almost concomitantly with the increase in enzyme  $G_z$  (Cohn and Torriani, 1953). However, the increase in enzyme was far greater than the fall in  $P_z$ .

That  $P_z$  is not a precursor of  $G_z$  was clearly demonstrated by the  $S^{35}$  isotope experiments of Hogness *et al.* (1955), discussed in Section IV, G, 1 on protein turnover, wherein it was shown that less than 1% of  $G_z$  could be derived from a preformed cellular precursor. Rotman and Spiegelman (1957) have presented comparable evidence with the same system, using  $C^{14}$ -labeled cells. These data eliminate a protein-conversion hypothesis and it seems possible that the situation may be represented as shown in formula (VIII).



(VIII)

This system is formally analogous to that which has been proposed for antibody and normal  $\gamma$ -globulin production in the mammal, since these

proteins also have marked similarities in primary structure and serological cross-reactivities (Cohn, 1954). Thus, as has been suggested, the cell may be producing a fixed polypeptide, whose secondary and tertiary structure may be determined by the presence of antigen in one case, and inducer in another.

However, Schweet and Owen (1957) have recently summarized the similarities and differences between inducible biosynthesis and antibody production. They have stressed particularly the observed penetration of antigens into nuclei and have suggested that antigen modifies the genetic apparatus of cells capable of producing globulins. These cells on duplication produce new genetic codes, new templates which direct the synthesis of new globulin configurations. In these senses, then, antibody production may be more closely related to some types of virus production, since data have been obtained to indicate that the RNA viruses of influenza and poliomyelitis also stimulate marked nuclear changes, reflected in alterations of DNA metabolism.

Spiegelman and his collaborators (1955) have studied the problem of the nature of intermediates between amino acids and enzymes. They have shown that a single amino acid analog which blocks enzyme synthesis does not produce an accumulation of endogenous peptides in the microorganisms or the incorporation of other amino acids to cell structure. They have concluded that the first stable intermediate on the way to protein synthesis requires the simultaneous presence of a large number of different amino acids. The implication is therefore clear that protein synthesis does not involve a stepwise formation of larger and larger peptides, the formation of each of which requires its own specific catalyst.

### 3. Role of Nucleic Acids in Inducible Systems

Enzyme synthesis in inducible systems, even as protein synthesis in general, is intimately tied to the presence and synthesis of nucleic acid. However, it is not yet known whether the presence of DNA is or is not required for induced biosynthesis to take place. That DNA synthesis is not essential to the induced biosynthesis has been indicated by the following evidence:

a. X-irradiation of yeast and ultraviolet irradiation of yeast and bacteria in quantities sufficient to block DNA synthesis without affecting RNA and protein synthesis does not affect the induced biosynthesis of enzymes (Baron *et al.*, 1953; Halvorson and Jackson, 1956).

b. A thymineless mutant of *E. coli* can synthesize various inducible enzymes, RNA, and protein in the absence of exogenous thymine and of a significant synthesis of DNA (Cohen and Barner, 1954).

c. *E. coli*, treated with mustard gas, cannot make DNA but can be induced by lactose (Pardee, 1954).

d. Treatment of protoplasts of *B. megatherium* with DNAase selectively removed DNA in considerable amounts without affecting their ability to make  $\beta$ -galactosidase (Landman and Spiegelman, 1955).

On the other hand, the presence and concomitant synthesis of RNA are variously reported to be essential to the production of particular enzymes, although both requirements may not be necessary to all such syntheses.

a. In the study of ultraviolet-irradiated yeast (Halvorson and Jackson, 1956), a dose that inhibited synthesis of  $\alpha$ -glucosidase also inhibited protein and RNA synthesis.

b. An analog of uridine, 5-OH uridine, selectively inhibits  $\beta$ -galactosidase production in *E. coli* without affecting over-all protein synthesis (Spiegelman *et al.*, 1955). Furthermore, this compound interrupts enzyme synthesis, even after its onset, suggesting a requirement for continuing RNA synthesis for enzyme production.

c. A number of uracil-requiring bacterial mutants are unable to be induced in the absence of this pyrimidine (Pardee, 1954). In one instance, where a uracilless mutant can synthesize enzyme in the absence of exogenous uracil, extensive turnover of RNA has been demonstrated (Barner and Cohen, 1958).

d. Treatment of protoplasts of *B. megatherium* with RNAase removes 80 to 90 % of the RNA without significant loss of DNA and protein;  $\beta$ -galactosidase production is also markedly inhibited (Landman and Spiegelman, 1955). Similar results are obtained with osmotically shocked protoplasts still capable of enzyme synthesis (Spiegelman, 1957).

e. Fractions of sonically disrupted *staphylococci* are able to synthesize various enzymes, such as catalase and  $\beta$ -galactosidase. Removal of the nucleic acids by nucleases reduces or abolishes enzyme production. Addition of intact RNA does promote the synthesis of catalase but not of  $\beta$ -galactosidase; however, provision of a mixture of purines and pyrimidines, permitting  $C^{14}$ -uracil incorporation into RNA, did stimulate synthesis of  $\beta$ -galactosidase (Gale, 1956), although it did not stimulate catalase synthesis. It was concluded that a concomitant synthesis of RNA is essential to protein synthesis in the latter case.

f. In *Staphylococcus aureus*, RNA synthesis is not inhibited by azaguanine. The induced formation of various enzymes is nevertheless strongly inhibited by the presence of this analog (Creaser, 1955), and it is found that this compound is incorporated into RNA. The formation of  $\beta$ -galactosidase is far more strongly inhibited by this agent than is catalase, particularly at certain stages of bacterial growth. Gale has interpreted this to mean that existing RNA templates may be adequate for the synthesis of some enzymes, such as catalase, but that *de novo* RNA synthesis is essential for others, such as  $\beta$ -galactosidase. This difference may conceivably parallel the differences between constitutive and inducible enzymes.

Chantrenne (1956a) has observed the incorporation of adenine and uracil in yeast nucleic acids during the formation of catalase and some other enzymes which are induced by oxygen in a respiration-deficient, anaerobically grown yeast.<sup>1</sup> In this system the enzymes produced do not contribute to cell growth. In additional experiments, it is reported (Chantrenne, 1956b) that the increased incorporation of adenine caused by the presence of the inducer is observed even when enzyme formation is blocked by *p*-fluorophenylalanine, suggesting that the tooling of the enzyme-forming site may be separated from actual enzyme production.

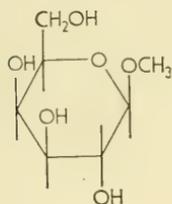
Finally, several reports have appeared indicating that polynucleotide fractions from induced cells may be added to uninduced cells in the absence of inducer and thereby stimulate enzyme production. Reiner and Goodman (1955) have reported that lactate-grown *E. coli* will produce gluconokinase if incubated with ATP and polynucleotide prepared from gluconate-grown cells. RNAase does not destroy the activity of the polynucleotide but renders it dialyzable. Kramer and Straub (1956) have observed that an RNAase-sensitive nucleic acid preparation from penicillin-induced *B. cereus* was capable of stimulating penicillinase production in uninduced organisms. In the latter case of the addition of the specific nucleic acid to the uninduced bacterium, enzyme production started at a high rate and decreased after 20 minutes, suggesting the possible degradation of the template during enzyme formation.

#### 4. On Induction

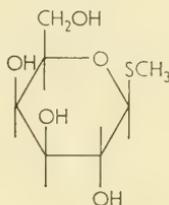
Several hypotheses have appeared on the nature of inducer action which relate the biosynthesis of the enzyme to the presence or activity of the enzyme. One of these supposes that an inducer is a substrate of the enzyme; another, that enzyme production is shifted toward synthesis by complexing with the enzyme and removing it from the synthetic system. The first of these hypotheses has been excluded by demonstrations that induction can occur under conditions in which the inducer is not metabolized. For example, thiomethyl- $\beta$ -D-galactoside and melibiose, inducers of  $\beta$ -galactosidase in *E. coli* strain ML, are not cleaved by the enzyme (Monod *et al.*, 1951). In the same study it was shown that a substance which complexes with the enzyme is not an inducer, thereby excluding the second so-called "mass action" hypothesis, at least in its simplest form. Phenyl- $\beta$ -D-thiogalactoside has such

<sup>1</sup>The effect of oxygen in stimulating cytochrome synthesis in anaerobically grown yeast is quite obscure at present. The synthesis of many other enzymes, e.g., enzymes of the tricarboxylic acid cycle, is also stimulated, and unless one conceives of an elaboration of all of these enzymes occurring by some special type of reverse sequential induction, it is difficult to classify this pattern of induced enzyme biosynthesis with the others discussed above.

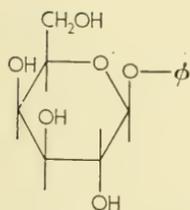
properties. Structures of these compounds and their activities are given in formula (IX).



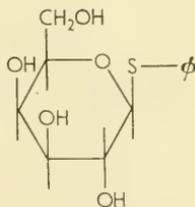
Methyl  $\beta$ -D-galactoside  
is hydrolyzed,  
is inducer.



Methyl  $\beta$ -D-thiogalactoside  
is not hydrolyzed,  
is inducer.



Phenyl- $\beta$ -D-galactoside  
is hydrolyzed,  
in a weak inducer.



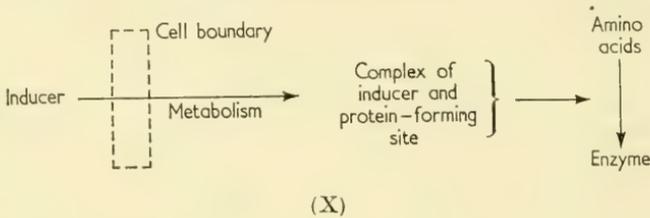
Phenyl- $\beta$ -D-thiogalactoside  
is not hydrolyzed, has a strong  
affinity for the enzyme,  
is not an inducer.

(IX)

It may be noted that although the phenylthiogalactoside is not an inducer in *E. coli*, it is an inducer of *B. megatherium* (Landman, 1957). In this organism, however, as in *E. coli*, the types of inducers suggest that inducer action depends on a combination with the enzyme-forming system rather than with the enzyme.

Using the penicillinase-forming system of *B. cereus*, Pollock (1950) was able to show that the induction of enzyme production was a catalytic phenomenon. In this system  $S^{35}$ -labeled penicillin was fixed specifically by the bacteria at  $0^{\circ}\text{C}$ . to the extent of about 80 molecules per cell. Such cells could be washed free of exogenous penicillin and would produce penicillinase at a constant rate in the presence of oxygen, glucose, and amino acids. This is the only instance known in which the presence of free inducer is not necessary for continued enzyme production; in other cases enzyme production stops when inducer is removed. The penicillinase was purified and its

activity was established on a molecular basis, i.e., its turnover number was determined. It was thereby shown that each molecule of penicillin fixed was not consumed in induction and could induce the formation of at least 10 molecules of enzyme. In this instance an inducer molecule appears to be metabolized and organized within an enzyme-forming site in such a way that the amino acids which are assembled emerge as a protein with a unique catalytic specificity. This phenomenon may be represented as shown in formula (X).



The linear rate of penicillinase synthesis in the *B. cereus* system and the linear "differential" rate of enzyme synthesis may be taken to suggest that, in induction, the protein-forming sites are fully formed and merely activated by the addition of the appropriately metabolized inducer. This interpretation has been questioned by Spiegelman and Campbell (1956), who have critically discussed the existing data. Their own genetic studies had led to the view that induction, at least in its early stages, involves an autocatalytic synthesis or activation of templates at the enzyme-forming site. It is considered to be significant in this regard that once enzyme formation has begun it becomes increasingly more resistant to ultraviolet irradiation than the effect of this treatment when inducer has just been added (Pollock, 1953; Halvorson and Jackson, 1956). This result implies that the process of organization of the enzyme-forming system is more sensitive to ultraviolet irradiation than the direct synthesis of enzyme from precursors. It is possible then that the nucleic acids are more concerned with the development of the former stage than in the latter. On the other hand, since nucleoproteins are more resistant to such irradiation than the free nucleic acids (Siegel *et al.*, 1956) this result may merely reflect the change in the template from a free to combined state.

##### 5. On Cell Permeability and Enzyme Synthesis

The analysis of the formation of enzyme-forming systems in microorganisms has recently been complicated by the discovery that an intracellular accumulation of inducer must precede enzyme synthesis and that the development of an accumulating system has in its turn all of the features of the induced biosynthesis of proteins. This discovery, although complicating some problems, clarifies others, such as the question of why many bacteria which

contain a functioning tricarboxylic acid cycle cannot handle exogenous components of the cycle until after a period of exposure to these substances (Karlsson and Barker, 1948; Repaske and Wilson, 1953). Davis (1956) has discussed the evidence which reveals this particular case as a problem in the inductive formation of a transport system. Thus, for adaptation to citrate utilization in *Aerobacter*, net protein synthesis is required and does not occur in a tryptophan-requiring mutant in the absence of the amino acid. Furthermore, growth in the presence of glucose inhibits the synthesis of the citrate-permeability factors, a particularly interesting point since it has long been known that glucose inhibits the induced biosynthesis of enzymes for many kinds of inducers and appears to implicate this effect in the initial stage of concentrating inducer.

Monod and his collaborators have studied the formation of permeability factors (variously called "y" factors or "permeases"<sup>1</sup>) in induction to  $\beta$ -galactosides (Monod, 1956; Rickenberg *et al.*, 1956). Using S<sup>35</sup>-labeled methyl  $\beta$ -D-thiogalactoside which induced  $\beta$ -galactosidase production but is not hydrolyzed by the enzyme, it was found that this material could be concentrated intracellularly to 4 % of the bacterial dry weight (100 times the concentration in the medium). The concentration was energy-dependent and was accompanied by an increase in oxidative metabolism (Kepes, 1957); it was inhibited by dinitrophenol or azide. Addition of the inhibitor after concentration released the inducer, as did other  $\beta$ -galactosides. The concentrated inducer did not appear to be in a metabolically altered state and the rate of its release from the cell was a function of intracellular concentration (Kepes and Monod, 1957). As noted earlier, the permease systems are also found in protoplasts and are therefore not cell wall components (Rickenberg, 1957).

Two classes of mutants unable to use lactose as carbon sources (lac-mutants) could now be distinguished. These were (1) the "absolute" mutants, which are unable to produce the  $\beta$ -galactosidase, but are able to develop the concentrating system and (2) "cryptic" mutants, which produce normal amounts of enzyme in the presence of large exogenous concentrations of the methylthiogalactoside (but not lactose), but cannot develop the permease for  $\beta$ -galactosides, and thereby lack the ability to handle lactose effectively. The existence of these classes of mutants is thought to be a relatively general phenomenon.

The permease system must be induced before galactosidase production. If glucose is normally added simultaneously with inducer, neither permease

<sup>1</sup> Although the name "permeases" has been given to the concentrating systems, it is not known that these systems are indeed protein catalysts. Accordingly, it has been suggested by some workers that the use of the term "permease" may be somewhat premature.

nor enzyme is made. If the permease is preinduced and glucose is added in the presence of low amounts of inducer, enzyme formation is not affected for many generations, since preinduced bacteria will be able to maintain a high inducer concentration and will make permease and galactosidase at a high rate despite the presence of inhibitory glucose. The permease systems once formed thus tend to perpetuate themselves under suitable conditions. Pardee (1957) has recently shown that the formation of the galactoside-permease but not of  $\beta$ -galactosidase can be induced by certain  $\alpha$ -galactosides in *E. coli* strain B, but not in strain ML. Stoeber (1957) has recently described a  $\beta$ -glucuronide-permease distinct from the galactoside system.

G. N. Cohen and Rickenberg (1956) have shown the existence of a series of specific systems (presumably permeases) for the reversible concentration of exogenous amino acids, prior to incorporation into proteins (see also Britten *et al.*, 1955). It is thought that each amino acid may have its specific permease. A number of interactions of amino acids inhibitory to growth may be explained by their effects on these systems. As with other permease systems, the oxidation of glucose inhibits penetration of amino acids into bacteria (Gale, 1947; Mandelstam, 1956a). The latter worker has also shown that the penetration of basic amino acids is competitively inhibited by the corresponding diamines (Mandelstam, 1956a,b). A review of bacterial permeases has recently appeared (Cohen, G. N., and Monod, 1957).

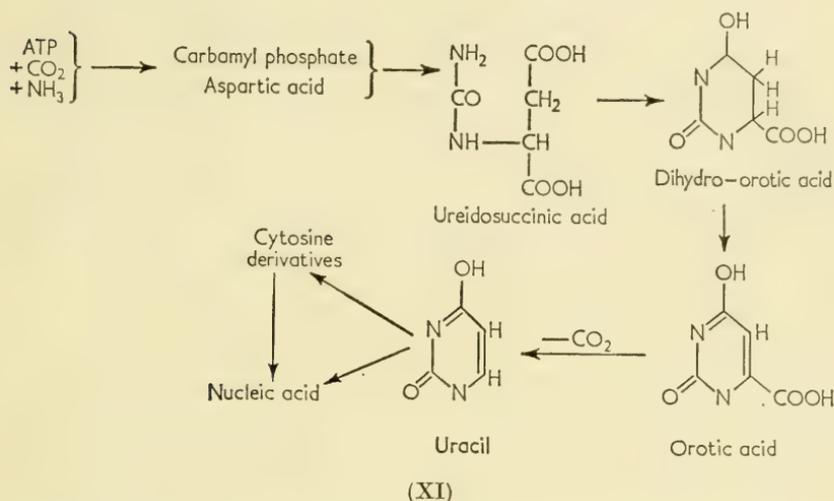
### 6. On the Inhibition of Enzyme Formation

As we have seen, inducers need not be substrates, but most often a substrate of an inducible enzyme is structurally very similar to an inducer. In addition, in a given sequence of reactions, a precursor or a reaction product will also resemble the substrate structurally and the latter, particularly, may even act as an inhibitor of induction. As an instance in which a precursor is inhibitory in a reaction sequence may be mentioned the conversion of dehydroshikimic acid  $\longrightarrow$  shikimic acid  $\longrightarrow$  phosphoshikimic acid  $\longrightarrow$  aromatic amino acids. In this system the accumulation of dehydroshikimic acid inhibits the subsequent metabolism of shikimic acid.

Many cases are known in which reaction products inhibit enzyme formation. This phenomenon is most readily demonstrated for constitutive systems. Thus, the synthesis of tryptophan desmolase in *Aerobacter aerogenes* is strongly inhibited by both tryptophan and by indole (Monod and Cohen-Bazire, 1953) and the synthesis of methionine synthetase in *E. coli* is inhibited by the presence of methionine (Cohn *et al.*, 1953a). Such effects take on the aspect of negative feed-back systems and have the general consequence of preventing a cell from forming an enzyme when it is not needed.

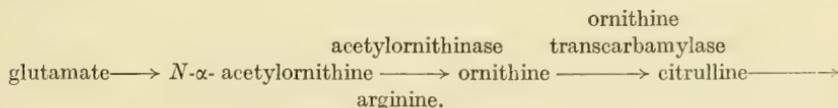
Yates and Pardee (1956) have particularly explored this phenomenon for the control of pyrimidine biosynthesis among pyrimidine-requiring mutants of *E. coli*.

Thus, in the sequence studied by these authors, as shown in formula (XI)



mutants were obtained which required uracil or cytosine for growth. In the presence of  $10^{-5} M$  concentrations of these pyrimidines, the production of ureidosuccinate was inhibited, as well as the subsequent products, dihydro-otrotic acid and orotic acid. In one mutant the presence of orotic acid inhibited formation of dihydro-otrotic acid. Cytosine nucleosides and nucleotides were found to inhibit the condensation of carbamyl phosphate and aspartic acid to form ureidosuccinic acid. In ultraviolet irradiated cells, in which the cytosine compounds were not removed into nucleic acid, the formation of ureidosuccinate was inhibited. In these systems there was apparently inhibition of both enzyme and of its formation.

Similar phenomena are known in the area of purine and amino acid biosynthesis. The sequence of arginine biosynthesis in *E. coli* may be represented as follows:



The presence of exogenous arginine represses the formation of acetylornithinase in a mutant blocked in the formation of  $N\alpha$ -acetylornithine, although the presence of the latter compound induced the formation of the enzyme (Vogel, 1957).

Most recently it has been shown that the synthesis of ornithine transcarbamylase is also inhibited by arginine in a mutant blocked in the conversion of acetylornithine to ornithine. If such a mutant is grown under conditions in which arginine is immediately assimilated into protein, i.e., in a chemostat in which the supply of arginine is carefully regulated, the amount of the transcarbamylase formed attains a level 25 times that of the steady state concentration in the wild type bacterium. Thus, wild type cells have a markedly greater potential than is permitted to operate for the synthesis of enzymes controlling the production of essential constituents; these potentials are repressed by negative feedback mechanisms. In the absence of such repression the initial rate of synthesis can be far more rapid than that of cell mass; a constant differential rate of synthesis obtains only when the accumulation of product and inhibition of enzyme synthesis establish steady state conditions (Gorini and Maas, 1957).

### 7. *Inducible Phenomena in Higher Forms*

Knox *et al.* (1956) have written an extensive summary of enzymatic and metabolic adaptations in animals. In many instances recorded by these authors the metabolic change resulting from some environmental effect may well represent phenomena of induced biosyntheses of enzymes. Thus, one of the earliest examples is the formation of  $\beta$ -galactosidase in the pancreas of dogs after ingestion of lactose. On the other hand, the ability of rats to handle galactose is not affected by galactose intake, and this ability is maintained despite a prolonged absence of galactose from the diet (Feigelson and Conte, 1954).

A most careful demonstration of the induced biosynthesis of an enzyme in the mammal has been that of the increase of liver tryptophan peroxidase in response to the injected tryptophan and some other substances, such as adrenalin or histamine (Knox and Mehler, 1951). In rats this phenomenon proved to be due to two independent mechanisms, of which one was specific for tryptophan and resembled the mechanism observed in microorganisms. The other relatively nonspecific increase in the enzyme arose as a result of stimulation of the pituitary-adrenal system and was abolished by adrenalectomy. The latter revealed a hormonal control, not of enzymatic reactivity, but of the amount of an enzyme elaborated (Knox, 1951). In this instance the increase in level of tryptophan peroxidase is attributable to cortisone release from the adrenal (Knox and Auerbach, 1955).

The instances of metabolic adaptation surveyed by Knox *et al.* (1956) have not yet been reproduced in isolated cell systems in tissue culture, in which a constant genetic background can be maintained. There is little doubt but that the major advances in technique in tissue cultures will produce investigations of this character. In one study of this type, Lieberman (1957) explored

the effect of growing mouse fibroblasts (strain L) in suspension in the presence of DPN. Such cells have a considerably depressed DPNase activity, apparently resulting from the formation of stable, inactive, enzyme-nicotinamide derivative complexes. It appeared that exogenous DPN could enter these cells. Other enzymes levels were not affected and, indeed, there was no indication that the synthesis of DPNase was affected, since DPNase disappeared in far less than a division time. Thus, the first examination of mammalian systems from this point of view has revealed a new type of control of enzymatic activity in these cells. This instance may be of particular interest to our understanding of the origin of enzymes, such as neuraminidase, which have only been found in infected cells. Does the virus introduce the enzyme which, in cleaving substrate, releases inhibited neuraminidase? Or does virus infection in some way lead to the induction of the synthesis of the enzyme? The very formulation of these problems in these terms should facilitate their analysis.

## VI. BIOCHEMICAL MECHANISMS OF POLYMER FORMATION

The clarification of mechanisms of polymer formation has barely begun. Only the most recent textbooks in biochemistry contain significant information in this area. However, a number of reviews are useful in surveying recent thought and experimentation with respect to problems of the synthesis of protein (Borsook, 1954, 1956; Fruton, 1954), of nucleic acid (Ochoa and Heppel, 1957; Kornberg, 1957a,b), of polysaccharides (Hassid, 1954; Kalckar, 1954; Stacey, 1954; Edelman, 1956), and of phospholipids (Kennedy, 1957).

As noted in earlier sections, the formation of the complex substances indicated above is dependent upon active metabolism. More particularly, the formation of the peptide bonds of proteins, the phosphodiester bonds of nucleic acids, the glycosidic linkages of polysaccharides, and the ester linkages of the phospholipids requires the intermediary development of high-energy bonds in compounds such as adenosine triphosphate (ATP) and acyl-S<sub>Co</sub>A. The concept that such bonds comprise the operating currency and driving substances of the energy-requiring reactions of the cellular economy was developed largely by Lipmann (1941) and its material bases have been growing continuously since his initial presentation of the theory.

It should be noted that a "high-energy bond" has a different significance from the concept of "bond energy" in the field of energetics. The latter signifies the energy which must be introduced into a molecule to break a bond between two atoms. Thus, the disruption of a P to O bond in ATP may require an energy input of 50 to 100 kilocalories per mole. On the other hand, the biochemist is concerned primarily with the change in chemical

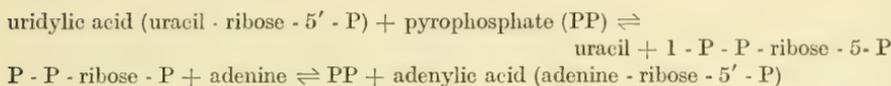
potential, or  $\Delta F$ , when the transfer of the phosphoryl group containing this phosphorus is effected from ATP to water or some other acceptor, such as glucose, a nucleotide, etc. In this case, the reaction proceeds spontaneously, and under standard conditions the reaction results in a change of chemical potential or of free energy ( $\Delta F$ ) of  $-7$  kilocalories per mole.



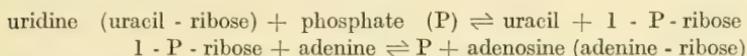
In such reactions, then, we are concerned primarily with group transfers and indeed the energetics of polymer biosynthesis are primarily concerned with group-transfer potentials. Although the reaction presented above is essentially irreversible in practice, group-transfer reactions are frequently readily reversible. The extent of such reversibility is determinable from the relation of  $\Delta F$  to the equilibrium constant ( $K$ ) of any reaction, i.e.,  $-\Delta F = RT \ln K$ . The energetics of group transfer has been clearly discussed by Klotz (1957).

Compounds embodying high group-transfer potentials serve to activate amino acids, nucleotides, sugars, fatty acids, etc., and thereby permit their utilization in biosynthesis. Compounds such as ATP arise in the breakdown of foods; their formation constitutes mechanisms for the conservation of the energy contained in the bonds of these nutrients. Thus, the conversion of glucose to 2 moles of lactate via the Embden-Meyerhof pathway of anaerobic glycolysis leads to the net production of 2 moles of ATP. The oxidation of acetate to 2 moles each of  $\text{CO}_2$  and  $\text{H}_2\text{O}$  leads to the formation of 15 moles of high-energy phosphate. General aspects of these routes of energy metabolism are discussed extensively in modern texts of biochemistry (cf. Fruton and Simmonds, 1953; White *et al.*, 1954); comparative aspects of these reactions in animals and microorganisms have been summarized by Krebs (1954).

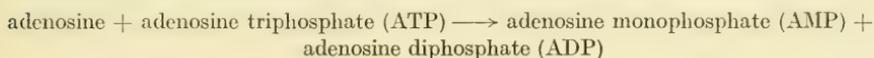
Assuming the ready availability of some high-energy intermediates, such as ATP, we shall outline the known mechanisms of activation of intermediates of polymer biosynthesis and proceed to a brief summary of the apparent course of these syntheses. However, the activation of components derived from nutrients does not always require the degradation of metabolites to the smallest possible components and the resynthesis from scratch of the essential activated molecules. Thus, many group-transfer reactions, e.g., phosphorylytic, pyrophosphorylytic, and hydrolytic reactions, are useful in the scavenging of transferable groups from foods in the fabrication of essential metabolites suitable for polymer biosynthesis. For example, an ingested nucleic acid may be degraded to nucleotides, perhaps providing a surfeit of uridylic acid, when adenylic acid is needed for biosyntheses. A pyrophosphorylytic cleavage of uridylic acid permits the formation of a pyrophosphoryl sugar phosphate which can react with adenine to form the required nucleotide:



In a variant of this scavenging reaction, if uridylic acid has been hydrolyzed to the nucleoside, uridine, the ribose portion can also be shuttled about by a phosphorylytic mechanism which can conserve the special properties of the ribosyl linkage. Thus:



In this case, ATP is necessary in the conversion of the nucleoside, adenosine, to the nucleotide via an adenosine kinase:



It is of the utmost interest that many compounds, e.g., the tri- and diphosphates of the ribose nucleosides, important in the activation of other molecules, are themselves the activated intermediates of polymer synthesis. It follows, therefore, that by affecting the utilization of one of these compounds it is sometimes very difficult to separate relatively distant areas of metabolism. For example, the synthesis of cerebroside in brain tissue requires galactose derivatives, the activation of which requires uridine triphosphate and other uridine derivatives. Inhibition of RNA synthesis by the use of some uracil analog may well affect cerebroside formation, as well as polysaccharide synthesis and protein synthesis. Furthermore, since uracil is the precursor for cytosine, whose nucleotide derivatives are active in phospholipid biosynthesis, an effect may also be obtained in this area of metabolism. It is evident, then, that problems of polymer biosynthesis cannot be too readily compartmentalized as a consequence of the existence of exclusive systems of reactions. Quite the contrary, we are confronted by an interpenetrating network of related reaction systems whose existence compels the development of the most detailed information, if the phenomena of cellular biosyntheses are to be understood and are to be controlled. If such information is available, it may become possible to make use of the special properties of these reaction systems which relate to specific aspects of metabolism.

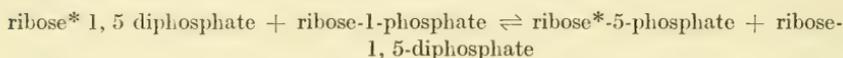
### *A. Phosphate Transfer*

By far the major part of activation reactions involves the utilization of phosphate compounds. The role of inorganic phosphate was discovered by Harden and Young (1905) in the fermentation of glucose in yeast extracts. Since that finding, it has been learned that almost all, if not all metabolites, are formed or function as phosphate derivatives or in association with such

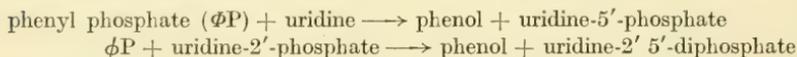
compounds. Although we know of instances, e.g., acetyl-SCoA, in which important facets of metabolism appear to bypass phosphate metabolism, such known reactions are still proportionately few and, in any case, soon merge with aspects of phosphate metabolism in the metabolism of the living cell. The mechanisms of the continuous attachment and transfer of phosphate must evidently be of great concern in our understanding of biological systems.

### 1. *Hydrolases and Transferases*

Mention has been made above of mechanisms by which inorganic phosphate and pyrophosphate may be used to form low molecular organic phosphates. Numerous examples will be given below of these reactions in coenzyme and polymer formation. In addition, numerous enzymatically catalyzed reactions of phosphate exchange have been recognized among the low molecular organic phosphates. For example, ribose 1, 5 diphosphate will transfer the 1 phosphate to the 5 position of ribose-1-phosphate, as follows:



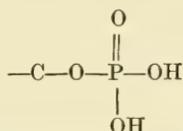
An enzyme can thereby facilitate the transfer of phosphate to the primary hydroxyl of a sugar moiety. It can be imagined that if the transfer of the group had been effected to water, the enzyme would be considered to be a phosphatase, which is commonly thought of as a hydrolytic enzyme. Thus, the type of reaction produced would be determined in part by the specificity of the requirement of the enzyme for certain acceptors. Conversely, then, it can be supposed that some hydrolytic enzymes might effect group transfers to acceptors other than water. Such reactions have indeed been found and many so-called phosphatases are now known to catalyze phosphoryl transfer, as summarized by Axelrod (1956b). For example, alkaline phosphatase can transfer phosphate from a sugar phosphate, such as glucose-6-phosphate, to alcohols and phenols. In extending this phenomenon, it has been found that phosphates can be transferred from phosphate esters of various alcohols to nucleosides and nucleotides by phosphotransferases of a wide variety of tissues and organisms:



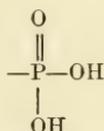
Although the role of such systems in metabolism is not clear, it is significant that 5' nucleotides can serve as phosphate donors and, in transfer of the phosphate from these nucleotides to nucleosides with plant and bacterial enzymes, the products are predominantly 5' nucleotides (Tunis and Chargaff, 1956). Pancreatic ribonuclease, a diesterase, also effects nucleotide transfer as a result of phosphate transfer, as well as hydrolytic cleavage. The existence

of the former reactivity for this enzyme thereby poses the possibility of its role in syntheses and rearrangements of polynucleotide chains.

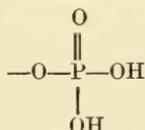
Two types of phosphate transfer are recognized at present. In examining the formula



it can be seen that two bonds may be severed, that between O and P to liberate the phosphoryl moiety



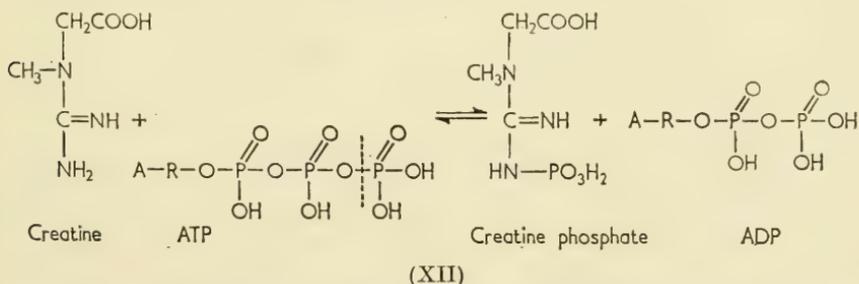
and that between  $\text{---C}$  and O to liberate the phosphate group



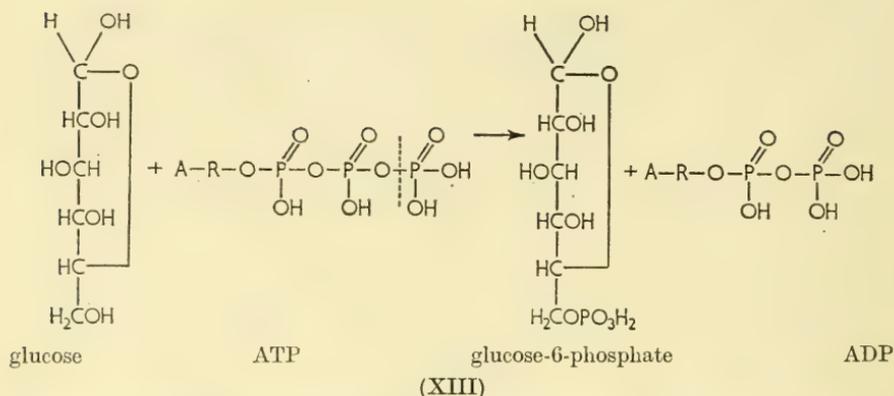
The use of  $\text{O}^{18}$ -labeled compounds in the cleavage of the bond and the detection of the isotope in the product have permitted the analysis of the reaction type. Most phosphatases have been shown to effect phosphoryl transfer. On the other hand, muscle phosphorylase and sucrose phosphorylase are examples of cleavage at the C—O bond.

## 2. Kinases

With compounds such as creatine phosphate, in which the moieties are linked through an N-P linkage, obviously only phosphoryl transfer is possible. Thus, the kinase, creatine transphosphorylase, affects phosphoryl transfer in the reaction, as shown in formula (XII).

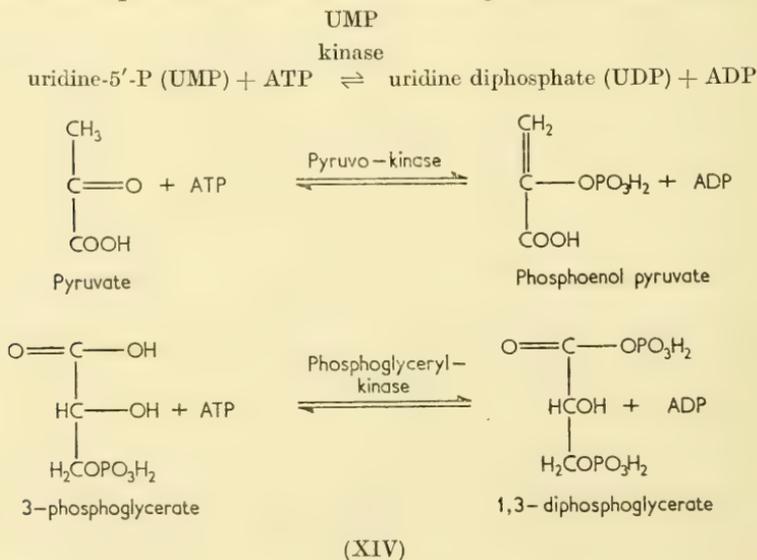


Indeed, all kinases tested by the  $\text{O}^{18}$  method appear to catalyze this type of phosphoryl cleavage, as in the hexokinase reaction shown in formula (XIII).



As a result of this reaction, glucose is converted to a charged derivative, a form in which association with enzymes is thereby facilitated. In this case, ATP is a phosphoryl donor to an alcohol and fulfills a role comparable to the phosphorylation of adenosine to adenylic acid, mentioned earlier. Compounds in which the phosphoryl group is combined to alcohols or hemiacetals, as in the formation of sugar-1-phosphates, e.g., galactose-1-phosphate, are considered to possess relatively low group-transfer potentials. Their formation from ATP therefore involves relatively irreversible reactions.

The reactions of ATP to form pyrophosphates, enol phosphates, and carboxyl phosphates are readily reversible, a fact which relates to the relatively high group-transfer potentials of the latter compounds. In some cases the reversibility is more easily demonstrated by the addition of coupled reactions. Examples of the initial reactions are given in formula (XIV).



Enzymes have been found for converting two moles of nucleoside diphosphate to one mole of triphosphate and one mole of monophosphate. The first of these was myokinase which catalyzes the reversible reaction  $2 \text{ADP} \rightleftharpoons \text{AMP} + \text{ATP}$ . It can be seen that the forward reaction is merely the reversal of a typical kinase-catalyzed reaction in which ATP is a phosphoryl donor. Similar reactions are known for UDP, guanosine diphosphate (GDP), and cytidine diphosphate (CDP), as in the system:  $2 \text{UDP} \rightleftharpoons \text{UMP} + \text{UTP}$ . Thus, the triphosphates of each ribose nucleotide can be formed and in turn act as a phosphoryl donor in kinase reactions. Deoxynucleoside monophosphates can also be converted to di- and triphosphates in enzyme systems containing ATP as a primary phosphoryl donor. The mechanisms of the latter reactions have not yet been determined in detail.

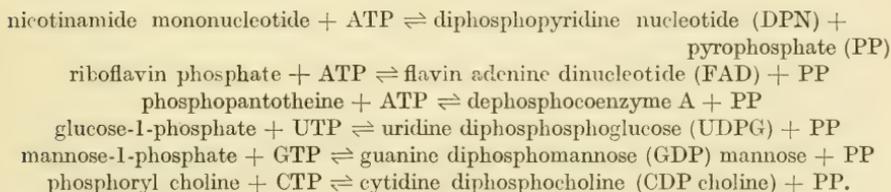
In each of the cases indicated above, the terminal phosphoryl group of the triphosphate has been transferred. In some instances a pyrophosphoryl group is itself transferred, as in the reaction:



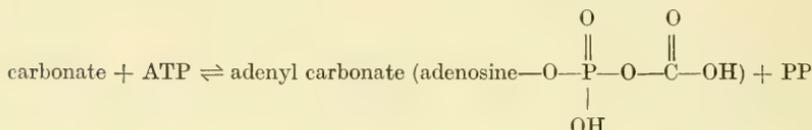
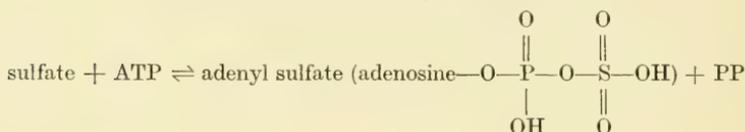
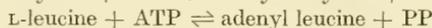
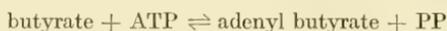
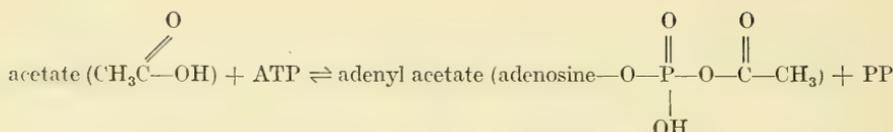
in which the activated sugar derivative essential to several routes of nucleotide synthesis is generated. The role of this compound in the scavenging recovery of ingested bases has been recorded earlier; other aspects of its essential role in *de novo* metabolic paths will be described below.

### 3. *Pyrophosphorylases*

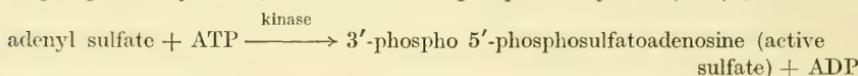
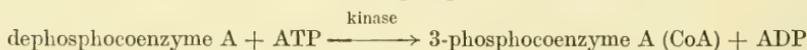
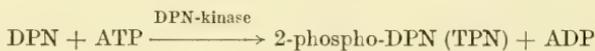
In the formation of the coenzymes, a reaction occurs in which the nucleotide monophosphate is transferred from a triphosphate to form a new pyrophosphate linkage and inorganic pyrophosphate. The following exemplify this reaction:



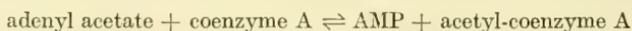
It can readily be seen in these instances that the reversal of these reactions constitutes a pyrophosphorylytic cleavage of a pyrophosphate to form a triphosphate and a monophosphate metabolite. All four ribosenucleoside triphosphates participate in these reactions and are relatively specific for particular acceptors. Comparable reactions to form mixed phosphoanhydrides are now known for fatty acids, amino acids, sulfuric acid, and carbonic acid, as follows:



In many of these cases, the active coenzyme of a reaction system is a further phosphorylated derivative of these metabolites, as produced in the reactions:



In other instances, the adenylyl anhydride is a precursor to the formation of the immediate metabolite, as in the reactions in which the acyl moiety is transferred from phosphoanhydride to form the reactive thioester.

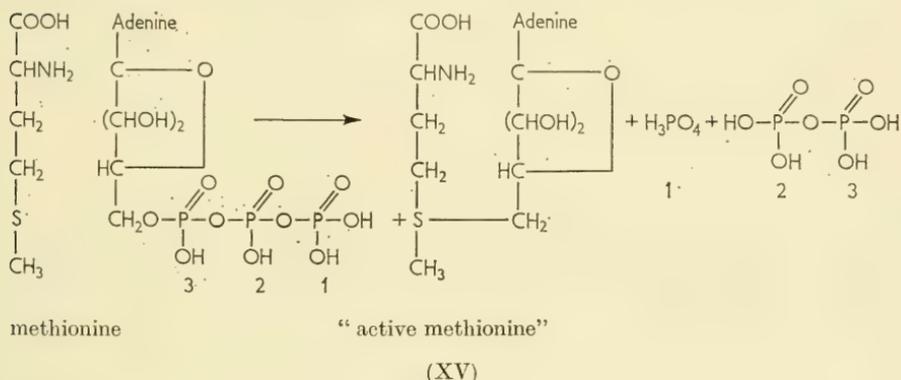


The ready reversibility of some of the reactions listed above attests to the high group-transfer potential of the activated compounds involved, i.e., they are designated as high-energy compounds.<sup>1</sup>

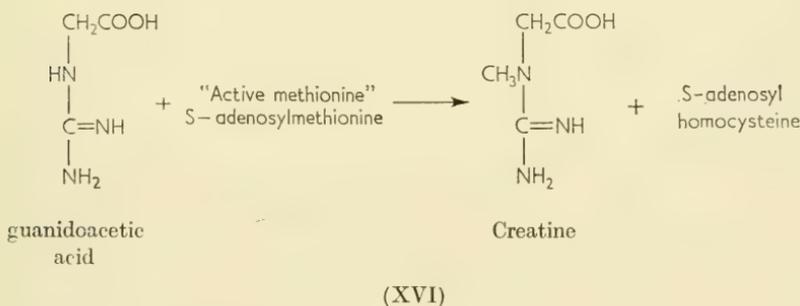
We can note that the reactions described for acetate and butyrate constitute the probable mechanisms of their *in vivo* activation in preparation for various acetylations and acyl transfers. These will be summarized below, as will the subsequent reactions of compounds such as UDPG, CDP choline, active sulfate, adenylyl carbonate, and adenylyl leucine (leucine adenylylate).

<sup>1</sup> Gulick (1955) has suggested that life originated under conditions in which phosphorus was only partially oxidized, permitting the formation of compounds such as guanidine phosphite. Dehydrogenation in this instance might then lead to the formation of a high energy compound, phosphoguanidine, formally analogous to the phosphagens, phosphocreatine, and phosphoarginine.

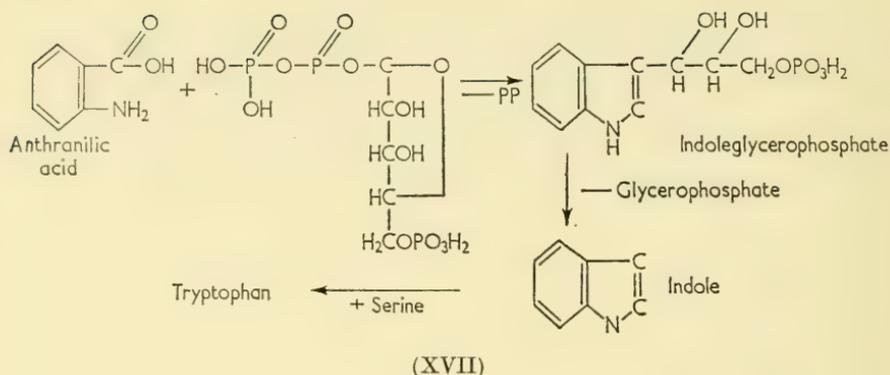
Other related reactions have been summarized by Kornberg (1957b). Of these the most important include the formation of active methionine, or S-adenosylmethionine, first described by Cantoni (1953), in a reaction liberating P from the terminal group of ATP and pyrophosphate from the innermost phosphates of the nucleotide (Cantoni and Durell, 1957). The details of this complex reaction are presented in formula (XV), and



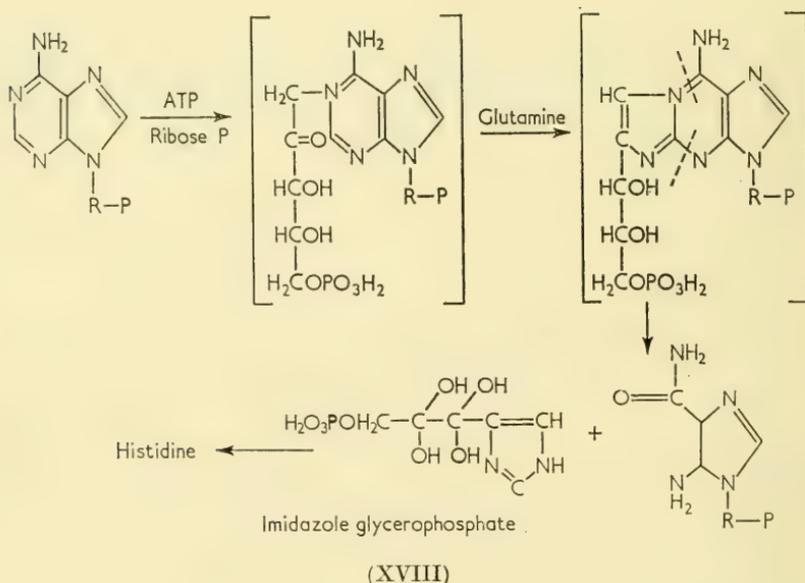
have not yet been thoroughly clarified. However, active methionine embodying a methyl sulfonium group of high group-transfer potential is now recognized to be an active methyl donor in the formation of a wide variety of N- and S-methyl-containing compounds, as in the reaction to form creatine shown in formula (XVI).



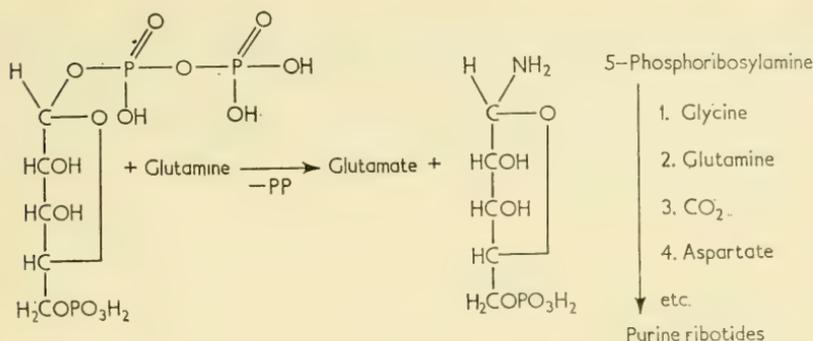
In the formation of tryptophan and histidine, reactions have recently been described whereby pyrophosphoryl ribose-5-phosphate participates in the formation of the indole and imideazole rings, respectively. Yanofsky (1957) has demonstrated a series of reactions, beginning with this compound and anthranilic acid, as shown in formula (XVII).



In the formation of histidine (Moyed and Magasanik, 1957) the reaction involves the participation of adenylic acid and glutamine, as shown in formula (XVIII).



Pyrophosphoryl ribose phosphate also reacts with glutamine to form the starting phosphoribosyl amine on which purine bases are constructed, as shown in formula (XIX).

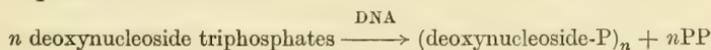


It is evident then that inorganic pyrophosphate is an important intermediary metabolite. Pyrophosphatases exist to cleave pyrophosphate to inorganic phosphate. However, the conservation of the energy of this linkage is desirable and a number of reactions may exist to fulfill this requirement. For example:  $PPP + AMP \rightleftharpoons PP + ADP \rightleftharpoons P_1 + ATP$ . The equilibrium would shift to the right as ATP is used in other metabolic events.

During the respiration of rat liver homogenates, inorganic pyrophosphate is produced from orthophosphate (Cori *et al.*, 1951). The PP formed does not appear to be a direct product of oxidative phosphorylation. The PP-forming system is primarily in the microsomal fraction and derives the pyrophosphate from ATP and other nucleoside triphosphates (Kenney *et al.*, 1957). It has been suggested that in this system the primary product is the nucleoside monophosphate:  $ATP \longrightarrow AMP + PP$ .

This microsomal system is clearly different from the system postulated by Kornberg (1957b) as a major source of PP. He has supposed that this metabolite may arise mainly from the reaction of ATP with the coenzyme-forming pyrophosphorylases. However, these enzymes are mainly concentrated in nuclei. It is possible that the ATP essential for these systems is generated almost entirely via anaerobic glycolysis rather than from oxidative phosphorylation.

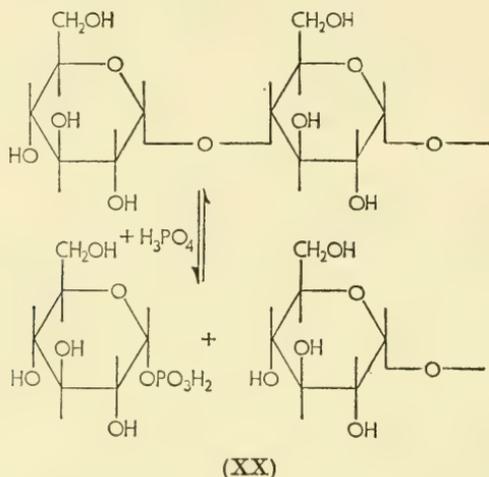
Of particular interest to our discussion is the recent discovery of the *in vitro* synthesis of DNA by a pyrophosphorylase (Kornberg *et al.*, 1956; Kornberg, 1957a,b). This reaction, to be discussed in greater detail below, may be represented as follows:



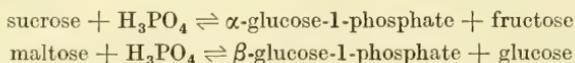
#### 4. Phosphorylases

The Coris (1936) discovered the conversion of glycogen to  $\alpha$ -D-glucose-1-phosphate by muscle phosphorylase in the presence of inorganic phosphate.

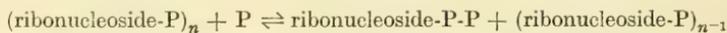
In this reaction, glucose held in 1, 4 $\alpha$ -glycosidic linkage is transferred to inorganic orthophosphate, thereby preserving the energy of the organic linkage. The group transfer potential of the C—O—P linkages of glucose-1-phosphate is approximately the same as that of the glucosidic linkages of the polysaccharides; glycogen (glucose)<sub>n</sub> + P  $\rightleftharpoons$   $\alpha$ -glucose-1-P + (glucose)<sub>n-1</sub>, as shown in formula (XX).



Phosphorylytic cleavages by specific enzymes have been observed for only a few other saccharides, such as sucrose and maltose:

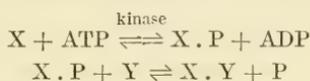


Comparable reactions also exist for the cleavage of N-glycosides, which exist in purine and pyrimidine nucleosides. Separate enzymes are responsible for these activities; indeed, in *E. coli*, separate pyrimidine nucleoside phosphorylases exist for the ribosides and deoxyribosides. Of particular significance to our discussion has been the recent discovery of the phosphorylytic cleavage of the nucleotide phosphodiester of RNA. The equation for this reaction which will be considered below in greater detail can be represented as follows:

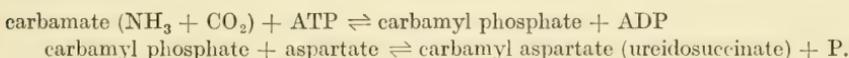


It is clear then that the phosphorylytic and pyrophosphorylytic reactions indicated above are of the greatest importance. They are involved not only in the mobilization and activation of small molecules so that they may participate in polymer syntheses but also in the terminal steps of the polymer biosyntheses themselves.

Mention may be made of a group of reversible phosphorylytic reactions of this type in which ATP is in equilibrium with ADP and P. It may be supposed that kinases and phosphorylases act in sequence as follows:



For example:



Intact mitochondria have an apparent adenosine triphosphatase which catalyzes an exchange of the terminal phosphate of ATP and inorganic phosphate, possibly via a phosphorylytic mechanism. A similar soluble enzyme has been isolated from these structures (Plaut, 1957). It is suspected that such an enzyme may be involved in the phenomenon of oxidative phosphorylation.

### 5. On Phosphoproteins

A number of proteins have been found to contain phosphorus bound directly to amino acids. For example, for many years casein has been known to contain P bound to the primary alcohol of serine, i.e.,  $\text{HOOCCHNH}_2\text{CH}_2\text{OPO}_3\text{H}_2$ . Most recently, serine has been implicated in the active site of several esterases and proteases. Most significantly, serine phosphate has also been isolated from hydrolyzates of yeast hexokinase and from phosphoglucomutase, as summarized by Kennedy and Koshland (1957). In the case of hexokinase, it is supposed that the phosphorylated protein is in fact the active form of a phosphotransferase (Ågren and Engstrom, 1956a) as the phosphorylated form of phosphoglucomutase has been shown to be (Najjar and Pullman, 1954; Sidbury and Najjar, 1957). Engstrom and Ågren (1956) have similarly isolated radioactive phosphoserine from phosphorylase incubated with glycogen and radioactive  $\text{P}^{32}$ . Ågren (1956) and Ågren and Engstrom (1956b) have also found major fractions of the phosphoproteins in the cell walls of *E. coli* and erythrocytes. It seems possible that these cell wall proteins, embodying phosphoserine, are the active metabolic centers of phosphate transfer for many enzymatic systems.

## B. Polysaccharide Biosynthesis

### 1. Phosphorylases and Other Enzymes

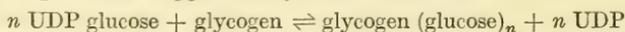
The reversible phosphorylase reaction of glycogen biosynthesis has been noted in a previous section. The enzyme occurs in many animal tissues, higher plants, and some bacteria, and does not act unless a small amount of

starch, glycogen, or other dextrin is present as a primer. Phosphorylases of different origin possess different primer requirements. The primer must be larger than a disaccharide and provides terminal nonreducing end groups to which glucosyl moieties may be added, permitting the stepwise extension of the chain. These phosphorylases are exceedingly specific for the substrate,  $\alpha$ -D-glucose-1-phosphate. However, the mechanism of activation of the glucosyl moieties is far from clear. Pyridoxal phosphate, of unknown function, has recently been identified in muscle phosphorylase (Cori and Illingworth, 1957), an enzyme which also contains bound adenylic acid.

However, glycogen is a complex, branched polysaccharide, composed of both straight chain amylose molecules in which glucosidic linkages are of the maltosidic  $\alpha$  1, 4 type, sensitive to phosphorylase, and phosphorylase-insensitive branch points in which the glucosidic linkage is of the 1, 6 type. Purified phosphorylase synthesizes amylose chains alone from  $\alpha$ -glucose-1-phosphate. In the degradation of glycogen, phosphorylase will degrade amylose chains to the branch points. An amylo-1, 6-glucosidase is then required to cleave the 1, 6 bonds; such cleavage then provides an entry for the further phosphorylytic attack of underlying amylose chains. Thus, the polysaccharide contains many layers of amylose chains which are approached by degradation with successive treatments of phosphorylase and glucosidase. With this technique it was found that the peripheral glucose moieties of the glycogen of animals (Stetten *et al.*, 1956) and bacteria (Holme and Palmstierna, 1956) are metabolically more active than internal glucose.

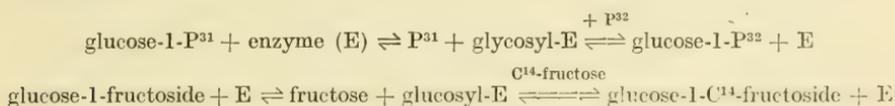
In addition to the phosphorylases, two other types of enzymes have been implicated in glycogen synthesis and in polysaccharide synthesis in general. The first of these are the transglycosidases in which sugar phosphates are not intermediates. The second group involve the intermediation of the coenzymes containing diphosphouridine.

As will be seen below, glycosyl transfer of hexoses, amino sugars, uronic acids, and pentoses may be effected via the intermediation of nucleoside diphospho-1-glycosides. One of the most important of this group of metabolites, which were discovered by Leloir and his collaborators, was uridine diphosphoglucose (UDPG). Leloir and Cardini (1957) have recently described the transfer of glucose from UDPG to glycogen in the presence of a liver enzyme. That the reaction does not involve the intermediary generation of glucose-1-phosphate is suggested by the stoichiometric formation of UDP.



The analysis of the action of sucrose phosphorylase revealed the transglucosidatic activity of this enzyme (Doudoroff *et al.*, 1947). This enzyme, isolated from *Pseudomonas saccharophila*, was capable not only of exchanging the phosphate of glucose-1-phosphate with inorganic  $P^{32}$  without releasing glucose, but could also exchange the unlabeled fructose of sucrose with

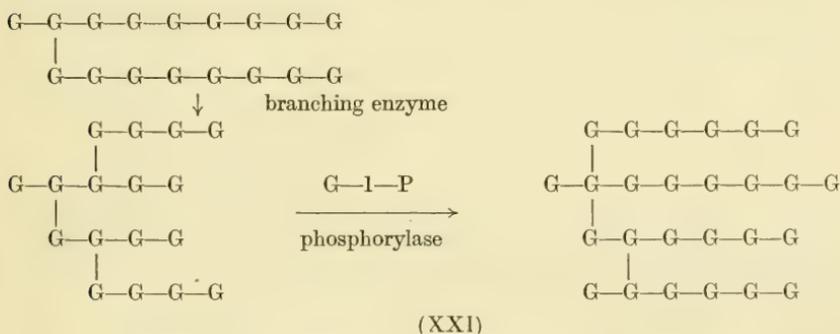
$C^{14}$ -fructose in a phosphate-free medium. These reactions may be written as follows:



Thus, glycosyl-enzyme complexes are considered to be important intermediates in the action of this and other transglycosidases.

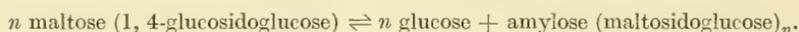
## 2. Transglycosidases and Hydrolases

Extracts of animal organs contain a branching enzyme, which, acting in conjunction with phosphorylase, produces a branched polysaccharide resembling glycogen. The branching enzyme removes short terminal chains of 1, 4 linked glucose units and links them to free primary hydroxyls at the  $C_6$  of glucose moieties within amylose chains, thereby establishing branch points and multiple sites for the further action of phosphorylase, as shown in formula (XXI).



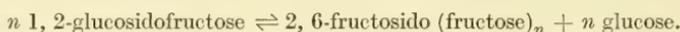
A somewhat similar branching enzyme, Q enzyme, is also known in bacteria (Hassid, 1954). As summarized by Hassid (1954) and Kalekar (1954), a large number of nonphosphorylytic transglycosidases have now been recognized. These include *trans-O*-glycosidases and *trans-N*-glycosidases, in which glycosyl-enzyme complexes are believed to be the critical intermediates. In the former group are enzymes which operate on disaccharides and longer polysaccharide chains. Of the enzymes operating on disaccharides may be listed a number of hydrolases, such as invertase and lactase, which may not only cleave their respective substrates to free monosaccharides but are also capable of transferring one component to another mole of acceptor, e.g., invertase will cleave sucrose (1, 2 glucosyl fructoside) to glucose and fructose, but is also capable of acting both as a transfructosidase and a transglucosidase. Preparations of these enzymes will also transfer moieties to amino

sugars, which then may also become components of oligosaccharides (Srinivasan and Quastel, 1958). The enzyme, amyloamylase, catalyzes the following reaction, which may build very long amylose chains:

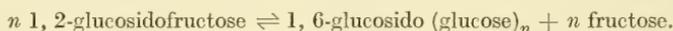


Transglycosidases operating on polysaccharides include both transglucosidases and transfructosidases. In the former series, enzymes probably exist to transfer glucose to 1, 3, 1, 4, 1, 5 and 1, 6 linkages from  $\alpha$ - or  $\beta$ -1, 4 linkages and from 1, 2-fructosides.

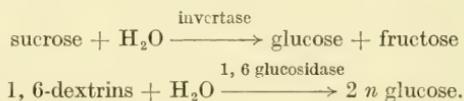
Among the transfructosidases, transfer of fructosyl moieties are known to occur from the 1, 2 to 2, 6 linkages as in the reaction catalyzed by levan sucrose.



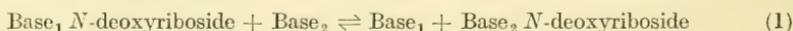
The corresponding transglucosidation is catalyzed by dextran-sucrase.



In the latter case, many kinds of glucosyl acceptors can initiate the formation of alternative chains. In the case of the hydrolases, water acting as an acceptor can block the formation of chains.



The counterpart of the purine and pyrimidine nucleoside phosphorylases exists in the *trans-N*-glycosidases (1) and hydrolases (2) for some nucleosides. These are not too widely distributed, existing only in a few microorganisms, and catalyzing reactions of the following types:



Reactions of both types are also known in the cleavage of DPN at the nicotinamide-*N*-riboside linkage.

### 3. Systems Utilizing Nucleoside Diphosphosugars

As mentioned above, Leloir discovered the coenzyme uridine diphosphoglucose (UDP glucose) in studying the interconversion of glucose and galactose (see summary by Cohen, 1954). Galactose was phosphorylated to galactose-1-phosphate and reacted with UDPG as follows:

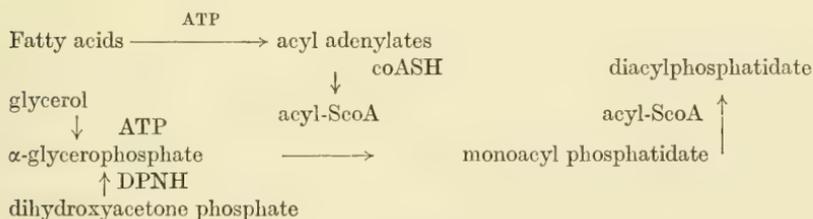


An isomerase then converted UDP-galactose to UDP-glucose, regenerating

this coenzyme to handle another molecule of DPH galactose. The isomerization of UDP-glucose appears to involve a cyclic oxidation and reduction with enzyme-bound DPN as coenzyme (Maxwell, 1957). In addition to UDP-coenzymes containing glucose and galactose, the following sugars have also been observed bound via 1-glycosidic linkage to UDP: *N*-acetyl glucosamine, *N*-acetyl galactosamine, glucuronic acid, galacturonic acid, D-xylose, L-arabinose. The UDP-sugars have been shown to be the intermediates in glycosyl transfer to form a large number of complex polysaccharides, such as hyaluronic acid (Cifonelli and Dorfman, 1957) and chitin (Glaser and Brown, 1957). In addition, UDP glucuronide is the coenzyme of glucuronide formation in detoxication reactions. A similar coenzyme consisting of guanosine diphosphomannose is involved in the transfer of this hexose.

### C. Phospholipid Synthesis

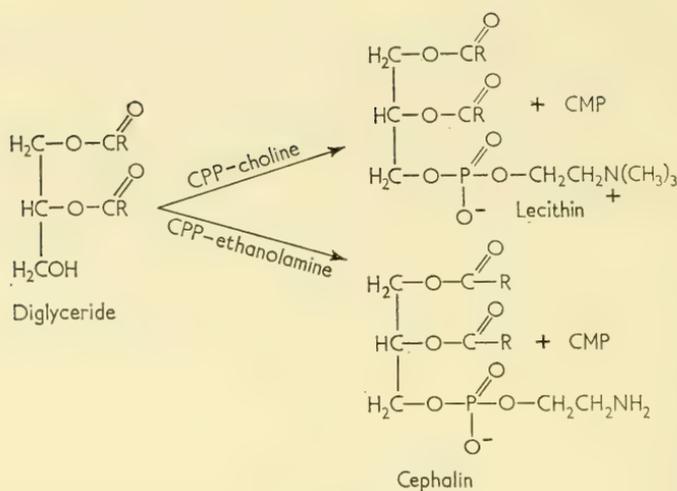
The role of coA and the formation of acyl-ScoA derivatives have been discussed earlier (p. 83). The successive reaction of such acyl-coA derivatives with  $\alpha$ -glycerophosphate to form mono- and diesterified phosphatidates has been presented in the discussion of Enzymatic Systems of Cytoplasm (Section III, D, p. 84). These reactions may be summarized as shown in formula (XXII).



(XXII)

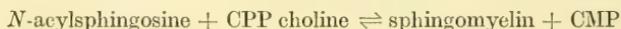
Mammalian tissues contain an enzyme which converts the phosphatidate to an  $\alpha$ - $\beta$ -diglyceride. This may react with another acyl-ScoA to form a triglyceride or with one of the forms of the cytidine-containing coenzymes to form lecithins or cephalins, as described by Kennedy (1957) and Rossiter *et al.* (1957). These reactions may be represented as shown in formula (XXIII).





(XXIII)

Sphingomyelin is synthesized in a similar reaction involving CPP-choline (Kennedy, 1957):



Although  $\alpha$ -glycerophosphoryl choline has been found in significant amount in liver (Schmidt *et al.*, 1955) and in seminal vesicle (Williams-Ashman and Banks, 1956), there is no evidence that this compound is other than a degradation product of lecithin, rather than an intermediate of lecithin biosynthesis (Dawson, 1955). A number of additional cytidine diphosphate derivatives have been recognized in bacteria. In general, it may be stated that a chemical understanding of the intermediary metabolism of the more complex lipids and phospholipids has fairly begun. Nevertheless, the chemical description of these compounds, an essential prerequisite to metabolic studies, has tended to lag behind the study of many other natural products.

#### D. Mechanisms of Peptide Synthesis

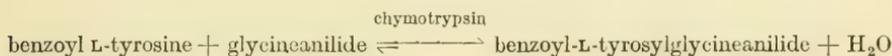
Previous sections have discussed the distribution of protein-synthesizing enzyme systems and their general properties and operation, with particular reference to the interrelations with the nucleic acids and nucleic acid-synthesizing systems. We shall be concerned in this section with problems of the

formation of peptide bonds, including the preliminary activation of the amino acids. An increasing number of workers have begun to explore the problem of the geochemical origin of amino acids, peptides, and proteins (Oparin, 1957; Fox, 1956) and the evolution of biological polymers, but we shall not consider these questions here.

Protein synthesis may be considered to involve at least three major problems: (1) the mechanism of formation of the peptide bond; (2) the determination of specificity in the order of amino acids along the peptide chain; and (3) the determination of the folding and interrelations of peptide chains. Very little is known about the last question; the second question, that of amino acid selection, possibly on a template, will be considered only briefly in a later section.

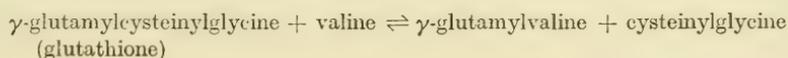
### 1. *Transpeptidation*

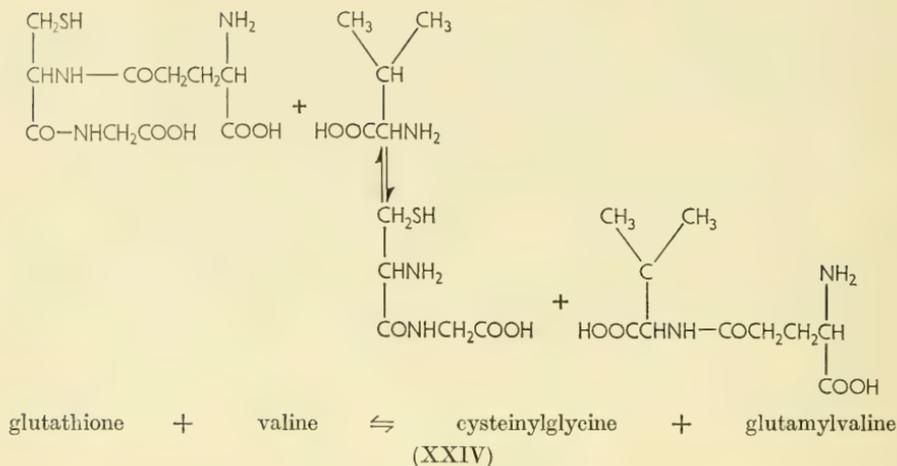
A reaction between the amino group of an amino acid and the carboxyl of another does not occur to a significant extent in solution. In the presence of a proteolytic enzyme, which only increases the rate at which equilibrium is reached, the equilibrium point is far over to hydrolysis rather than toward synthesis. However, conditions may occasionally be set to favor synthesis, as in the reaction



In this case, the insolubility of the peptide and its removal from solution provides the driving force for the reaction. It can therefore be asked if such reactions are not biologically important in the deposition of amino acids and peptides on insoluble particles. In other instances it is conceivable that the removal of the reaction products by other reactions may also assist in shifting the direction of the reaction towards synthesis.

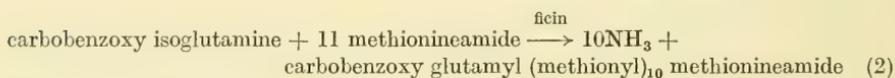
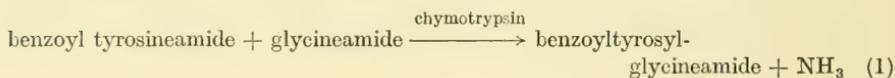
A number of transpeptidation reactions are known in which new peptides may be accumulated (Fruton, 1954). Of particular interest in this connection have been reactions of glutathione, which participates in transpeptidations of the glutamyl portion of the molecule, as in the reaction represented in formula (XXIV).





It has been suggested that a  $\gamma$ -glutamyl group may be a removable physiological acyl substituent at the amino group of an amino acid in the course of biosynthesis of a peptide involving the carboxyl of the amino acid.

The extension of peptide chains may be effected at the expense of amides in the presence of a proteolytic enzyme, as follows:



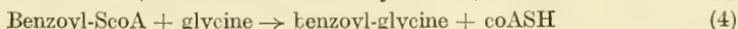
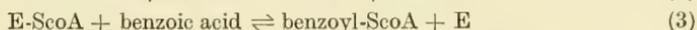
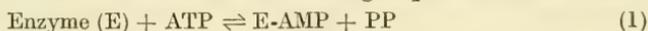
The initial intermediate in the latter reaction is probably carbobenzoxy glutamyl methionineamide. Such reactions have produced an increased appreciation of the possibility of a relatively high free energy of hydrolysis of some amide bonds. The existence of terminal amide bonds in hormones like vasopressin and oxytocin suggests the possibility of a role of these bonds in an initial formation of a peptide bond in the physiological functioning of these hormones. It should be noted that the heat of hydrolysis (free energy data are unavailable at present) of the  $-\text{CONH}_2$  bond is of the same order of magnitude as that of inorganic pyrophosphate. The enzymatic exchange of the  $\text{NH}_3$  in the amide of glutamine with hydroxylamine ( $\text{NH}_2\text{OH}$ ) does require an initial activation by ATP. Thus, although it is conceivable that proteolytic enzymes or transpeptidases may play some role in the extension of polypeptide chains, nevertheless the initial formation of glutamyl bonds or amides as in exchangeable glutamine or isoglutamine may be expected to require an activation of the carboxyl group following some other energy-yielding reaction, such as the hydrolysis of a pyrophosphate group in ATP.

## 2. Carboxyl Activation in Model Systems

The evidence for such a carboxyl activation of free amino acids has been summarized by Borsook (1956), Novelli and DeMoss (1957), and others. A number of systems were studied initially in which the  $R_1CO-NHR_2$  bond was made in nonprotein compounds. Although some differences were observed from reaction to reaction, it was evident that in each case the carboxyl group was activated by a mechanism involving the cleavage of one of the pyrophosphate bonds of ATP. Details of the reaction mechanisms for the following systems have been discussed by Borsook (1956).

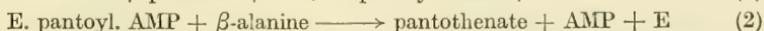
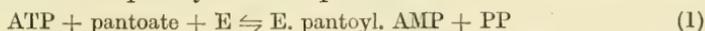
1. In the synthesis of hippuric acid in mammalian liver the over-all reaction was:

benzoic acid + glycine + ATP  $\longrightarrow$  benzoyl glycine (hippuric acid) + AMP + PP  
 Dissection revealed an intermediary role for coenzyme A. The mechanism of the reaction is considered to involve the following sequence of reactions:<sup>1</sup>

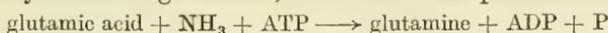


2. In the synthesis of pantothenic acid by *E. coli*, the overall reaction is  
 pantoic acid +  $\beta$ -alanine + ATP  $\rightleftharpoons$  pantothenic acid + AMP + PP

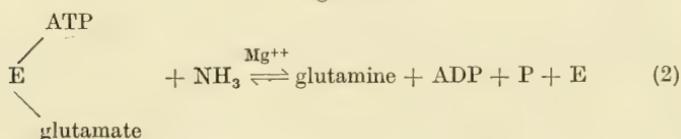
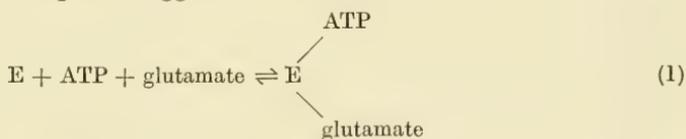
In this case, however, coenzyme A is not involved. A ternary complex containing enzyme-bound pantooyl AMP is postulated.



3. In the synthesis of glutamine, ADP and P are products as follows:

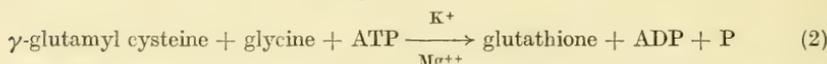
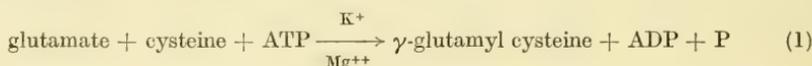


Some intermediate steps are suggested to be:

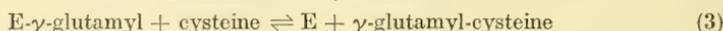
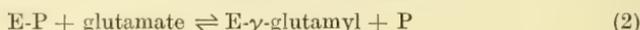


<sup>1</sup> In the enzymatic synthesis of phenylacetyl glutamine by enzymes of human liver (Moldave and Meister, 1957), phenylacetate and glutamine condense in the presence of ATP and coA. The same mixture of enzymes and cofactors can form hippuric acid from benzoic acid and glycine. The analysis indicated the initial formation of acyl adenylates which exchange with coA to form phenylacetyl-S coA, which then reacts with the amino acid. The hippurate system then in its initial step is perhaps more comparable to pantoate activation than suggested by the mechanism proposed in the text.

4. The synthesis of the tripeptide glutathione, involves a stepwise formation of dipeptide and tripeptide, as follows:



In each reaction, the first step is postulated to be:



Thus, four different courses have been observed for the four model systems studied. Only one was found to involve coenzyme A. In two instances, the cleavage of ATP yielded  $\text{ADP} + \text{P}$ ; in two others,  $\text{AMP} + \text{PP}$  were formed. Nevertheless, the carboxyl group, later to be incorporated into the amide bond, was activated in each case.

Of the four systems studied, that of carboxyl activation in pantothenate synthesis has been suggested most often to bear the closest similarity to the activation of amino acids, since the latter also involves the liberation of PP from ATP, as mentioned in an earlier section. The case of pantoate activation is also superficially analogous to the activation of other acyl moieties (acetate, and a number of other groups, sulfate, carbonate, etc.), although a pantoyl adenylate has not yet been described. As noted earlier, acetate catalyzes the exchange of pyrophosphate with ATP in yeast extracts with the intermediate formation of adenyl acetate, which then reacts with coA to form acetyl-ScoA (Berg, 1955, 1956a). A comparable sequence and role is recognized for butyrate (Talbert and Huennekens, 1956) and higher fatty acids (Jencks and Lipmann, 1957).

In the latter case, the same enzyme system from liver capable of activating fatty acids also was able to activate one amino acid, phenylalanine. The formation of the phosphocarboxyanhydride of the amino acid was indicated both by the release of PP and by a reactivity with hydroxylamine to form a characteristic hydroxamate, useful in the detection and estimation of this class of compounds. In this study the existence of an acyl adenylate deacylase was also detected.

The role of sulfate in pyrophosphate exchange with ATP to form adenyl sulfate, has been described by Hilz and Lipmann (1955). This compound is an intermediate in the formation of adenosine-3'-phospho-5'-phosphosulfate, the coenzyme for the transfer of sulfate in the synthesis of molecules like chondroitin sulfate (D'Abraham and Lipmann, 1957) and phenol sulfate (Robbins and Lipmann, 1957).

According to Bachhawat and Coon (1957) an AMP-CO<sub>2</sub> compound reacts with  $\beta$ -hydroxyisovaleryl-ScoA to form  $\beta$ -hydroxy- $\beta$ -methylglutarylcoenzyme A. The adenylate is similarly generated by a CO<sub>2</sub>-activating system which catalyzes the exchange of PP and ATP.

### 3. Carboxyl Activation of Amino Acids

In similar reactions, amino acids catalyze the exchange of PP with ATP in nonparticulate extracts of animal tissue (Hoagland, 1955; Cole *et al.*, 1957), bacteria (DeMoss and Novelli, 1956), and yeast (Berg, 1956b). A similar mechanism of amino acid activation appears to exist in plants (Webster, 1955, 1957a and b; Stephenson *et al.*, 1956).

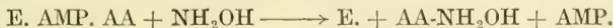
In these reactions it has not been possible to demonstrate a net formation of amino acid adenylate and it has been postulated that, as in pantoate activation, the enzyme reacts with ATP:



Pyrophosphate is displaced by the amino acid to give the bound amino acid



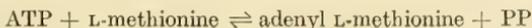
The amino acid adenylate does not dissociate from the enzyme to a significant extent. However, hydroxylamine in some instances forms a dissociable hydroxamate, dissociating this product plus AMP.



Regenerated enzyme is freed to recycle ATP and amino acid. Thus, in these instances, in the presence of NH<sub>2</sub>OH, the net formation of activated amino acids is indicated by the formation of amino acid hydroxamates and a stimulated release of AMP and PP. The irreversibility of hydroxamate production accounts for the inhibition of PP exchange under these conditions. The validity of this postulated mechanism in this series of reactions has recently been confirmed in a study involving O<sup>18</sup>-labeling and transfer of this oxygen from tryptophan carboxyl to AMP (Hoagland *et al.*, 1957b).

Although it has not been possible to find a free intermediate as a result of amino acid carboxyl activation and it is considered that the appropriate adenylate is strongly attached to the enzyme surface, exogenously supplied synthetic amino acid-AMP derivatives, such as leucyl adenylate, are converted to ATP in the presence of PP (DeMoss *et al.*, 1956).

The initial studies of Hoagland *et al.* (1956) indicated the existence of separate enzymes for the activation of the amino acids. These workers detected a specific system for methionine activation in rat liver, a result soon obtained with yeast extracts (Berg, 1956b). This enzyme catalyzed the following reaction:



Davie *et al.* (1956) have isolated a relatively pure, soluble, specific, tryptophan-activating enzyme from the pancreas. Schweet (1957) has obtained a tyrosine-specific enzyme from pancreas.

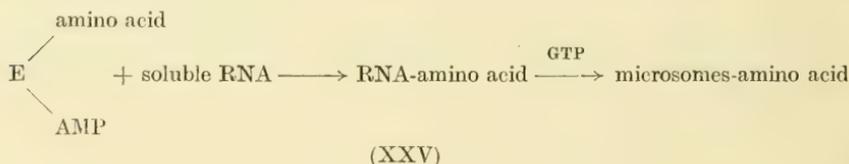
By means of the former enzyme it has been possible to show that some tryptophan analogs, such as azatryptophan and tryptazan, are activated, incorporated into protein, and then block growth (Sharon and Lipmann, 1957). Other analogs, such as 5-methyl tryptophan, inhibit the enzyme by competing with tryptophan at the activation step and appear to block growth in this manner.

In the studies of Novelli and DeMoss (1957), several activating enzymes for amino acids were also found in *E. coli* extracts. These workers have found in a wide variety of bacterial and animal extracts that only eight amino acids are activated in a comparable PP exchange. The others were presumed to be activated in as yet undetermined transacylation reactions. However, Nisman *et al.* (1957) have recently observed that lysis of protoplasts, a milder method of cell rupture, permits the demonstration of activating systems for many more amino acids.

#### 4. Intermediate Reactions

Since the amino acid activation and binding are affected by a soluble enzyme and the deposition of the amino acid occurs most actively at the ribonucleoprotein centers of the microsomal fraction, the nature of the transfer from the enzyme-AMP-amino acid complex is posed. Guanosine di- or triphosphate is essential in some as yet undetermined fashion to this transfer (Keller and Zamecnik, 1956; Littlefield and Keller, 1957). Liver microsomes derived from vitamin B<sub>12</sub>-deficient rats are ineffective in the incorporation of amino acids in these systems, but become effective on supplementation with exogenous vitamin B<sub>12</sub>, thereby implicating this substance as a cofactor as well (Wagle *et al.*, 1957).

Most recently it has been found that a low molecular fraction of RNA present in the supernatant fraction reacts with the complex and incorporates the amino acid (labeled leucine) as a soluble RNA-amino acid compound. (Hoagland *et al.*, 1957b). This reaction is sensitive to ribonuclease. In the presence of GTP a deproteinized RNA-amino acid derivative can transfer its bound amino acid to the microsomal fraction. This is the first direct demonstration of a role for polynucleotides in protein synthesis. The course of these reactions may be represented as shown in formula (XXV).



It may be asked if these facts indicate a stepwise addition of amino acids to form peptides and proteins. It is significant that in these isolated systems individual amino acids can be incorporated into peptide linkage without the simultaneous presence of other amino acids and their simultaneous incorporation into protein. However, there is no indication at the present time of the size of the newly formed peptide units in the ribonucleoprotein of the microsomal fraction, nor is the relation of such peptide formation to normal protein synthesis entirely clear. It is known in animal cells and microorganisms that deficiencies with respect to single essential amino acids block all amino acid incorporation (Borsook *et al.*, 1957) suggesting either the essentiality of integrated simultaneity in protein synthesis or the existence of requirements for specific amino acids as acceptors in the development of peptide chains. Thus, the so-called protein syntheses studied so far with single amino acids may be more akin to the polymerization of leucine to polypeptides of 3 to 20 amino acids when the phosphate anhydride of leucine is permitted to stand in an aqueous solution at room temperature (Katchalsky and Paecht, 1954).

The problem of whether amino acids form low molecular peptides on the way to protein or are simultaneously zippered to form a polypeptide chain is an outstanding problem of protein synthesis. In problems such as the apparent conversion of milk protein to plasma protein in the rat, a great deal of work has been done to determine whether peptides may be derived intact from the milk protein. As reviewed by Campbell and Stone (1957), it is considered that there is little evidence that such an interconversion occurs at a level above that of the free amino acids. However, contrary views are held by a variety of workers. For example, Ebert (1954) and Walter *et al.* (1956) consider that large specific peptides and proteins are derived from the organ fragments used as transplants in studies of grafts and embryogenesis.

No abnormal accumulation of intermediate peptides has yet been seen under experimental conditions of amino acid deficiency (Halvorson and Spiegelman, 1952) although the existence of soluble peptides in various organisms, e.g. blue-green algae, has long been recognized. However, the existence of very small amounts of soluble intermediates in fatty acid metabolism could not be detected for many years. As an alternate hypothesis it may be supposed that low molecular peptides are formed at the microsome surfaces. Koningsberger *et al.* (1957) have recently described the existence of nucleotide-bound carboxyl-activated peptides on ribonucleoprotein particles of yeast. The fact that in some instances peptides support growth requirements of mutant organisms somewhat better than do free amino acids may bear on this problem (Simmonds and Fruton, 1949; Simmonds and Miller, 1957).

One approach to this problem has involved an attempt to see if an amino acid derived from different parts of a protein possesses identical isotope

contents when the protein has been synthesized in an organism incubated with a radioactive amino acid. This problem has been discussed in detail by Steinberg *et al.* (1956). Although in some cases nonuniform labeling has been observed, suggesting the possible stepwise formation of intermediate peptides, it is not yet known how long it takes to make a protein molecule (Dalglish, 1957). Thus, it is conceivable that nonuniform labeling may occur as a result of the changes in the free amino acid pool, if it takes an appreciable time to produce a polypeptide chain, even when this is being elaborated at a single fixed template by a so-called zipper action.

Considerable lags have been observed in the elaboration and appearance of some proteins, such as serum albumin (Peters, 1953), suggesting the existence of many intermediates. However, other workers have found very short periods of this kind, as for ferritin production in liver (Loftfield, 1956). Indeed, Peters, T. (1957) has recently reported that during the lag, serum albumin-like proteins may be detected bound to cytoplasmic particles, from which they are then presumably released. Although the problem of the time of synthesis of a protein molecule has not yet been satisfactorily solved (Craddock and Dalglish, 1957), it seems possible that it is indeed very short.

Before turning to questions of the specificity of peptide organization, presumably as directed by templates, we shall first consider existing data on nucleic acid formation.

### *E. The Biosynthesis of Nucleic Acid Intermediates*

In the intermediary metabolism of protein biosynthesis it was seen that the focal building blocks were the amino acids. The formation of each of these may involve a long and characteristic sequence of reactions, leading to the existence of several hundred intermediates for this general area of metabolism. In contrast to this situation, the intermediary metabolism of the nucleic acids has many fewer independent reaction sequences. These may be grouped, as indicated earlier, into scavenging reactions permitting the recovery of ingested nucleic acid and reactions of *de novo* biosynthesis (Kornberg, 1957a).

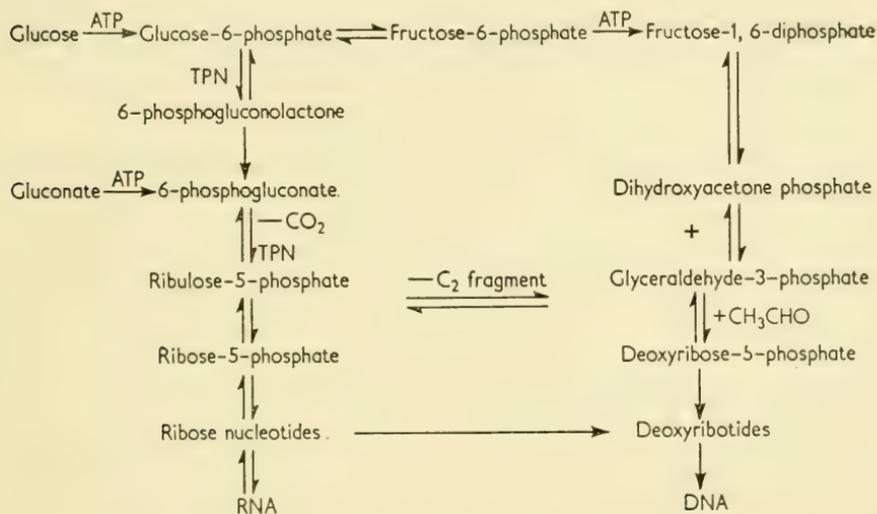
#### *1. Scavenging Reactions*

In the former category are reactions which, for the most part, have been indicated earlier: (a) linkage of free bases to ribose-1-phosphate or deoxyribose-1-phosphate to form a ribo- or deoxyribonucleoside, respectively; (b) the phosphorylation of the nucleoside to nucleotide; (c) the linkage of free base (purine or pyrimidine) to 1-pyrophosphoryl ribose-5-phosphate to form a purine or pyrimidine ribonucleotide. In addition, transglycosidations via nonphosphorylytic reactions permit formation of new nucleosides from bases and nucleosides. In all of these reactions, the organism is called upon

most often for the synthesis of the appropriate pentose phosphate derivable from glucose metabolism.

## 2. "De Novo" Synthesis

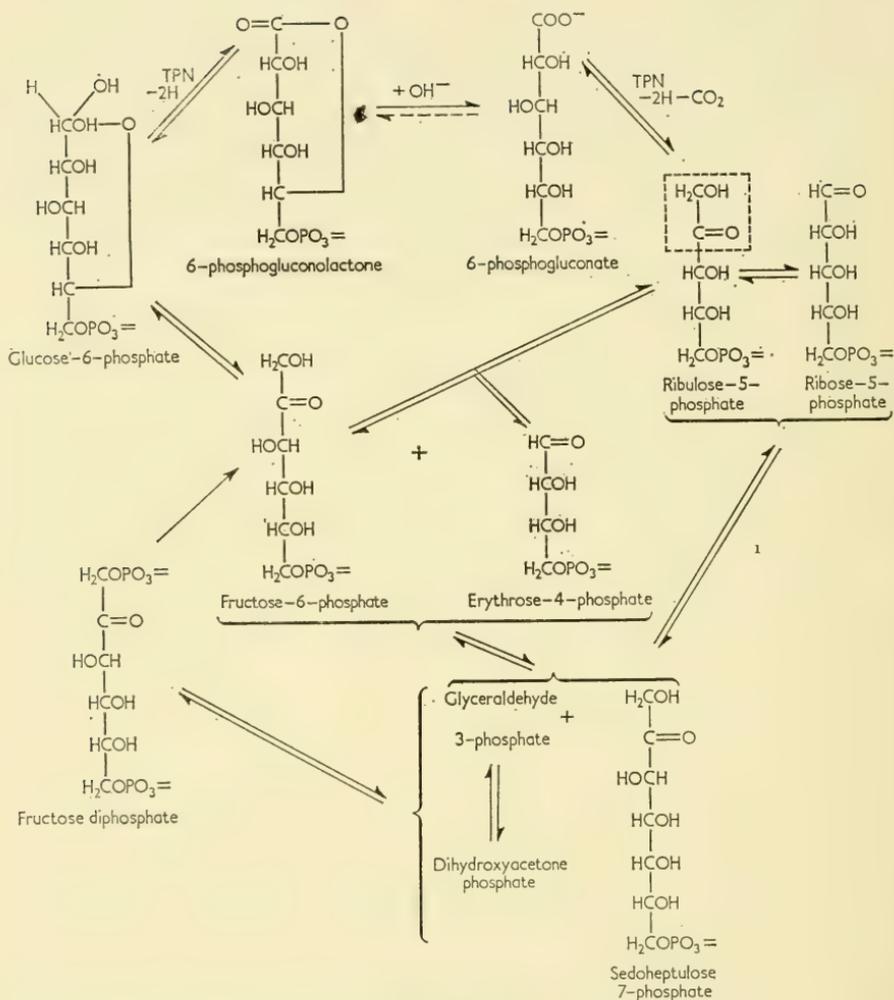
*a. Pentose and Deoxyypentose Synthesis.* The rapid growth of knowledge in this area began in 1948 with a resumption of study of the oxidative pathway of phosphogluconate metabolism. The development of this subject through 1952 has been discussed by the author (Cohen, 1954). Pathways existing in *E. coli* and indeed in most cells for the origin of ribose and deoxyribose can be schematized as shown in formula (XXVI).



Two alternative paths of glucose utilization present in *E. coli* and in most other cells.  
(XXVI)

It can be seen that each of the alternative paths stemming from glucose-6-phosphate may produce the carbon atoms which form the sugars of the nucleic acids. The determination of the quantitative relations of these possible pathways in different organisms under different physiological conditions has become a serious effort in many laboratories (Wood, 1955; Axelrod, 1956a). The effect of virus infection on these pathways has been studied in phage-infected *E. coli* (Cohen, 1954).

The detailed enzymology of the pathways of pentose phosphate metabolism has been discussed in many recent reviews, e.g., Axelrod (1956a), which elaborate more recent information of the complex cycle involved. A scheme of the reactions involving the pentose phosphates is presented in formula (XXVII) (Horecker and Mehler, 1955).



(XXVII)

Given ribose-5-phosphate via the mechanisms outlined above, this substance may be isomerized to ribose-1-phosphate in preparation for the condensation with bases to form nucleosides, or pyrophosphorylated in preparation for the condensation with bases to form nucleotides.

Very little is known of the mechanism of deoxyribose formation. Although an enzyme, deoxyribose-5-phosphate aldolase, is known to catalyze the synthesis of this substance from glyceraldehyde-3-phosphate and acetaldehyde, the properties of the enzyme are such as to suggest that it can be more

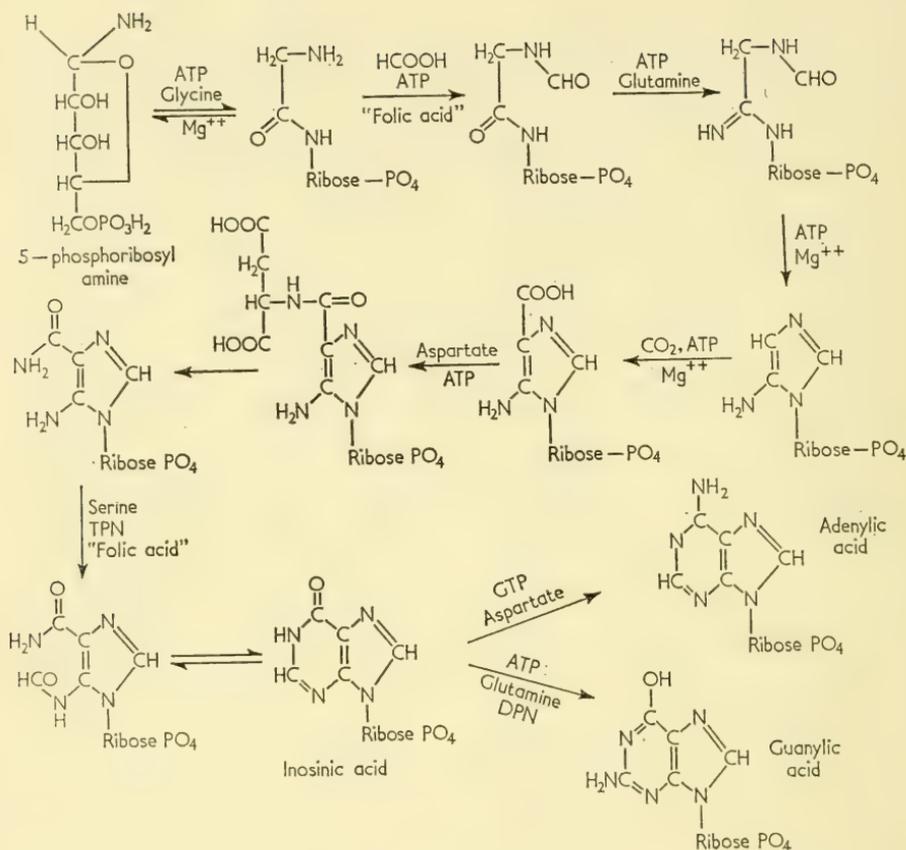
<sup>1</sup>The substrate activated by transketolase to form sedoheptulose-7-phosphate has now been shown to be D-xylulose-5-phosphate, which is formed by epimerization from ribulose-5-phosphate.

active in degradation than in synthesis. However, even if given deoxyribose-5-phosphate, a mutase for conversion to deoxyribose-1-phosphate is not present in all organisms, thereby obviating a phosphorylase route to nucleosides for some organisms. In addition, the pyrophosphorylation of deoxyribose-5-phosphate has been sought without success, making it very dubious that deoxyribose-5-phosphate is on a more direct route to deoxyribotide synthesis. Isotope studies of deoxyribose synthesis have not revealed a single instance wherein deoxyribose could arise by this aldolase mechanism. On the other hand, existing isotope studies in intact organisms have indicated the direct reductive conversion of ribosyl derivatives to deoxyribosyl derivatives (Brown, 1956; Loeb and Cohen, 1957; Reichard, 1957).

Isotopic competition experiments have indicated that the pyrimidine deoxyribosides are not intermediates in the formation of DNA pyrimidines in virus-infected *E. coli* (Cohen *et al.*, 1957). Although significant amounts of pyrimidine deoxyribosides have been isolated from a number of normal and tumor tissues (Schneider, 1955), it was not felt that these components rather than the nucleotides were important intermediates in DNA synthesis in regenerating liver (Hecht and Potter, 1956c). Thus, it has been thought that the conversion occurs most actively at the nucleotide level. However, in a recent report, evidence has been presented for an enzymatic conversion of a ribonucleoside to deoxyriboside in bacterial extracts (Grossman and Hawkins, 1957).

*b. Synthesis of Purine Ribotides.* The outstanding work of Buchanan, Greenberg and their collaborators has resulted in an almost complete dissection of the routes of purine biosynthesis. Unlike pathways of carbohydrate metabolism, for which variants are known at almost every step, this pathway appears essentially the same in all organisms tested. As indicated earlier, 1-pyrophosphoryl ribose-5-phosphate is converted by reaction with glutamine to the 5-phosphoribosylamine and this compound becomes the foundation on which the purine superstructure is built. The reaction sequence is presented in formula (XXVIII) at top of p. 180.

It can be seen that inosinic acid is the branch point for the formation of both adenylic acid and guanylic acid. The conversion of inosinic acid to adenylic acid is effected by the intermediary formation of adenylosuccinic acid as a result of condensation of aspartic acid with inosinic acid and deacylation of adenylosuccinic acid. This deacylase is apparently the same as the deacylase involved in the cleavage of the earlier succinyl-derivative formed in the reaction chain (Miller *et al.*, 1957; Gots and Gollub, 1957). The formation of guanylic acid from inosinic acid requires an initial dehydrogenation with DPN to xanthylic acid (Lagerkvist, 1955; Magasanik *et al.*, 1957) which is then aminated by glutamine in the presence of ATP to guanylic acid.

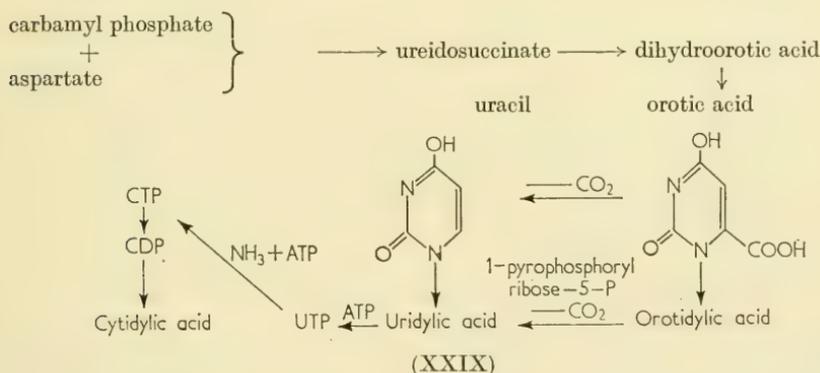


(XXVIII)

The critical role of glutamine in at least three steps of purine biosynthesis suggests the potential role of glutamine analogs in blocking nucleic acid biosynthesis. Of the several glutamine-requiring reactions known, one is particularly sensitive to the two glutamine analogs, aza-L-serine and 6-diazo-5-oxo-L-norleucine (Levenberg *et al.*, 1957). This is the conversion of formyl glycinamide ribotide to formylglycineamidine ribotide, as indicated in the schema, in which the enzyme is irreversibly inactivated by the antibiotics. That azaserine can effect this inhibition has been demonstrated with intact organisms, e.g., *E. coli* (Tomisek *et al.*, 1956). The possible activity of azaserine has not yet been adequately explored in virus-infected cells; it should be noted that the growth of mammalian cells in tissue culture requires glutamine, as does the production of certain viruses in such cells (Eagle and Habel, 1956).

Mechanisms for the production of di- and triphosphates of the ribonucleotides have been described earlier. The mechanism of the formation of the purine deoxyribotides is as yet quite obscure. Degradative mechanisms for purines are described in all textbooks of biochemistry.

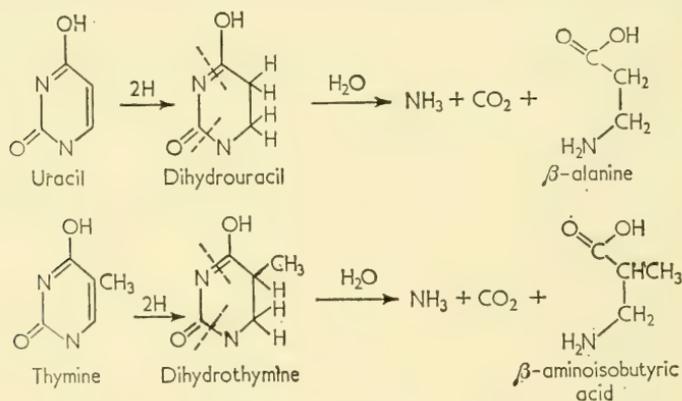
*c. Biosynthesis of Pyrimidine Nucleotides.* The formation of orotic acid and uracil via the condensation of carbamyl phosphate and aspartic acid to ureidosuccinate and dihydroorotic acid has been presented earlier, in the discussion of feedback mechanisms in the control of enzyme production. The condensation of orotic acid and uracil with 1-pyrophosphoryl ribose-5-phosphate to form orotidylic acid and uridylic acid, respectively, has been described (Kornberg, 1957a,b). These are separate reactions whose existence and use are determined in particular organisms by the presence or absence of an orotic acid decarboxylase or an orotidylic acid decarboxylase. Thus, organisms are known in which orotic acid is converted first to orotidylic acid and decarboxylated to uridylic acid. In others, orotic acid is first decarboxylated to uracil prior to condensation to uridylic acid. In still other organisms, both pathways exist side by side. These relations are presented in formula (XXIX).



As presented in the schema, the formation of the cytosine ribonucleotide series occurs at the triphosphate level in the amination of UTP to CTP (Lieberman, 1956). This phenomenon virtually isolates cytosine metabolism at the end of a reaction chain which must be approached through uracil derivatives. At the present time, no mutants are known to have cytosine requirements without concomitant uracil requirements. Cytosine will not react with ribose-1-phosphate to give a nucleoside or with 1-pyrophosphoryl ribose-5-phosphate to give a cytosine nucleotide. Thus, the pyrimidine ring of cytosine must be deaminated to uracil before scavenging is possible. However, some slight evidence exists to indicate that cytosine nucleosides may be phosphorylated to the nucleotide.

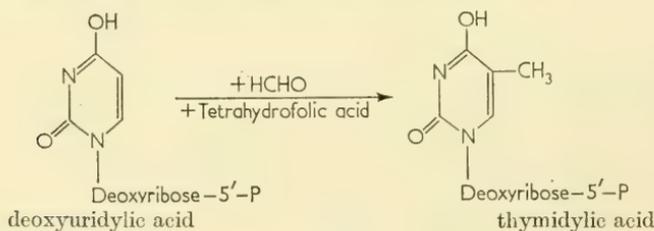
The synthesis of thymine, or 5-methyl uracil, is of critical interest in DNA metabolism, since this base is found uniquely in this nucleic acid. Existing evidence (Friedkin and Kornberg, 1957; Cohen *et al.*, 1957) indicates that in *E. coli* the methyl group is added to uracil only at the nucleotide level.

It appears likely that this is true in other organisms as well, although an enzyme is known for the phosphorylation of thymidine; the latter may be thought of as a scavenging system. The utilization of pyrimidine nucleosides via a comparable kinase action is well known, although few organisms are capable of extensive incorporation of free pyrimidines to nucleic acid. Tumors are an exception to this rule, although it is not entirely clear whether this reflects an augmented complement of pyrimidine phosphorylases or pyrophosphorylases in tumor cells or a reduced rate of degradation of free pyrimidines (Canellakis, 1957c). In the mammal, uracil and thymine are rapidly reduced and the pyrimidine ring is then cleaved, as shown in formula (XXX).



The product of thymine degradation,  $\beta$ -aminoisobutyric acid, is often excreted and may prove to be a sensitive measure of thymine metabolism and DNA degradation resulting from radiation therapy and cellular necrosis.

The enzymatic synthesis of thymidylic acid has been shown to occur as shown in formula (XXXI) (Friedkin and Kornberg, 1957).



Although the one carbon source, formaldehyde, is initially at a hydroxymethyl level, the final product is reduced to a methyl compound. It has been suggested that the coenzyme, tetrahydrofolic acid, provides the hydrogen atoms for this reduction. The intermediates and mechanism of the reaction have not yet been described. Folic acid analogs are known to block the formation of thymine, presumably in this reaction. In addition, 5-fluorouracil deoxyriboside is phosphorylated in *E. coli* and the deoxyribotide is also a potent inhibitor of this enzyme (Cohen, Flaks, and Barner, unpublished data), presumably accounting for the potent antitumor activity of fluorouracil deoxyriboside.

As presented in Fig. 1, a comparable hydroxymethylation occurs in virus-infected *E. coli* in the conversion of deoxycytidylic acid to hydroxymethyl deoxycytidylic acid (Flaks and Cohen, 1957). However, in this case the reduction to the methyl level does not occur. The DNA of higher animals and plants contains 5-methyl deoxycytidylic acid but it is not known whether this is made via an amination of thymidylic acid or via the reduction of the intermediate formed at the hydroxymethyl level.

## F. Biosynthesis of the Nucleic Acids

### 1. The Structure of the Nucleic Acids

It is beyond the scope of this discussion to provide a detailed analysis of nucleic acid structure. The data on the composition and metabolism of the nucleic acids have been summarized and analysed in many reviews. Of these one important reference work is the two-volume treatise on "The Nucleic Acids" (Chargaff and Davidson, 1955) which covers the field until 1954. Nevertheless it will be useful to present a very brief survey of present views of the polymeric nature of the naturally occurring nucleic acids, to provide a suitable backdrop for the current efforts at biosynthesis.

It is now recognized that in cells both RNA and DNA represent heterogeneous mixtures of polynucleotide polymers of considerable size. The heterogeneity of nucleic acid has been established in a variety of ways. Thus, a sample of DNA or RNA isolated from cells may display physical heterogeneity under conditions of sedimentation in the analytical ultracentrifuge or may be separated in chemically and physically distinct fractions by column chromatography (Bendich *et al.*, 1953; Chargaff *et al.*, 1953; Bradley and Rich, 1956; Miura and Suzuki, 1956). Metabolic heterogeneity of RNA has been recognized in cells, since various rates of incorporation of isotopes into RNA fractions of nuclei and cytoplasm have been observed. Although a similar type of metabolic heterogeneity has been reported for DNA (Bendich, 1952), this has not been supported (Osawa and Sakaki, 1957). The latter workers have shown that when DNA is synthesized in rabbit

appendix in the presence of  $P^{32}$  and chromatographed, separated fractions possess essentially identical specific activities, a result which also extends to fragments of the DNA molecules.

Finally, chromatographic procedures can separate biologically active molecules of DNA possessing specific transforming ability for *Pneumococcus* from DNA inactive in comparable tests (Bendich *et al.*, 1956; Lerman, 1955). Thus, the biological heterogeneity of the genetic material of cells reflects in some part the physical and chemical heterogeneity of its constituent polymers. The existence of this heterogeneity of cellular nucleic acid defines in considerable measure the experimental advantages of chemical study of the inheritance of a virus whose complement of nucleic acid may be of the order of 1/25 to 1/500 that of a bacterium. Biological, physical, and chemical heterogeneity are thereby effectively reduced by whole orders of magnitude.

The particle sizes of the cellular nucleic acids have not yet been satisfactorily determined. An RNA from calf liver had an average molecular weight of the order of 300,000 (Grinnan and Mosher, 1951; Magasanik, 1955); DNAs from various cell sources have been reported to have molecular weights of from  $1 \times 10^6$  to  $10 \times 10^6$  (Jordan, 1955). However, all of these cellular nucleic acids are heterogeneous and the molecular weights in the literature are averages of different kinds, depending on the methods of estimation. The ranges of molecular weights for particles within each sample have not been determined; this problem is only now becoming recognized as important.

A few estimates of particle size are available for the biologically more homogeneous viral nucleic acids; in general these appear to possess a greater degree of physical homogeneity than do the cellular nucleic acids. Most careful work has been done on plant virus RNA, starting with that of tobacco mosaic virus (Cohen and Stanley, 1942; Hopkins and Sinsheimer, 1955; Schuster *et al.*, 1956). The RNA of this virus may exist as a single particle of about  $1.9-2.1 \times 10^6$ , which degrades to a particle of  $2.5-2.9 \times 10^5$ , which in turn decomposes to units of weights  $6 \times 10^4$  and  $1.5 \times 10^4$ . On the other hand, the RNA of a spherical virus, turnip yellow mosaic virus, appears to consist of many units (25 to 50) of 36,000 to 100,000 molecular weight, which interact readily to form larger units (Cohen and Schachman, 1957). Isolated DNA of T-even phages has an average molecular weight of 19 to  $25 \times 10^6$  (Cohen, 1957; Meselson *et al.*, 1957). Nevertheless, the physical characterization of these substances can still be considered to be in a primitive state.

Analysis of the distribution of the component nucleotides along polynucleotide chains has barely begun. As summarized by Markham (1956) and most recently by Heppel *et al.* (1957a,b), techniques for the stepwise analysis of RNA chains have been developed; hardly any comparable approaches work for DNA. In both substances, the polynucleotide chains are recognized

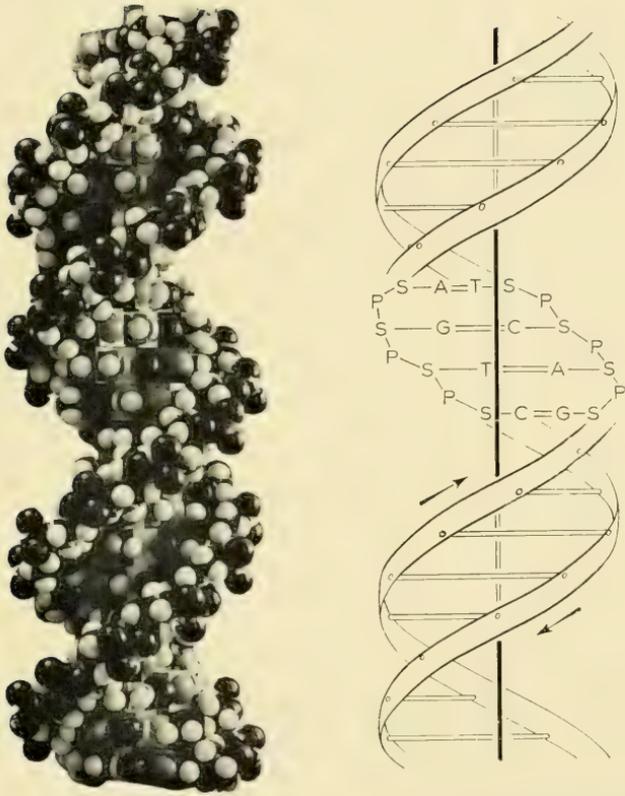


FIG. 27. Molecular models of DNA embodying complementary base pairing. Both a shallow and a deep groove are to be noted in the DNA helix. In the model on the left may be seen the compact fitting of polyarginine in the lower groove (Wilkins, 1956).



to consist of nucleotides linked by phosphodiester bonds from the C<sub>3</sub>-hydroxyl of one nucleoside sugar residue to the C<sub>5</sub>-hydroxyl of another nucleoside sugar, as depicted in Fig. 26.

Gross analysis of base composition, largely a result of the exploitation of paper chromatography,<sup>1</sup> has produced a number of suggestive generalizations for all DNA so far analyzed and some less satisfactory generalizations for most RNA. In samples of DNA, bases containing 6-amino groups exist in amounts equivalent to bases containing 6-hydroxyl groups; in addition, purines = pyrimidines, adenine = thymine, and guanine = cytosine. These results have suggested a complementarity of purine and pyrimidine bases, producing interaction by hydrogen bonding along parallel nucleotide chains. The model based on these equivalences was developed by Watson and Crick (1953) (also see Pauling and Corey, 1956) and is presented in Fig. 27. It has been amply supported by X-ray analysis of many DNA preparations, as summarized by Wilkins (1956). This general structure has proved to be a useful frame of reference for consideration of the properties of DNA, particularly its denaturation and degradation (Doty, 1956). Of at least equal interest has been the hypothesis that the existence of complementary chains in DNA provides a mechanism for the duplication of DNA. It is supposed that the separated chains can serve as templates for the organization of new complementary polynucleotide chains. The evidence concerning this hypothesis will be considered in a later section.

Base analyses for RNA samples of entire cells have also revealed an apparent equivalence of 6-amino groups and 6-hydroxyl groups, although not of individual bases (Elson and Chargaff, 1955). A number of samples of viral RNA, e.g., the RNA of turnip yellow mosaic virus, provide exceptions to even this pairing rule. X-ray crystallographic study of RNA samples has not been as fruitful as for DNA, although it has been suggested that the X-ray patterns suggest a DNA-like structure for RNA (Rich and Watson, 1954; Crick, 1957a). Evidence for the hydrogen bonding of polynucleotide chains of RNA have stemmed in largest part as a result of studies on the biosynthesis of ribose polynucleotides, to be discussed below. Despite the absence of complementary bases in RNA for pairing in the Watson-Crick model, in which two polynucleotide chains run in opposite directions, it is possible to devise other paired structures, as, for example, the model of Donohue and Stent (1956) in which a duplex may be built up of two polynucleotide chains of identical base sequence.

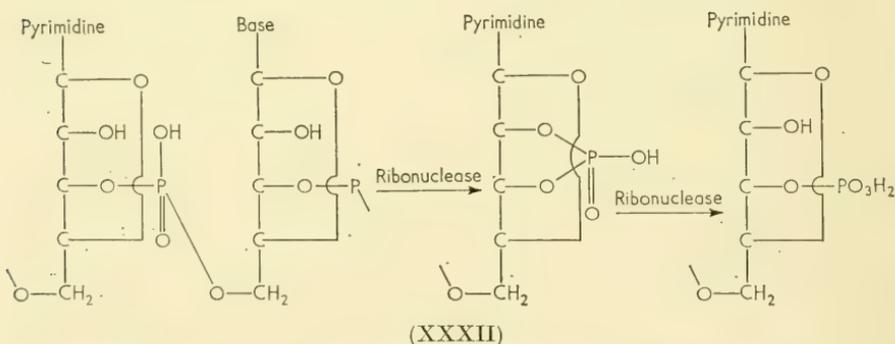
Evidently such a structure of RNA provides a mechanism for replication, even as does the Watson-Crick model for DNA. That the requirement for a

<sup>1</sup> In recent years, paper chromatography and ion exchange techniques have revealed trace elements of new bases in both RNA and DNA (Davis and Allen, 1957; Dunn and Smith, 1955).

mechanism for specific replication exists for RNA, at least as well as for DNA, is of course affirmed by the specific activities of the RNA of tobacco mosaic virus and its strains in initiating viral infection, although, as in the case of DNA, it is not yet proved that nucleic acid replicates itself without a non-nucleic acid intermediary. A few other instances of specificity of RNA and RNA-like structures had been observed before the recent work with virus RNA. For example, it had been shown that certain bacterial RNAs inhibited a bacterial deoxyribonuclease, which was not inhibited by RNA from the mammal, plant, or yeast (Bernheimer, 1953). In addition, poly-ribophosphate, a double chain of polymerized ribose phosphate linked  $C_1-O-C_1$ , thereby eliminating purines and pyrimidines, has been recognized as the type-specific substance of *Hemophilus influenzae*, type B (Zamenhof *et al.*, 1953).

## 2. The Biosynthesis of RNA

*a. Transesterification with Ribonuclease.* Digestion of RNA with pancreatic ribonuclease results in the splitting of all internucleotide linkages which involve  $C_3'$  of a pyrimidine nucleotide. The first step appears to involve the formation of cyclic nucleotides, i.e., pyrimidine nucleoside  $2' : 3'$  phosphates and the simultaneous cleavage of the linkage to the  $5'$  hydroxyl of the next nucleotide. The cyclic phosphates then hydrolyze to form the  $3'$  nucleotide, as represented in formula (XXXII).



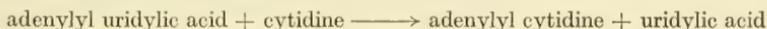
The first reaction is thus seen to involve a transfer of phosphate from the  $C_5'$  hydroxyl of one nucleotide to the  $C_2'$  hydroxyl of the pyrimidine nucleotide. In the second hydrolytic reaction, transfer is made to water. Various inhibitors, such as heparin, appear to affect the second reaction. A spleen ribonuclease or phosphodiesterase produces this end result without the intermediation of the cyclic nucleotide.

Both enzymes have now been demonstrated to participate in synthetic and exchange type reactions. Ribonuclease can catalyze the formation of esters of  $3'$  nucleotides from cyclic nucleotides and alcohols. Spleen phospho-

diesterase can exchange one alcohol for another on such a phosphate ester (Heppel and Whitfield, 1955).

When ribonuclease is incubated with cytidine-2' 3'-phosphate under suitable conditions, polynucleotides are formed, made exclusively of cytidylic acid linked through 3' and 5' hydroxyls. Cyclic cytidine nucleotide could also be linked in this way to adenosine or cyclic adenosine nucleotide. The other cyclic pyrimidine nucleotide, uridine-2'3'-phosphate, could also be used as a donor in synthetic reactions with ribonuclease. The acceptor in these reactions must be a primary alcohol.

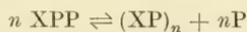
Although spleen phosphodiesterase was inactive with cyclic nucleotides, it could transfer nucleotides, as in the reaction:



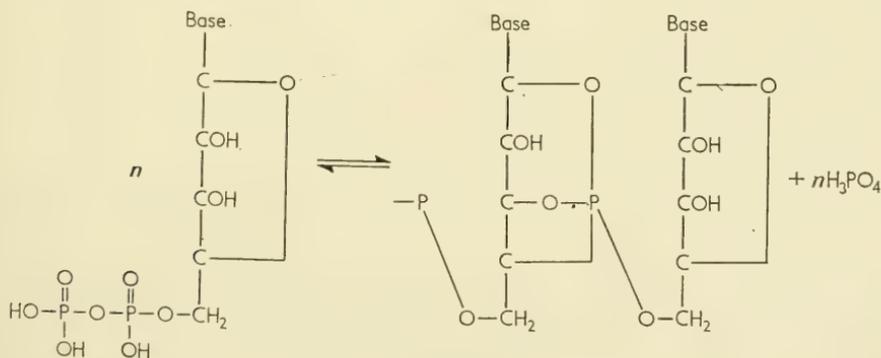
When benzyl esters of mononucleotides were incubated with the enzyme, monobenzyl esters of dinucleotides were formed. These synthetic reactions and their possible significance have been summarized and discussed by Heppel *et al.* (1955).

Crystalline pancreatic ribonuclease has now been separated into two fractions, one of which catalyzes a reversible exchange or transesterification between cyclic pyrimidine nucleotides and nucleosides. The other fraction irreversibly synthesized dinucleoside monophosphates from cyclic nucleotides and a nucleoside (Hakim, 1957).

*b. Polynucleotide phosphorylase.* The problem of the mechanism of RNA synthesis was signally advanced by the discovery of a polynucleotide phosphorylase in bacteria, e.g., *Azotobacter vinelandii*, by Grunberg-Manago and Ochoa (1955). The enzyme catalyzes the reversible synthesis of polyribonucleotides from 5'-nucleoside diphosphates, releasing inorganic ortho P in the process:



The enzyme may be assayed by observing the exchange of  $\text{P}^{32}$  with the terminal P of nucleoside diphosphates.

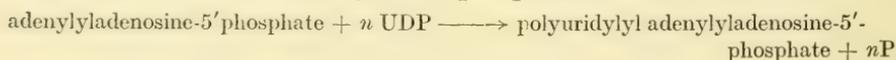


(XXXIII)

The reaction, as shown in formula (XXXIII), will proceed with each of the nucleoside diphosphates, with the exception of guanosine diphosphate, or with a mixture of all the diphosphates, including that of guanosine. In the former instance, the synthetic product is a high molecular polynucleotide comprised of a single type of nucleotide, bound in the chain by 3'5' phosphodiester linkages (Heppel *et al.*, 1957a). In the latter case, the synthetic polynucleotide product contains a mixture of all of the bases, as in natural RNA (Heppel *et al.*, 1957b). Synthetic polynucleotides containing a single base are rapidly phosphorylated in the presence of the enzyme; natural RNA and synthetic polynucleotides containing four bases are slowly phosphorylated (Ochoa, 1957). In the latter case it has been suggested that the enzymatic resistance of these substances arise from their existence in solution as multi-stranded chains.

The ready reversibility of the polynucleotide phosphorylase reaction revealed that the free energy change ( $\Delta F$ ) in hydrolysis of the pyrophosphate of the nucleoside diphosphate is of the same order as the  $\Delta F$  of cleavage of the phosphodiester linkage in RNA. The existence of the hydroxyl at C<sub>2</sub> of ribose presumably determines this property of the polyribotides, since the enzyme is inactive with DNA or deoxynucleoside diphosphates. In addition to its presence in bacteria, the enzyme has been found in small amounts in spinach leaf, and yeast (Heppel and Rabinowitz, 1958) and a suggestion of its presence in the nuclei of guinea pig liver has been reported (Hilmoe and Heppel, 1957). In the latter instance the phosphorylation of adenine polynucleotide was detected but, starting with ADP, a net synthesis was not observed.

The purification of the enzyme has revealed that the addition of a nucleoside diphosphate to form polynucleotide requires the presence of a primer (Mii and Ochoa, 1957; Singer *et al.*, 1957). Thus, the polymerization of UDP showed a lag, abolished almost specifically by polyuridylic acid. Polymerization of ADP and CDP required polyadenylic acid and polycytidylic acid, respectively. The primer must be at least a dinucleotide in length and appears to be incorporated into the polymer that is produced. The primer provides the terminal nucleotide ending in a 5'-phosphate. Thus:



In a comparable phenomenon, polynucleotides were degraded by the enzyme to "limit polynucleotides" consisting of a dinucleotide or a dinucleoside monophosphate, depending on the substitution on the terminal nucleoside.

Physical measurements of the synthetic polynucleotides have revealed particle weights in the range of  $10^5$  to  $10^6$ . However, end-group analyses have indicated markedly lower weights for the same polymers. This appears to arise as a result of aggregation in solution, a result confirmed by study of the

ultraviolet absorptive properties of the natural and synthetic nucleic acids (Warner, 1957). Hydrogen bonding between bases in the polynucleotides restricts resonance in these structures. As shown by Rich and Davies (1956) and Warner (1957), synthetic polyadenylic acid and polyuridylic acid in 1 : 1 ratio spontaneously aggregate to form a stable, double-stranded compound of lowered absorptive properties, which provides an interesting model for natural, double-stranded helical DNA.

If the proportions of polyadenylic acid and polyuridylic acid are varied in the presence of 0.01 *M* MgCl<sub>2</sub>, it was observed that a stable complex forms at a ratio of 1 polyadenylic acid to 2 uridylic acid (Felsenfeld and Rich, 1957). Thus, a triple stranded compound may be formed in which poly U may fill the helical groove of the normal double-stranded helix. It has been suggested that this may be a prototype for the entrance of a single RNA chain into the helix of a double-stranded DNA, and perhaps even for its formation at such a site.

If functional within the cell, the polynucleotide phosphorylase may be expected to produce free RNA polymers. As noted earlier, with very few exceptions the nucleic acids are associated with protein within the cell. Only a single case is known of the accumulation of free RNA polymers; relatively large particles of free RNA have been found in some plants (Lindner *et al.*, 1956).

### 3. DNA Synthesis

Kornberg (1957a,b) and his collaborators have isolated and purified an enzyme from *E. coli* which converts a mixture of deoxynucleoside triphosphates to DNA, splitting off terminal pyrophosphate in the process (Kornberg *et al.*, 1956). The reaction will not occur with diphosphates and requires the triphosphates of all four nucleotides found in DNA. It will not occur to a significant extent in the presence of a single nucleoside triphosphate nor will ATP replace deoxyATP. Of great interest is the requirement for a primer of high molecular DNA. It is thought that such primer DNA may be acting as a template in the model proposed by Watson and Crick. Also that the nucleoside triphosphate may add to the sugar end of the polynucleotide with displacement of inorganic pyrophosphate, in a manner analogous to the relation of primer to the synthetic reaction catalyzed by polynucleotide phosphorylase. Unlike the latter reaction however, DNA synthesis is essentially irreversible, although a very slight exchange of P<sup>32</sup>-P<sup>32</sup> with triphosphate occurs in the presence of DNA, Mg<sup>++</sup>, and enzyme. Synthetic DNA may attain a particle size comparable to natural DNA.

The requirement for deoxynucleoside triphosphate has been suggested to account for the absence of uracil in DNA. Thus, thymidylic kinase, which converts thymidylic acid in the presence of ATP to thymidine triphosphate, is inactive with deoxyuridylic acid (Friedkin and Kornberg, 1957).

The synthesis of the nucleic acids by the two enzymes described above may be compared in Table XII.

TABLE XII  
SYNTHESIS OF NUCLEIC ACIDS

Substance synthesized	RNA	DNA
Substrates	One or more ribonucleoside diphosphates	All four deoxyriboside triphosphates
Energetics of the reaction	Readily reversible	Essentially irreversible
Other requirements	Mg <sup>++</sup> , low molecular poly-ribotide	Mg <sup>++</sup> , high molecular DNA
Product sensitive to	RNAase	DNAase

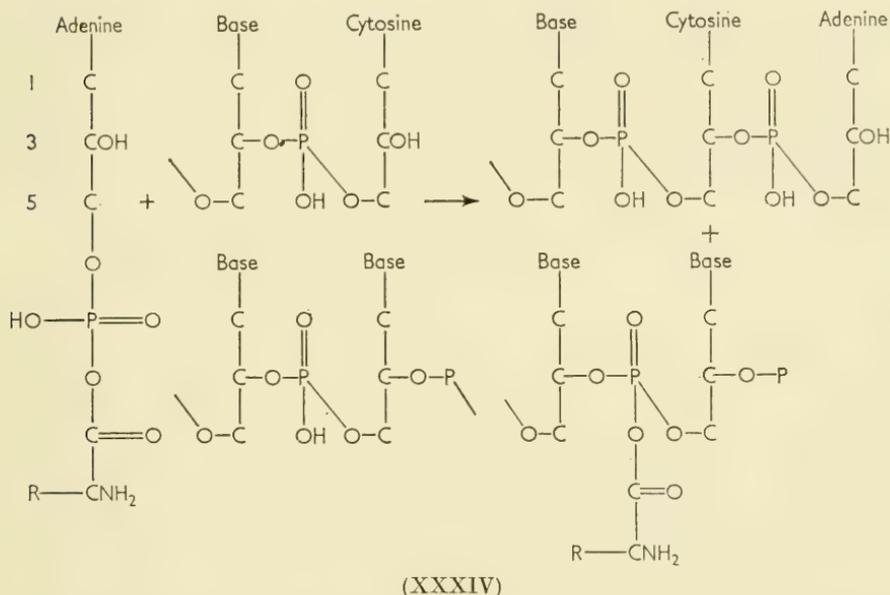
#### 4. Nucleic Acid Synthesis in Extracts of Animal Tissues

Although thymidine may be incorporated into DNA by thymus nuclei (Friedkin and Wood, 1956) and by homogenates of regenerating rat liver (Bollum and Potter, 1957), it is not known if these reactions involved the intermediation of the triphosphate mechanism described above. The purine precursor, 4-amino-5-imidazolecarboxamide, was also shown to be incorporated into the purines of DNA and RNA in a pigeon liver homogenate (Noguchi and Miura, 1956).

A number of other workers have demonstrated the incorporation of nucleotides into RNA in cell-free homogenates of animal tissues. In the incorporation of orotic acid into rat liver homogenates, a 5' nucleotide rather than a 3' nucleotide appeared to be a precursor (Herbert *et al.*, 1957). Low levels of ATP were essential and incorporation was proportional to the amount of microsome fraction added. Mitochondria were unnecessary, if ATP were supplied exogenously. Canellakis (1957a) has observed an incorporation of uridine-5'-phosphate into RNA in a particle-free cytoplasmic fraction of rat liver.

Several groups have demonstrated a terminal incorporation of ATP into RNA in soluble systems. Canellakis (1957b) has shown that AMP was linked exclusively to a terminal monoesterified cytidylic acid of RNA, a result obtained earlier by Heidelberger *et al.* (1956), who demonstrated that such a moiety is liberated as adenosine following alkaline cleavage. The terminal addition to RNA chains of AMP derived from ATP was similarly observed by Zamecnik *et al.* (1957) and Paterson and LePage (1957). Edmonds and Abrams (1957) have evidence for two types of incorporation of AMP from ATP into RNA.

In experiments involving the incorporation of ATP, it is suspected that this reaction is the consequence of the intermediate formation of the adenylates of amino acids. In the utilization of such compounds, the transfer of amino acids to the intermediate soluble RNA fraction is perhaps accompanied by a simultaneous transfer of the adenylic acid to the end of another RNA chain, as shown in formula (XXXIV).



In support of this view may be mentioned the experiment to show that trace amounts of amino acid are required in chloramphenicol-inhibited bacteria for the synthesis of RNA, but not of DNA (Gros and Gros, 1956). In an interesting study by Webster (1957b), it was found that ribonucleoprotein particles isolated from pea seedling incorporate various RNA precursors, such as adenine and uracil, in the presence of mitochondria, an oxidizable substrate, ribose-5-phosphate, ATP, and  $Mg^{++}$ . Incorporation was promoted by a mixture of amino acids and was inhibited by amino acid analogs and by hydroxylamine. These inhibitors did not prevent the early stage of nucleotide formation.

## VII. PROBLEMS OF POLYMER DUPLICATION

For the purposes of this discussion we shall confine our attention to the duplication of nucleic acids and proteins. Viral nucleic acids, both DNA and RNA, can be infectious, and in infected cells compel the synthesis of viral nucleic acid and viral protein. Therefore, these nucleic acids contain, within

their arrays of chemical structures and interconnecting bonds, codes which orient in some more or less direct mechanism the specific assembly of nucleic acids like themselves and specific proteins, as well.

### *A. DNA Duplication and Partition*

In the duplication of DNA, the Watson-Crick hypothesis postulates that the array of nucleotides in one helical chain of a duplex determines the alignment of complementary nucleotides, which then combine to form another helical chain to complete the duplex. A number of chemical facts have appeared which present some difficulties for this duplication mechanism. It is known that in the DNA of animal and plant cells, both 5-methyl cytosine and cytosine fill the cytosine complement of the polynucleotide chain. Furthermore, 5-methyl cytosine is usually associated with guanine in dinucleotide sequences. The problem of forming a chain in which these bases are specifically placed has evoked the hypothesis that in some instances the DNA precursors may be di- or polynucleotides (Crick, 1957b); such a postulate has been challenged in turn (Chargaff, 1957).

In T-even phage DNA, cytosine is completely replaced by 5-hydroxymethyl cytosine (HMC). However, in these viral DNAs, the hydroxymethyl group is glucosylated to varying degrees, depending on the inheritance of the phage. Thus, although bacteriophage T4 appears to contain only a monoglucosylated HMC, T6 contains HMC nucleotides with 0, 1, or 2 glucose moieties (Cohen and Lichtenstein, unpublished data). There is no indication how the existence of a 6-hydroxyl on complementary guanine can select among these three types of HMC nucleotides, if indeed such a selection is essential in the determination of genetic specificity of T6 DNA.

Watson and Crick (1953) have suggested that the synthesis of the complementary chain to form the duplex may occur along both unwinding chains, in a kind of zipper action at the unwinding point. One of the difficulties involved in this hypothesis is the difficulty of separating chains, since these are wound around each other in many turns. A number of ways of separating the chains has been suggested (Delbrück and Stent, 1957), including unwinding by a variety of methods or by breaks and reunions. In the latter case, one might even imagine whole lengths of one chain digested away to permit synthesis on the exposed chain; however, this is difficult to reconcile with the observed conservation of DNA.

A proposal of Delbrück (1954) conceives of breaks and rejoining to occur in limited segments of the duplex during the replication process. However, his mechanism involves a thorough mixing of sections of parental and daughter nucleotides in the same chain, and recent data appear to exclude this possibility (Meselson and Stahl, 1958).

In the experiments of Meselson and Stahl, *E. coli* was grown in a medium containing  $N^{15}$ , and then transferred to an  $N^{14}$  medium to continue its growth. The culture was sampled (1) when the bacteria were fully labeled in  $N^{15}$ ; (2) when they had divided once in  $N^{14}$ ; and (3) when growth continued for a number of divisions in  $N^{14}$ . DNA samples were isolated from each of these types of bacteria and analyzed by a sensitive density-gradient ultracentrifugation technique capable of distinguishing  $N^{15}$ - and  $N^{14}$ -DNA. In the fully labeled bacteria, only  $N^{15}$ -DNA was found. After one division, only one species of DNA was obtained containing equal amounts of  $N^{15}$  and  $N^{14}$ , implicating the involvement of both parental chains in the formation of both daughter duplexes. However, in all subsequent divisions, two species of DNA were obtained, one containing  $N^{14}$  alone, and one containing equal amounts of  $N^{15}$  and  $N^{14}$ . Thus, it appeared that single chains remained intact from generation to generation, or, if parts of a strand were broken during duplication, they rejoined to form the original strand.

Although these experiments tend to support the picture of the duplication of DNA as projected by Watson and Crick, it is not yet entirely certain that such duplication does not involve the intermediation of a second template. A direct duplication mechanism is also supported by the experiments of Hershey and Melechen (1957) on phage DNA production in the relative absence of protein synthesis. However, it may be suggested that if DNA can orient the synthesis of a specific protein or RNA, it is conceivable that these structures in turn may provide a template for the synthesis of DNA chains. The data to support the latter possibility are very sparse and stem primarily from an experiment on the effects of infection with  $P^{32}$  phage containing very highly radioactive  $P^{32}$  (Stent and Fuerst, 1955). When  $P^{32}$ -labeled cells were infected in  $P^{32}$  medium with  $P^{32}$  phage, on immediate storage at  $-197^{\circ}\text{C}$ . the ability of the infected cells to produce phage could be shown to decrease as a function of radioactive decay of the  $P^{32}$  within the phage. After a few minutes of intracellular phage development, however, the ability of these cells to produce phage became independent of radioactive decay. One hypothesis to explain this result suggests that the code for phage production was transferred to moieties free of decaying  $P^{32}$ , perhaps to protein.

A number of workers have attempted to implicate protein in the replication process in another way. The X-ray analyses of DNA, as summarized by Wilkins (1956) and Crick (1957a), have revealed a configuration (the B configuration which exists *in vivo*) in which there are two smooth helical grooves, one shallow and the other deep. In nucleoprotamine, the polypeptide chain of protamine is regarded as winding helically in the shallow groove around the DNA molecule, which maintains the double helical structure, as presented in Fig. 28a. The known heterogeneity of histone necessarily complicates the determination of structure of nucleohistone, but a somewhat similar view of

the structure of nucleohistone has been obtained by X-ray analysis. However, Wilkins (1956) believes that in this instance histone is wrapped around both grooves of the DNA, as indicated in Fig. 28b.

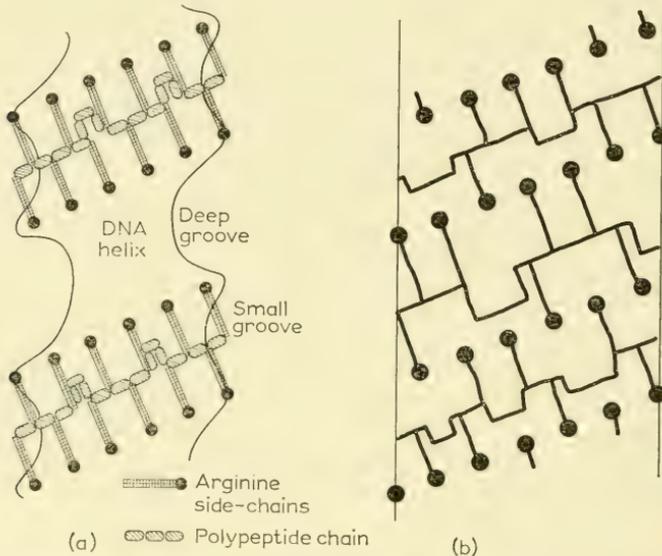


FIG. 28. (A) shows how protamine binds to DNA. The polypeptide chain winds around the small groove on the DNA helix. Phosphate groups are at the black circles and coincide with the basic ends of the arginine side chains (Wilkins, 1956).

(B) shows a possible way histone might bind to DNA. The lines trace the polypeptide chain, the folds in it, and the side chains of basic amino acids which are associated with phosphate groups (Wilkins, 1956).

Starting with this view of deoxyribonucleoprotein structure, Bloch (1955) has proposed a mechanism whereby there are a separation and rotation of bases prior to replication, with the histones holding the separated deoxynucleotide chains of the parent duplex in position during the alignment and polymerization of complementary nucleotides. It will be recalled that it has been shown that the synthesis of histone occurs simultaneously with that of DNA (Bloch and Godman, 1955; Alfert, 1957), and this would lead to the formation of four polynucleotide plus polypeptide strands. It is suggested that the specific configuration of the DNA chain may also thereby determine the structure of the newly synthesized histones. Separation of these relationally coiled complexes organized in two twisted strips can be effected as a result of phenomena comparable to the chromosome coiling which takes

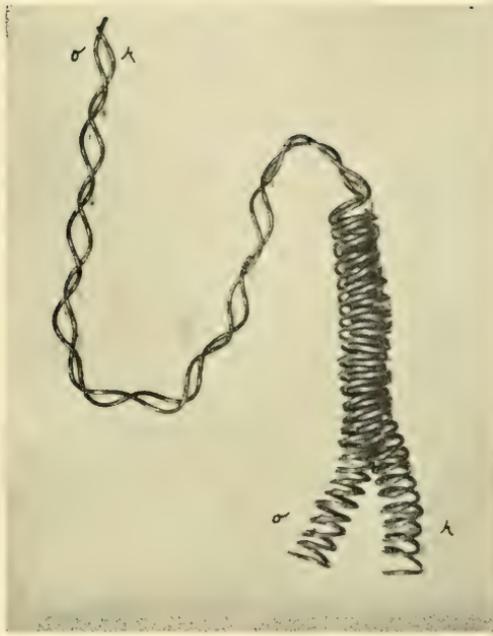


FIG. 29. Possible relationship between original and replicate complexes during the late interphase stage after duplication but before separation (upper left) and the mitotic stages as separation takes place (lower right) demonstrating how chromosome coiling could effect the separation of the two strands (Bloch, 1955).



place during prophase prior to cell division. Thus, the twisted strips can be separated from each other by winding the helical strips about an axis perpendicular to the axis of the helix, as presented in Fig. 29.

One consequence of such a mechanism is the suggestion that during separation of daughter chromosomes newly synthesized DNA goes to one chromosome and the original to the other. The absence of mixing, indeed, has been demonstrated by the experiments of Meselson and Stahl. However, an astonishing set of observations has revealed that in the division process all of the newly synthesized DNA of a chromosome enters one chromosome and is separated from the parental DNA in another. Such a result has been obtained by Taylor *et al.* (1957), who labeled the DNA of bean root by growth in tritium-labeled thymidine and followed the fate of the chromosomes by autoradiography after subsequent growth in unlabeled media. Thus, it is clear that chromosome structure involves functional association of the separate nucleoproteins in such a way as possibly to extend the Bloch molecular mechanism to the replication and division of the entire organelle.

Kacser (1956) has suggested a mechanism for the replication of nucleoprotein in chromosomes, in which parental protein is combined with new nucleic acid and parental DNA is combined with new protein. A double-labeling experiment, performed as described by Taylor, should be able to test this hypothesis.

One possible role of the basic protein in chromosomes has been considered to be that of neutralizing the charge of the polyanion nucleic acid. Anderson has discussed this question in great detail (Anderson, 1956a,b), suggesting that the division mechanism arose initially as a consequence of the cyclic changes in the rate of formation of nucleic acids and proteins. He has proposed that the fundamental mechanism of cell division is a consequence of the cyclic changes in the ratio of charges on cellular colloids and has shown that much of the data on division is compatible with this view. Of interest from this point of view are the well-known effects on division and nuclear structure of a variety of polycations, such as protamine, spermidine, etc., and polyanions, such as heparin. Of great importance in this connection is the developing concept in virology of the synthesis of independent pools of viral polymers, as in the proposal of the independent duplication of phage DNA by Hershey and Melechen (1957) and of tobacco mosaic virus RNA and protein. In the latter case, a mechanism of interdigitating polymerized protein and polymeric RNA has clearly been developed; however, comparable mechanisms of terminal organization are not yet known in other systems. The normal roles of basic compounds, such as putrescine, spermine, etc., in cell division and virus multiplication might well be explored from this point of view.

## *B. The Transfer of Biological Information*

### *1. DNA as a Template*

How is the code contained within DNA used as a template for the synthesis of RNA and protein? It has been seen that protamines and histones may wrap around DNA in such a way as to suggest a selection of residues which will fit the grooves made available in the helical coil. The closeness of fit which this complementarity requires is not clear; no evidence for the biological specificity of protamines or histones has yet been obtained. In any case, there appears to be a synthesis and turnover of these basic proteins to an extent no greater than that of DNA, and the chromosomal location of these substances would seem to limit their utility in extending the control of genetic material to the physiological sites.

It has been seen that a third strand of RNA may form a triplex within a duplex of RNA, and it has been suggested that the synthesis of specific RNA occurs in this manner within the duplex of DNA at the chromosome site. Such a hypothesis is usually extended to imply that the nuclear genetic substance (DNA) controls the production of RNA in the nucleus, which then determines the synthesis of essential proteins in other parts of the cell, notably in the cytoplasm.

This mechanism of RNA synthesis does not necessarily require a concomitant synthesis of DNA during the reproduction of the carriers of physiologically useful information. One particularly good case in support of this concept is known in the work of Watanabe (1957) on the control of synthesis of phage protein. If T2-infected bacteria are irradiated with ultraviolet light a few minutes after infection and insertion of phage DNA, DNA synthesis does not occur but the synthesis of phage antigens continues. Initial irradiation of phage or infected bacteria eliminates the ability to synthesize these proteins. Thus, the information necessary for protein synthesis has been transferred to some unit of lower sensitivity to ultraviolet irradiation than has phage DNA, and this transfer occurs before the duplication of DNA.

In general, it is supposed that the use of DNA as a template will require the complementarity or closeness of fit discussed, for example, in connection with the Watson-Crick model or by Pauling (1955) and other workers in considering antigen-antibody reactions and antibody synthesis. These may lead to the formation of complementary nonidentical structures, such as the strands of DNA, and possibly to the formation of complementary identical structures, as in the aggregation of chains of polyadenylic acid which hydrogen bond through adenine moieties, leading to a restriction of resonance and ultraviolet absorption of these residues. The latter mechanism has been discussed, not only by Donohue and Stent (1956) for RNA, but had been proposed many years earlier in connection with possible mechanisms of protein

duplication. More recently, the theoretical problem of the specificity of interaction between identical molecules in general has been explored as a possible function of the charge interaction between electric dipole oscillators (London forces) (Jehle, 1950, 1957; Yos *et al.*, 1957).

It has been suggested that these forces lead to a template action resulting in the direct assembly of identical molecules and can help to explain gene stability, specificity, and assembly; in a word they may truly produce "self-duplication." However, it should be noted that, as a consequence of the work of Yos *et al.* (1957), they consider it unnecessary and undesirable to invoke the splitting of the DNA duplex into separate daughter chains; this result has already been obtained in the work of Meselson and Stahl (1958).

In earlier sections we have considered the question of the stability of DNA and its apparent lack of turnover. Notable exceptions are the cases described by Zamenhof *et al.* (1956a, b) and by Coughlin and Adelberg (1956) as a consequence of thymine deficiency. In an attempt to explain the obvious function of DNA and its apparent stability in some dynamic mechanism, a hypothesis was presented in Fig. 25 of a partial cleavage of phosphodiester bonds in portions of a polynucleotide chain, with the simultaneous formation of amino acid anhydrides. Such derivatives might subsequently condense with other amino acids, releasing phosphate for recondensation with the previously freed 3' hydroxyl in the polynucleotide chain.

That DNA does help directly to organize amino acids into proteins is suggested by: (1) the concomitant synthesis of histone during DNA synthesis in normal interphase, and (2) the abolition of nuclear incorporation of amino acids into protein as a result of the action of deoxyribonuclease. A remarkable related observation of Brunish and Luck (1952) has not yet been explained. Nucleohistone of liver was capable, albeit rather slowly, of incorporating carboxyl-labeled amino acids in a form which did not give rise to radioactive carbon dioxide through the use of ninhydrin, implying the formation of peptide bonds.

It is important to note that little or no evidence is available to support any of the possible mechanisms described above concerning the mode of action of DNA. However, these mechanisms, particularly modifications of that involving the formation of intermediate amino acid nucleotide complexes, are now being discussed widely to attempt to explain the way in which RNA directs protein synthesis.

## 2. RNA as a Template

That RNA can control the synthesis of RNA directly in all cases is not entirely certain. Although cytoplasmic RNA can be synthesized in enucleate *Acetabularia*, the data on enucleate *Amoeba* suggest the contrary conclusion.

In addition, increasing evidence, although as yet sparse and unconfirmed, have indicated a nuclear involvement in the multiplication of some RNA viruses, such as influenza and poliomyelitis. It would therefore be important to know if RNA viruses can multiply in enucleate cells or if other evidence can be obtained to eliminate the participation of DNA in such multiplication.

The conclusion that RNA can control the synthesis of protein stems from the following classes of data:

a. Protein is made in enucleate cells lacking DNA; its synthesis is inhibited by ribonuclease treatment of such cell fragments.

b. Inhibition of RNA synthesis in genetically blocked, nutritionally deficient, or otherwise inhibited systems blocks protein synthesis.

c. Protein synthesis is most active in cell organelles possessing the highest RNA content.

d. Protein synthesis *in vivo* involves the formation of amino acid adenylates, the intermediate transfer of the amino acids to RNA, which then shuttles them to the ribonucleoprotein portions of the microsomal fraction. Amino acid incorporation is prevented by the action of ribonuclease.

Amino acids play a catalytic role in RNA synthesis in cells and cell fractions. In cells a gross inhibition of protein synthesis by chloramphenicol does not inhibit RNA synthesis, but the lack of availability of an amino acid can eliminate this function. It may be inferred that the amino acids are essential for some stages of nucleotide activation or transfer, as has indeed been reported.

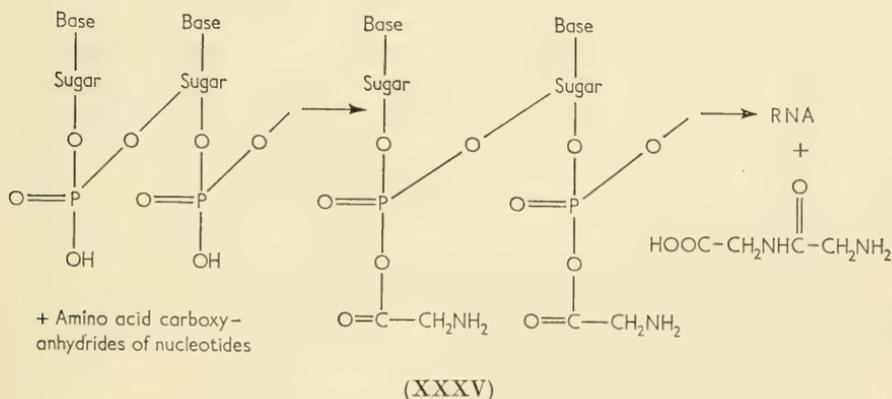
Specific enzyme production in cell fragments (protoplast membranes) almost devoid of DNA has been reported by Spiegelman (1957). Ribonuclease, but not deoxyribonuclease, destroys the ability of the fragment to produce  $\beta$ -galactosidase. However, since the fragments retain the ability to synthesize DNA under conditions of enzyme synthesis, the significance of these very interesting results is obscured.

Some workers have suggested that the primary template in protein synthesis is protein itself (Haurowitz, 1950). Since its duplication must occur in an unfolded state, it is supposed that the role of the nucleic acids is merely to maintain the template protein film in the expanded state. However, the activity of transforming DNA and the infectivity of viral nucleic acids affirms the specificity of these substances. It will be assumed in this discussion, then, that RNA may be a primary template and that the RNA in microsomal preparations plays a specific role in organizing the synthesis of specific arrays of peptides.

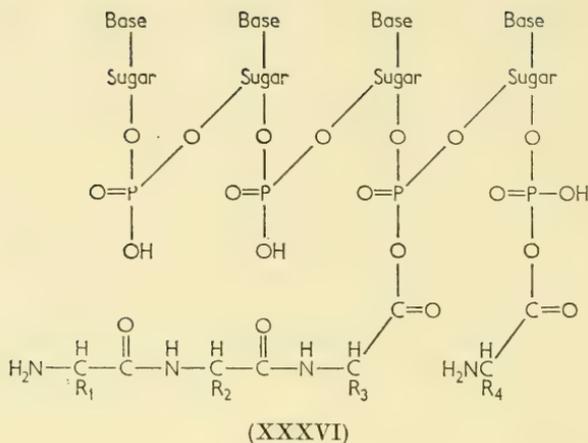
The following variations have been proposed concerning the mode of action of the RNA template in organizing protein synthesis:

a. Lipmann (1954) initially proposed an activation of specific sites of some unspecified template structure by transfer of the pyrophosphoryl group from ATP. This exchanged with the carboxyl of an amino acid whose nature was determined by the specific surface. In a later step, the bound carboxyl-activated amino acids spontaneously condensed to form a polypeptide chain, freeing the surface for a renewed activation.

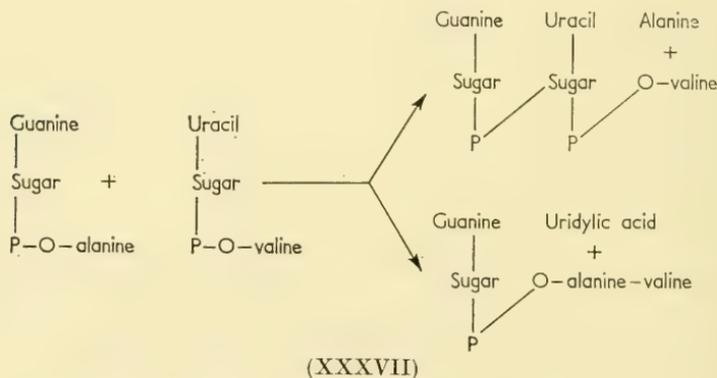
b. Borsook (1956) proposed that the amino acids were linked via carboxyl groups to the phosphates of the nucleic acid. The amino acids were then condensed to polypeptide by the action of some enzyme. In the similar mechanism of Novelli and DeMoss (1957), which takes into account the known data on initial amino acid activation and subsequent transport of bound intermediates, it is suggested that the bound amino acids spontaneously condense when the template is fully charged or a mechanism exists for unidirectional condensation (Dalglish, 1957). Spontaneous condensation is facilitated by the proximity ( $2.2\text{\AA}$ ) of a carboxyl group of one amino acid to the amino group of another and is presented in formula (XXXV).



The schema presented above indicates the formation of a dipeptide, glycylglycine. However, if the amino acids were present along the entire chain, the formation of peptides and consequent liberation of the amino acids from the polynucleotide chain might proceed with a single amino acid fixed to a nucleotide. In this way, it is conceivable that the RNA template might possess a number of polypeptides, in various stages of completeness, dangling from the same polynucleotide chain. Such a possibility, in part presented in formula (XXXVI), has been proposed by Dalglish (1953) and by Borsook (1956), and indeed might be thought of as supported by the work of Koningsberger *et al.* (1957).



The transfer of amino acid to template RNA from a bound amino acid anhydride of a nucleotide may also be conceived to proceed with the simultaneous transfer of nucleotide to the end of an unsubstituted polynucleotide chain. This mechanism is suggested in Fig. 25 for the closure of breaks in polynucleotide chains, but it may also be of primary interest in the extension of polynucleotide chains. Many variations of such a process can be postulated, although the existence of free intermediates, other than the adenylates, is not yet known. However, one such mechanism has been suggested by Simkin and Work (1957), and is shown in formula (XXXVII).



Implicit in this mechanism is the concept of a specific relationship between bound amino acid and nucleotide, as well as the idea that this relationship will permit the synthesis of specific RNA and polypeptide sequences. However, the number of different kinds of nucleotides (4) are evidently inadequate for the number of amino acids involved.

c. Potter and Dounce (1956), have reported on the existence of alkaline stable amino acid-nucleotide complexes in isolated RNA preparations and consider these to imply the presence of amino acids bound in phosphoamide linkage. Dounce (1952) has suggested that such compounds are the intermediates in polypeptide synthesis. The amide group of one bound amino acid would be transferred to the free carboxyl of a second, adjacent, bound amino acid by a mechanism analogous to that proposed by Borsook for carboxyl-bound amino acids.

### 3. *The Problem of Polypeptide Order*

The mechanisms of protein synthesis outlined above do not account for the specificity of the polypeptide chain and serious thought is beginning to be given to the problem of how a polynucleotide might determine amino acid sequence. Existing evidence appears to suggest that all possible dipeptide sequences may exist in proteins, although many fewer than the possible 400 dipeptides (assuming 20 different L-amino acids) have so far been found (Gamow *et al.*, 1955; Brenner, 1957). Similar results have been obtained with RNA and DNA, although, as noted earlier, in the latter case, 5-methyl cytosine and guanine deoxyribotides are associated as a dinucleotide in a decidedly nonrandom fashion. The lack of restriction to pairing of all amino acids or of all nucleotides imposes certain requirements on coding systems, while the frequency of pairing in these polymers may help in describing specific properties of both the polymers and the coding systems. It is of interest, in connection with the latter point, that the digestion of tobacco mosaic virus RNA by ribonuclease had led to the production of fragments whose relative contents of purine and pyrimidine residues are essentially similar to those expected if the same numbers of these bases were randomly ordered in a polynucleotide (Hart, 1957). This is only taken to mean that order in this RNA template involves something more complex and more difficult to discern than the repetition of a code of a few residues.

In nucleoprotamines, the ratio of amino acid to nucleotide approaches 1 : 1. However, in the more complex nucleohistone, it approaches 2 : 1. In the residual ribonucleoproteins of the microsomal fraction after treatment with sodium deoxycholate, the ratio also approaches 2 : 1. If it is assumed that in the latter case these amino acids are not part of dangling polypeptides but are as closely associated with RNA, as histone is with DNA (Chargaff *et al.*, 1956), such a relationship appears to exclude a simple 1 : 1 relationship whereby a given nucleotide in a sequence determines the fixation of a single amino acid. Such a relation, which does exist in nucleoprotamine, had been widely used as a working hypothesis because of the similarity of the internucleotide distance in DNA to the spacing between amino acid residues in an extended polypeptide chain.

In addition, if there were such a simple relation, it might be imagined that the RNA or even ribonucleoprotein of a cell elaborating proteins containing an abnormal concentration of some amino acids would display related abnormalities in nucleic acid composition. However, the silk gland of the silk moth *Philosamia ricini* contains proteins having about 45 % glycine. The RNA of this gland possessed a base composition very much like the RNA of the gut of the same animal, which had very different types of protein (Matthews, 1957). Similarly, although the RNA of turnip yellow mosaic virus contains 37 % cytidylic acid, the protein of this virus did not possess a particularly abnormal gross amino acid composition.

Although the ribonucleoprotein of the silk gland of the silkworm, *Bombyx mori*, has an amino acid composition unlike silk fibroin, which contains about 80 % of glycine, alanine, and serine, these amino acids are, nevertheless, *N*-terminal in the nucleoprotein (Shimura *et al.*, 1956). It is conceivable that only a part of the ribonucleoprotein isolated may act as template for the fibroin molecule and that one should not expect to detect a stoichiometric relationship between the final, dissociated, accumulated product and the working sequence of nucleotides which may exist as only a small part of a template.

As discussed by Gamow *et al.* (1955), more than one nucleotide must be used to code each amino acid, since there are only 4 nucleotides for 20 amino acids. A variety of triplet codes have been devised in which a set of three nucleotides determines a single amino acid, but a number of these were easily excluded (Gamow *et al.*, 1955). Brenner (1957) has now demonstrated that 64 overlapping triplets, made possible by the choices among 4 nucleotides taken 3 times, are insufficient to code the known amino acid sequences. This result also appears to exclude the concept of a 1 : 1 relation of amino acid to nucleotide along a single duplex of one chain each of polynucleotide and polypeptide, even as suggested by the analytical results.

A number of other approaches to the problem have been set forth. In one complex proposal, the use of nonoverlapping triplets of nucleotides as diffusible trinucleotides is suggested to code for and be associated with a single amino acid, the entire sterically specific complex of which might then attach to an RNA template, polymerize amino acids, and dissociate polypeptide (Crick *et al.*, 1957). There is no *a priori* reason at present to exclude complex mechanisms on this question, Occam's razor notwithstanding. Certainly the discovery of the role of soluble RNA in transferring amino acids to the microsomal fraction appears consistent with this proposal.

## VIII. CONCLUSION

This survey, as fragmentary and lacking in depth as it may be, provides the biochemical framework within and on which the modern virologist

should develop his data. The analysis of the trinity, DNA, RNA, and protein, in terms of structure, distribution, interactions, and biosynthesis, is clearly far from complete. Nevertheless, the rate of increase of detailed knowledge on these questions is almost virus-like in its rapidity. It should be evident from this discussion that biochemistry has moved considerably beyond the study of respiratory rates of intact cells, on the one hand, or the study of single enzymatic reactions, on the other. Biochemistry is no longer the chemistry of natural products or the mere dissection of enzymatic sequence. At the cellular level, both of these aspects are sufficiently developed to warrant an approach to the problem of molecular behavior and interaction in functional cellular systems.

No biological problem presents greater opportunities for such a biochemical analysis than does that of virus multiplication, in which the critical problems are those of polymer characterization, activity, and biosynthesis. As the preceding survey should indicate, these subjects are now among the most active areas of biochemical investigation with all types of cells, and in this sense the problems of biochemical virology have finally merged with the major problems of cellular biochemistry. However, few biochemists and few virologists operate in recognition of the existence of this development. Few biochemists have elected to use the splendid biological systems and biochemical opportunities provided by virus-infected cells. On the other hand, few virologists accept the view that many of their major biological problems can now be posed in chemical terms and require answers at this level. It is doubtful that exhortation of biochemists to learn biology or of biologists to attempt chemical investigation themselves or through collaboration with biochemists will prove particularly useful. Nevertheless, it is hoped that this survey will help somewhat to the end of enticing workers into biochemical virology by making available much of the relevant data on their common ground, the cell.

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## Chapter III

### The Physical Properties of Infective Particles

HOWARD K. SCHACHMAN AND ROBLEY C. WILLIAMS

*Virus Laboratory, University of California, Berkeley, California*

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## I. INTRODUCTION

When it became apparent about twenty-five years ago that the agents of virus diseases were submicroscopic particles which could be purified and studied by methods of colloid chemistry, interest was initiated in the determination of their physical properties. The subsequent years have seen the development of many new methods for the study of colloidal systems, and as these have appeared they have, without exception, been applied to the examination of virus particles. Some of the methods have proved to be particularly suitable to the study of viruses; these are the ones that will be

discussed here. Other methods, although interesting and promising, will be omitted, or discussed only briefly, because of the relative rarity of their application.

When virus particles are harvested from infected tissues and purified it is found that they are composed mainly of a nucleoprotein and that their size range is between about 200 and 3000Å. The particles associated with a given disease are frequently found to be so uniform in size and shape that they can be conveniently termed macromolecules, but the spatial arrangements of their protein and nucleic acid portions are mostly unknown. Although we may hope eventually to discover something about the structure of viruses at the level of their amino acid and nucleotide configurations by applying the methods of organic chemistry, it remains true that most of the physical examinations of viruses have dealt with their properties as colloidal particles, either in the intact or disintegrated form. In this domain of size interest centers primarily in three physical parameters: shape, size, and mass. However, as will be discussed later, when shape and size are referred to there is no universally accepted concept as to what is meant by the words. They are always circumscribed in their meaning by consideration of the ways in which they are measured. Thus, the X-ray crystallographer determines the size of a virus as it exists in a unit cell of a crystal; the hydrodynamicist determines the effective size of the particle as it plows its way through a solution; the electron microscopist finds its shape and size, when dry, by direct photography. If virus particles were hard objects, like marbles, these distinctions among methods would be irrelevant, but a virus particle may be more like a sponge with water bound both internally and externally. It has amphoteric properties which may confer upon it different effective diameters, depending upon concentration and the ionic environment. We should not be surprised if we find that the same virus particles apparently have different shapes and sizes in the hands of different experimenters, all of whom have performed their work correctly. In addition to these inherent discrepancies there are the uncertainties arising from the difficulties of producing truly monodisperse suspensions of viruses. The presence of impurities, of dimerization, disintegration, and aggregation provides ample opportunity for apparent disagreements among the values obtained for shape and size. There is no *a priori* reason to believe that a virus suspension can be prepared in a way such that it is composed only of physically identical particles. Although, for example, bushy stunt virus is known to have a molecular weight of  $9 \times 10^6$ , to contain 16 % by weight of ribonucleic acid, and 8.1 % of the amino acid, threonine, all we know is that these values are averaged over billions of particles. Individual particles may differ from the average figures by several per cent. but we would be unaware of this fact.

Another parameter of viruses that interests physical chemists is their

molecular, or particle, weight. The use of the term "molecular weight" as applied to viruses is not acceptable to all, but in those cases where a high degree of physical homogeneity has been demonstrated in a virus suspension the use of the term is a convenience. At least two kinds of molecular weight can be distinguished; an anhydrous weight, and a weight that includes bound water. But ambiguity exists as to how "bound" water is defined, and with some techniques there is uncertainty as to how its presence might affect the determination of a molecular weight. Hydration (and its hydrodynamic effects) is one of the physical properties of viruses that is still the subject of vigorous investigation and discussion.

Discussed only briefly is a consideration of the movement of particles in a solution subjected to an electric field. Despite the great utility of electrophoresis in both the isolation of viruses and in the analysis of preparations of purified viruses, little is known about the relationship between the rate of migration of charged macromolecules and the number, nature, and location of ionizing groups on their surface.

Despite the somber implications of the above remarks we can form some sort of picture of a virus particle in suspension. It is an object that generally has a symmetrical shape, frequently spherical or nearly so. Within it there are polypeptide chains and nucleic acid polymers in, presumably, some regular spatial array. Throughout the whole particle, in the interstices between the chains, there are water molecules and some ions which are influenced in their distribution by the charged groups on the polypeptides and nucleic acids. The central region of the particle is probably filled only with fluid. The outer surface is certainly not smooth, and may consist of deep groovings and knobs. The surface has many charge groupings, and the water and ions of the environment are locally bound with differing concentrations, depending upon the type and nature of the groups. Outside the particle as a whole there is an ionic atmosphere that is radially nonuniform, extending for a distance perhaps as large as the radius of the particle and depending upon the ionic strength of the solution. This is the sort of object whose physical properties we are going to consider.

The last ten years have witnessed revolutions in many of the disciplines used in the characterization of large molecules, with the result that precision not previously contemplated is now commonplace and measurements hardly visualized ten years ago are routine. Thus many of the results on many types of macromolecules are obsolete, and new data will rapidly supercede those compiled with great labor over many years. Simultaneous with these advances in theory and technique have been improvements in preparative procedures. The methods employed now for the isolation and purification of viruses are often milder and faster than those used years ago, with the result that the deleterious effects of various chemical reagents and cellular enzymes are

reduced. Our criteria of purity are much more stringent than heretofore. The time is ripe, it seems, for a reexamination of many viruses previously studied. Therefore, the major part of this chapter is devoted to the fundamentals of methods themselves rather than to a compilation and evaluation of the large amount of available data on different viruses.

## II. PHYSICAL METHODS

### A. Hydrodynamic and Thermodynamic Methods

#### 1. Introduction

Often in the study of viruses, and particularly with the larger animal viruses, cursory examination of electron micrographs or ultracentrifuge patterns reveals evidence of polydispersity with respect to the size of the virus particles. Such variations in particle size often are not evident from measurements by certain techniques like viscometry or light scattering, and it is necessary, therefore, to consider average properties such as an average diameter or an average molecular weight. (We shall temporarily ignore the semantic paradox implied by referring to the molecular weight of a polydisperse material.) Polydispersity may arise from the presence in a preparation of a discrete number of monodisperse fractions, or it may result from a collection of particles ranging in size in a continuous manner. For the former, methods are available whereby each component can be considered separately and its size determined. In the latter situation, however, physical methods give some kind of *average* molecular weight, which average we now consider.

When a solution is polydisperse, it is convenient to think of it as consisting of a number (from one to infinity) of monodisperse fractions. If we designate any such fraction as the  $i^{\text{th}}$  component, the *number average* molecular weight may then be defined as:

$$M_n \equiv \frac{\sum n_i M_i}{\sum n_i} \quad (1)$$

where  $n_i$  represents the number (or number fraction) of molecules of the  $i^{\text{th}}$  type and  $M_i$  is the molecular weight of that component. The symbol,  $\Sigma$ , is a summation sign; i.e.  $\sum n_i M_i = n_1 M_1 + n_2 M_2 + n_3 M_3 + \dots$ . Since  $n_i M_i$  is the weight (or weight fraction)  $c_i$ , of the  $i^{\text{th}}$  component, the number average molecular weight can be expressed in the alternative form

$$M_n \equiv \frac{\sum c_i}{\sum c_i / M_i} \quad (1a)$$

In the determination of a colligative property of a solution, such as the osmotic pressure, every molecule contributes equally, regardless of its weight, and hence the relevant molecular weight is  $M_n$ .

In some physical determinations of molecular weight (as will be shown later) the quantitative effect of the presence of a molecule depends on the weight of the molecule. We then refer to the *weight average* molecular weight, defined as

$$M_w \equiv \frac{\sum c_i M_i}{\sum c_i} \quad (2)$$

It is apparent that some properties may depend on the square of the molecular weight or the length, for example. For such a case the so-called *Z-average* molecular weight is referred to:

$$M_z \equiv \frac{\sum c_i M_i^2}{\sum c_i M_i} \quad (3)$$

There are ways of obtaining  $M_z$  although these are less popular than the methods which yield  $M_w$  and  $M_n$ . It is important to note here that the characteristic dimensions of large particles determined from light-scattering measurements correspond to the *Z-average* dimension.

If a solution is monodisperse, the three molecular weights just defined will be identical. Conversely the degree of agreement of two or more of the average molecular weights provides experimental evidence indicating that the preparation is monodisperse. For instance, the number average molecular weight is obtained by a quantitative application of electron microscopy while the weight average molecular weight can be secured by sedimentation equilibrium or light-scattering studies. The ratio of weight to number average molecular weight can be used to calculate parameters describing the spread in molecular weights for certain types of distributions.

An important consequence of the possibility of obtaining two or more kinds of molecular weight on the same material lies in the implications of such results with regard to the biosynthetic mechanisms involved in the syntheses of the macromolecule. It is evident that a polydisperse product will result from polymerization processes involving random condensation reactions. The distribution of molecular weights resulting from such a process is termed the most probable distribution, and it can be shown that the ratio of  $M_w : M_n$  is 2 : 1 for this mixture. Other polymerization mechanisms have been shown to yield distributions for which  $M_w/M_n$  approaches unity. It would be expected that biosynthetic mechanisms involving templates would lead to a homogeneous collection of macromolecules. Thus the determination of the different types of average molecular weight may provide some insight about the mechanism of synthesis of the polymer.

## 2. Viscometry

*a. General Considerations.* Despite gross inadequacies in our knowledge of the structure of liquids and of the factors responsible for the wide variations

among the viscosities of various fluids, the effect on viscosity caused by the addition of small amounts of macromolecules is well understood both qualitatively and quantitatively. For virologists, viscometry can be particularly rewarding. In conjunction with other measurements it provides information about the size, shape, and hydration of the virus particles or other materials being investigated. Under special circumstances it is useful in the detection of impurities whose presence can be revealed by other methods only with great difficulty. Measurements can be made simply and rapidly; viscometry, as a result, finds wide application in the study of the kinetics of synthesis or degradation of macromolecules like viruses or nucleic acids. From these kinetic data inferences can be drawn about the structure of the macromolecules. Unlike most other techniques now widely applied in the study of macromolecules, viscometry can be practised with inexpensive apparatus most of which is still designed and constructed in the investigator's own laboratory. For certain types of materials, of which deoxyribonucleic acid is the paramount example, such apparatus is inadequate and recourse is made to elaborate, intricate instruments just becoming available commercially.

Viscometry as applied to solutions of macromolecules involves a comparison of the solution of interest with the pure solvent. From such measurements, the increment in viscosity is determined and this is related to properties of the solute. Rarely, if ever, is the absolute viscosity of the solution of interest. All theories for the viscosity of solutions originate with the definition of viscosity as a measure of the amount of energy required to maintain a certain rate of flow of the liquid. More commonly, viscosity is considered a measure of the resistance to flow. In pure liquids this internal resistance or friction is a function of the attractive and repulsive forces among the molecules of the liquid; these cannot be specified with sufficient exactness to furnish a reliable theory for the viscosity of pure liquids. Such considerations do not enter treatments of the viscosity of solutions, however, as is shown by the following brief discussion.

*b. Viscosity of Solutions.* When macromolecules are added to a solvent, the flow patterns normally present during the movement of the liquid are disturbed. Instead of the adjacent layers of liquid gliding over one another at slightly different rates, as in so-called laminar flow, the layers are forced to move around the particles which may be considered as obstructions. Owing to the differences in velocities among neighbouring stream lines, the particles are caused to rotate. Particles which are not rigid may be deformed. Thus, in maintaining the flow of a solution there is a dissipation of energy over and beyond that required for the production of flow of a pure liquid; this additional energy is reflected experimentally as an enhanced viscosity of the solution relative to the solvent. This problem was first investigated

from a theoretical point of view by Einstein (1906, 1911), who showed that the viscosity increment caused by the addition of rigid, spherical particles was simply proportional to the total volume fraction of the added material. In some respects this was a surprising result, showing as it did that the size of the spheres was unimportant, for many small spherical particles were equivalent to a few large spheres having the same total volume.

Einstein's treatment is restricted to solutions at concentrations sufficiently low that the regions of disturbed flow from individual particles do not overlap. Subsequently more detailed investigations of this hydrodynamic problem have permitted the extension of the Einstein equation to solutions of higher concentration by the addition of terms containing the second and third power of the concentration. Although there is not as yet general agreement on the values of the coefficients of these higher terms, uncertainties here are obviated by the device of extrapolating measurements at various concentrations to infinite dilution. In this way interaction effects are avoided and data can be interpreted according to the Einstein equation or a suitable modification of it. Implicit in the Einstein treatment was the assumption that the particles are uncharged. Of course most macromolecules of biological interest contain ionizable groups, and these cause electrostatic forces leading to higher viscosities than would be observed for uncharged molecules. To eliminate this, the electroviscous effect, it is general custom to conduct experiments in solutions containing electrolytes, such as buffer salts or sodium chloride. For most materials, 0.1 molar salt solutions suffice to damp out electrostatic forces which would otherwise render the Einstein theory inapplicable. Until recently the validity of this fundamental theory had been demonstrated only with particles of microscopic size, such as glass spheres or yeast cells; but the availability of uniform, spherical, polystyrene latex particles has permitted a critical examination of the theory with particles only slightly larger than many of the spherical viruses. As with the suspensions of glass spheres, the results with the polystyrene latex particles (Cheng and Schachman, 1955b) were completely in accord with the Einstein equation, thereby lending confidence to the application of this equation to solutions of spherical virus particles.

With the development of colloid chemistry, viscosity measurements achieved wide popularity and considerations were given to rigid particles of other shapes. Most popular of these are the ellipsoids of revolution. A prolate ellipsoid, resulting from the revolution of an ellipse about its long axis, serves as a model for rodlike particles. Rotation of the ellipse about its short axis gives an oblate ellipsoid which is a platelike object. The complicated hydrodynamic problem of determining the viscosity increment caused by such particles was solved in rigorous form by Simha (1940). For the calculations of Simha to be applicable, it is mandatory that the particles be

randomly oriented. Actually the very process of making a viscosity measurement tends to orient anisometric particles because the streamlines of the flowing liquid move at different velocities relative to one another. A particle having its long axis oriented in the direction of flow creates less disturbance to flow than does a similar particle oriented perpendicular to the direction of movement of the liquid. Anisometric particles, such as rodlike or plate-like objects, are subjected to rotary Brownian motion in solution; because of this rotation the effective volume occupied by the particles is much larger than their geometric volume. As a consequence, anisometric particles which are randomly oriented in solution cause a greater increase in viscosity than do spherical particles of the same volume. Thus differentiation of particles of spherical shape from those which are elongated is readily achieved by viscosity measurements.

Flexible synthetic macromolecules resulting from the polymerization of small units have also been treated theoretically in terms of their contribution to the viscosity of solutions. For these substances both free draining and impermeable models have been considered. Since virus particles are generally thought to be rigid, only models for such undeformable, impermeable structures are considered here; but it should be emphasized that the equations for these models are likely to be inapplicable to certain constituents of viruses, such as ribonucleic acid, and perhaps even to some unusual viruses. For a critical and thorough discussion of the viscosity of solutions of flexible macromolecules, the reader should consult Flory (1953).

All of the theories outlined above require knowledge of the volume concentration of the solute in terms of the kinetic or hydrodynamic unit. Seldom is the concentration known in that form. Instead, only the dry weight concentration can be determined satisfactorily. This causes a dilemma which can be handled in different ways, depending upon the availability of auxiliary information. If, for example, the electron microscope shows that the particles are spherical and the material is pure, the viscosity data are used to compute the volume concentration. This in turn is combined with the dry weight concentration to calculate the amount of water associated with the dry material in the kinetic unit. Some workers refer to this value as the hydration. In the absence of evidence that the particles are spherical, both the shape and effective volume must be considered as unknowns each of which can be evaluated from the appropriate combination of different hydrodynamic measurements.

*c. Measurement of Viscosity.* Of the various techniques employed in the measurement of the viscosity of solutions, that method based on the capillary viscometer has achieved the widest popularity. It combines simplicity with accuracy in a manner seldom experienced in physical chemical practice. Various designs have been proposed but the operating principle in all is the

same. The instrument is composed of a long, narrow glass capillary, of uniform bore, which connects a drainage bulb to a large reservoir chamber. A determination with such an instrument consists of a measurement of the time required for liquid to flow from the drainage bulb through the capillary into the reservoir. Usually the flow is maintained by the hydrostatic head of liquid since, in the construction of the apparatus, the drainage bulb is placed above the reservoir. Through the use of the Poiseuille equation, the viscosity of the liquid can be calculated directly from the measured volume rate of flow, the pressure head, and the length and diameter of the capillary. Such a determination, of course, requires precise knowledge of the various parameters of the instrument. Alternatively, the solution of interest is compared with a reference liquid such as water, and the absolute viscosity of the solution is obtained without detailed knowledge of the dimensions. Only the relative outflow times and relative densities are required if a standard liquid is employed. There are many precautions to be exercised in the design, construction, and use of a viscometer of this type and these have been the subject of detailed investigations. Only one factor remains as a deterrent to acceptance of this type of instrument for all substances. This is an inevitable consequence of the nature of flow through a narrow tube. The rate of movement of the liquid in the different streamlines varies across the tube in a parabolic manner with the maximum rate at the center and zero at the wall. Thus the shear gradient, defined as the rate of change of velocity of the layers of the flowing liquid with respect to the distance separating the layers, varies from zero at the center to some maximum value at the wall. It is this shear gradient (or velocity gradient) which is responsible for the orienting force on the anisometric particles; a precise treatment of the consequent orientation is difficult because of the wide variation in shear gradient across the tube. For spherical particles this limitation is trivial because all orientations of a spherical particle are equivalent. However, rodlike particles may be oriented to different extents depending on their exact location relative to the distance from the center of the tube. It should be noted that sufficiently low shear gradients can be achieved in capillary viscometers so that even the anisometric tobacco mosaic virus particles are not oriented preferentially during flow. Deoxyribonucleic acid, however, is oriented and special precautions are needed so as to allow extrapolation of the experimental results to zero shear gradient.

Although another type of instrument, the Couette viscometer, meets satisfactorily this one limitation of the capillary viscometer, it suffers from other defects. In this instrument the liquid is placed between two concentric cylinders, one of which is caused to turn by an externally applied torque and the other is held suspended from a thin torsion wire. The liquid which is set in motion by the rotation of the outer cylinder imparts a torque on the

inner cylinder, causing it to turn through an angle until the restoring moment created in the torsion wire offsets the moment resulting from the viscous drag of liquid. The viscosity of the liquid is readily calculated from measurement of the angle of twist of the suspension wire and certain apparatus constants. As with the capillary viscometer, relative viscosities are readily calculated without knowledge of the dimensions of the instrument. Different devices are available for measuring the twist of the wire. Theoretical consideration of the flow pattern of the liquid between the two cylinders shows that the velocity gradient is essentially constant if the gap between the cylinders is small. Most important, extremely low shear gradients can be achieved with ease whereas corresponding values in a capillary viscometer are virtually unattainable. It is regrettable that the Couette viscometer is so intricate, and that most designs do not permit sufficient accuracy of measurements to warrant the widespread adoption of this instrument as the one of choice for viscometry. Recently (Frei *et al.*, 1957), important modifications in the design and construction have been introduced which change this picture radically, and it is likely that this modified instrument will find wider application in the future.

Viscosity measurements have also been made for many years by the application of Stokes' law, which describes the rate of fall of a spherical particle through a viscous medium. From knowledge of the densities of the particle and the solution, the radius of the sphere and the measured rate of sedimentation, the viscosity of the solution can be calculated directly. As in the other methods, knowledge of the exact size of the sphere is unnecessary if relative viscosities are desired. Only the relative times for the spherical particle to fall a fixed distance and the densities of the sphere and the two liquids are needed. This method has not been applied widely although the recent widespread production of minute glass and plastic spheres may make this technique more popular. Usually the ball is allowed to fall under the influence of gravity, but some applications have been reported wherein a centrifugal field was employed as a driving force for submicroscopic particles (Schachman and Harrington, 1952).

*d. Interpretation of Viscosity Data.* Most experimental data can be expressed in the form of a power series relating the relative viscosity,  $\eta_{r,1}$  or  $\eta/\eta_o$ , as a function of concentration,

$$\eta_{rel} = \eta/\eta_o = 1 + Ac + Bc^2 + \dots \quad (4)$$

In this equation  $\eta$  is the viscosity of the solution and  $\eta_o$  is the viscosity of the solvent. The term,  $\eta_{sp}$ , the specific viscosity, is used frequently and is defined as  $(\eta/\eta_o - 1)$ . Equation (4) can be rearranged to give

$$\eta_{sp}/c = A + Bc + \dots \quad (5)$$

where  $\eta_{sp}/c$  is termed the reduced viscosity. At infinite dilution, the second and higher terms in the power series become negligible and Equation (6) results.

$$\lim (\eta_{sp}/c)_{c \rightarrow 0} = A = [\eta] \quad (6)$$

This serves as definition of the intrinsic viscosity,  $[\eta]$ .

Note that it has the units of reciprocal concentration. If, as is rarely the case, the concentration,  $c$ , is in volume units, i.e. milliliters of solute per milliliter of solution, then  $[\eta]$  would be dimensionless. According to Einstein,  $[\eta] = 2.5$  for spherical particles if the concentration of the solute is expressed as volume fraction. It should be recognized that Einstein's result is a limiting form of Equation 4 and that the higher terms represent interparticle interactions. If the concentration is in grams per milliliter, the intrinsic viscosity will have the units of milliliters per gram. However, the literature often contains intrinsic viscosities as deciliters per gram because the concentration was expressed as gm./100 ml. In order to evaluate the experimental data in terms of the desired intrinsic viscosity, the quantity  $\eta_{sp}/c$  should be plotted against  $c$  to give a curve whose intercept at  $c = 0$  is the intrinsic viscosity. Unfortunately such plots usually show wide scattering of the points, especially at low concentrations, and it is often difficult to derive a reliable value of the intercept by extrapolation. For such situations the data should be plotted as  $\eta_{sp}$  versus  $c$ . In this way curves are obtained which are almost straight lines near the origin. The slope at  $c = 0$  then gives the intrinsic viscosity directly. Although this method of treating the data works satisfactorily, the former is preferred because it shows any trends in the data such as those resulting from concentration dependent equilibria. Plots of this type were used to demonstrate the dissociation of aggregates of tobacco mosaic virus into the characteristic monomeric units (Schachman, 1947).

As already indicated, both the Einstein equation and the Simha extension of it to ellipsoids of revolution require that the concentration be expressed as volume fraction. If the particles were analogous to glass beads, the conversion of weight to volume concentration would be straightforward since the volume of a bead is its weight divided by its density. With molecules which interact with water by virtue of strong attractive forces such a calculation is meaningless. This poses a problem that is not as yet satisfactorily resolved. Some workers have proposed that the volume fraction of solute be defined as  $\bar{V}c$  where  $\bar{V}$  is the partial specific volume of the solute. Its reciprocal is the analog of the density, but only in the absence of interactions can these be equated. The term,  $\bar{V}c$ , refers, of course, to the dry volume of the solute. Since many macromolecules are presumed to be swollen in solution, imbibing appreciable amounts of solvent, some correction for this

“hydration” is necessary. Hydration is customarily expressed in terms of the hydrodynamic volume fraction,  $\Phi$ , by the following

$$\Phi = \bar{V}_c(1 + w/\bar{V}_\rho) \quad (7)$$

where  $w$  is the number of grams of solvent of density,  $\rho$ , associated with one gram of dry solute. Combination of this with the Einstein equation, which for dilute solutions can be written

$$\eta_{sp} = \nu\Phi \quad (8)$$

gives

$$[\eta] = \nu\bar{V}(1 + w/\bar{V}_\rho) \quad (9)$$

In Equations (8) and (9),  $\nu$  is the value of the viscosity increment caused by rigid particles of different axial ratios, the so-called shape factor, and it is evaluated theoretically by Simha. If it is known that the particles are spheres,  $\nu = 2.5$  and the value of  $w$ , the hydration, is directly calculable from the measured value of  $[\eta]$ . Alternatively, knowledge of the hydration and the measured value of  $[\eta]$  permits the calculation of  $\nu$  for the material under investigation. From Table I, relating the function  $\nu$  to axial ratio, the shape of the hydrodynamic unit is determined. Since various types of data led to the view that tobacco mosaic virus was only slightly hydrated, Lauffer (1938a, 1944a) calculated the axial ratio of the particles directly from the viscosity data.

It is clear from the above discussion that the intrinsic viscosity depends on both the shape and the volume of the solute molecules and evaluation of either depends on knowledge of the other. Scheraga and Mandelkern (1953) considered the problem in a different way by writing the effective volume,  $V_e$ , of a molecule as an unknown without any reference to the partial specific volume. This is no doubt a more rigorous treatment and is to be preferred, since the hydrodynamic volume cannot be expressed as a function of the partial specific volume by any theoretical consideration. In conjunction with other data, both  $V_e$  and the shape of the hydrodynamic unit can be evaluated. This is treated more fully in the discussion of sedimentation and diffusion.

In the case of both PR8 influenza A virus (Lauffer and Stanley, 1944) and rabbit papilloma virus (Schachman, 1951a), electron microscopic evidence indicated that the virus particles were essentially spherical. In principle, therefore, the viscosity data provided a value of the volume concentration. In both cases the experimental values of  $[\eta]$  indicated hydrations which seemed moderately large. Upon close inspection it became clear that the high viscosities were attributable to small amounts of impurities which were difficult to detect by other methods. The impurities were apparently

fibrous materials, like nucleic acids or polysaccharides, and were largely responsible for the observed viscosities of the solutions. As preparations became more highly purified the observed intrinsic viscosity decreased. For the two plant viruses, southern bean mosaic virus (Miller and Price, 1946) and bushy stunt virus (Cheng and Schachman, unpublished), however, the intrinsic viscosities were much lower, as would be expected for spherical objects only moderately hydrated.

TABLE I

DEPENDENCE OF VISCOSITY INCREMENT, FRICTIONAL RATIO AND COMBINED PARAMETER,  $\beta$ , ON AXIAL RATIO

Axial ratio	Prolate			Oblate		
	$\nu^a$	$f/f_o^b$	$\beta \times 10^{-6}^c$	$\nu$	$f/f_o$	$\beta \times 10^{-6}$
1	2.50	1.000	2.12	2.50	1.000	2.12
2	2.91	1.044	2.13	2.85	1.042	2.12
3	3.68	1.112	2.16	3.43	1.105	2.13
4	4.66	1.182	2.20	4.06	1.165	2.13
5	5.81	1.255	2.23	4.71	1.224	2.13
6	7.10	1.314	2.28	5.36	1.277	2.14
8	10.10	1.433	2.35	6.70	1.374	2.14
10	13.63	1.543	2.41	8.04	1.458	2.14
12	17.76	1.645	2.47	9.39	1.534	2.14
15	24.8	1.784	2.54	11.42	1.636	2.14
20	38.6	1.996	2.64	14.8	1.782	2.15
25	55.2	2.183	2.72	18.2	1.908	2.15
30	74.5	2.356	2.78	21.6	2.020	2.15
40	120.8	2.668	2.89	28.3	2.212	2.15
50	176.5	2.946	2.97	35.0	2.375	2.15
60	242.0	3.201	3.04	41.7	2.518	2.15
80	400.0	3.658	3.14	55.1	2.765	2.15
100	593.0	4.067	3.22	68.6	2.974	2.15
200	2051.0		3.48	136.2		2.15
300	4278.0		3.60	204.1		2.15

<sup>a</sup>  $\nu$  refers to the viscosity increment and corresponds to the intrinsic viscosity when concentration is expressed as volume fraction [calculated from Simha (1940)].

<sup>b</sup> Calculated from Perrin's equation (1936) and tabulated by Svedberg and Pedersen (1940, p. 41).

<sup>c</sup>  $\beta = [N/(16200\pi^2)]^{1/3}(f_o/f)\nu^{1/3}$ ; calculated and tabulated by Scheraga and Mandelkern (1953).

### 3. Rotational Diffusion

*a. General Considerations.* As a result of thermal energy, large particles in a viscous medium undergo continual zig-zag movements known as

Brownian motion. Less well-known, although described in 1909 (Perrin, 1909), is the rapid rotational motion during the course of which an individual particle is exposed to various orientations relative to a fixed direction. If a given particle is observed over a long time period, the average angular displacement per unit time can be evaluated, and from this the rotational diffusion coefficient is determined. This is, of course, directly analogous to the determination of the translational diffusion coefficient by measurement of the displacement of a particle with time. In both cases it is the average of the squares of the displacement, translational or rotational, that leads to a diffusion coefficient. Observation of a given particle during either its translational or rotational motion is very tedious and, in fact, is not feasible for particles as small as many viruses. Therefore, alternative methods are mandatory if such motions are to be followed experimentally. For both types of movements, techniques have now been developed whereby some property of the system which is readily measurable in quantitative terms is used in an indirect way to provide details about the motion of the solute molecules. Just as the number of molecules in a given volume element is termed the concentration, so we can express the angular concentration as a representation of the number (or weight) of particles having a fixed orientation relative to certain coordinates. If all of the particles were oriented by some device and the restraining force were suddenly removed, the ensuing Brownian motion would lead quickly to a state in which the various orientations among the many molecules would be completely random. The molecules would continue, of course, to rotate vigorously after the random condition had been achieved; but such motions then would be difficult to detect. On the other hand, the motion immediately after the release of the restraint might be followed by investigating some property that is a function of the degree of orientation of macromolecules. From measurement of the rate of decay of that property, a rotational diffusion coefficient can be determined. The driving force for the rotational motion is thermal energy usually expressed as  $kT$ , where  $k$  is the Boltzmann constant and  $T$  is the absolute temperature. Opposing the rotational motion is the viscous drag of the liquid; this resistance to motion is usually expressed in terms of a rotary frictional coefficient. By means of the same type of hydrodynamic treatment employed in the interpretation of the viscosity of solutions, the frictional coefficients can be expressed, for example, in terms of the size and shape of rigid ellipsoids. For very elongated particles the diffusion coefficient for rotation about the short axis is inversely proportional to the third power of the length of the long axis. Therefore the study of rotational diffusion constitutes a powerful and sensitive method for the examination of the length (and homogeneity with regard to length) of elongated macromolecules. Workers in this field are confronted with the same dilemma facing those

employing viscometry, since theoretical investigations have shown that both the volume and shape of the hydrodynamic unit are involved in the frictional term for molecules that are almost symmetrical. Therefore, a unique answer as to the shape of hydration cannot be expected from rotational diffusion measurements alone. As with other hydrodynamic techniques, paired measurements are required if assumptions as to the shape or hydration are to be avoided.

*b. Experimental Methods.* In recent years both theoretical and experimental innovations have led to additional methods for the measurement of rotational diffusion coefficients. As a result there are now available five different techniques which will be outlined here. Some methods are more general than others. Some have attained a greater degree of success experimentally, whereas others rest on a firmer theoretical foundation. Some are more desirable for very elongated macromolecules while others are preferable for the more symmetric particles. Specialized apparatus not commonly available is required for some; whereas improvising with common laboratory equipment will suffice for others. Only infrequent application of rotational diffusion methods to viruses have thus far been reported; these have dealt with the rodlike viruses only, although the techniques are not restricted, in principle, to elongated particles.

*i. Electric Birefringence.* Most direct of the different methods is that involving a kinetic study of the rate of disorientation of molecules which had first been oriented by the imposition of an electric field. The pulse of current is exceedingly brief in duration and the dipolar macromolecules orient themselves according to their own distribution of charges and the polarity of the electric field. As soon as the electric potential is removed, the oriented or partially oriented molecules tend toward a more random distribution relative to the direction of the field. Not all of the molecules are aligned in the direction of the field during the brief interval ( $10^{-2}$  sec.) that the field intensity has its maximum value. However, a steady state is attained in which the tendency toward alignment by the impressed field is counterbalanced by the tendency toward randomness due to Brownian motion. Once the field is removed, the latter becomes dominant and the rate of decay is conveniently measured. Alignment or even partial orientation of anisometric particles like tobacco mosaic virus causes the solution to become birefringent, a state which can be detected and measured quantitatively through the use of crossed polarizers. With suitable apparatus that is complex both optically and electronically, the decay of birefringence with time is portrayed as a decay curve from which the rotary diffusion coefficient can be calculated accurately (Benoit, 1951; O'Konski and Haltner, 1956). Moreover, the shape of the decay curve is defined theoretically, so that mixtures of rodlike particles of different sizes can be differentiated from

a solution of uniform particles. In addition, information can be obtained about the existence of a permanent dipole moment in the particles as contrasted to one that is induced under the influence of the field. The production of birefringence in an electric field is known as the Kerr effect, and its demonstration with tobacco mosaic virus was first made by Lauffer (1939).

*ii. Streaming Birefringence.* Birefringence with rodlike particles can also be established by imposing a velocity gradient on a solution of such particles. As already indicated in the discussion on viscometry, the particles become oriented in a flowing stream as a consequence of the differing velocities of the streamlines. The resulting birefringence is known as streaming birefringence or double refraction of flow. It is of considerable interest historically to note that streaming birefringence was demonstrated with tobacco mosaic virus before the virus was isolated in pure form (Takahashi and Rawlins, 1932, 1933). When the juice extracted from mosaic-diseased tobacco plants was passed through a fine capillary placed at  $45^\circ$  between crossed Nicol prisms, the field of view corresponding to the flowing solution was bright against a dark background. The extract from normal plants was not birefringent. Although this birefringence in the juice extracted from diseased plants could result from various types of particles in the solution, it was shown even at that early date that rodlike particles were involved and that the solution of oriented macromolecules resembled a crystal. Further investigations (Lauffer, 1938b) after the virus was obtained in purified form revealed that the birefringence was not an intrinsic property of the particles; i.e., the virus is optically isotropic and the birefringence produced in the flowing stream is the result of alignment of nonbirefringent particles. Although this simple apparatus is satisfactory for demonstrating double refraction of flow, the measurement of rotational diffusion coefficients through the exploitation of a velocity gradient requires elaborate apparatus similar to a Couette viscometer. In effect, the average orientation of the macromolecules relative to the streamlines is determined by optical means. Measurements are made at a variety of shear gradients, and rotational diffusion coefficients are calculated from each measurement with the aid of an elaborate hydrodynamic theory (for review see Cerf and Scheraga, 1952). Here, too, information of value is provided about the homogeneity of the particles. This technique has been applied frequently by different workers to preparations of tobacco mosaic virus.

*iii. Anomalous Viscosity.* Recently a third method has become available as a result of theoretical investigations of the complex hydrodynamic problem relating the intrinsic viscosity to the shear gradient (Saito, 1951). It has been pointed out earlier that the contribution of anisometric particles to the viscosity is a function of the orientation of the particles relative to the direction of flow. Opposing the orientation caused by the velocity gradient

is the rotary Brownian motion, and a different balance is achieved at each shear gradient. A theoretical expression and tables of computed results can be used to relate the rotational diffusion coefficient to the observed dependence of intrinsic viscosity on shear gradient. Thus far this method has been used in only a preliminary way with solutions of tobacco mosaic virus (Wada, 1954). Ideally a Couette viscometer should be employed for the measurements.

*iv. Dielectric Dispersion.* One of the older methods for measuring rotational diffusion coefficients is based on the analysis of the contribution of the macromolecules to the dielectric constant of the solution (Oncley, 1942). In an alternating electric field of relatively low frequency the periodic oscillation of the field is matched by the rotation of the macromolecules, which are first oriented in one direction and then in the opposite sense in response to the alternating field. As long as the particles can reverse their orientation in keeping with the alternating field, a dielectric constant is obtained which includes a contribution from the charged particles. However, an increase in the frequency of the alternating current soon leads to a situation in which the macromolecules can no longer rotate sufficiently rapidly. As a consequence the observed dielectric constant decreases since fewer macromolecules make their maximum contribution. From the change in dielectric constant with frequency, known as the dispersion of dielectric constant, a value for the rotational diffusion coefficient can be derived. Complex apparatus is required for this technique, and rigorous limitations are imposed with regard to the types of solutions that can be examined.

*v. Polarization Fluorescence.* In each of the four methods discussed thus far some type of force, electric or hydrodynamic, is imposed in order to cause some degree of orientation of the macromolecules. The field may then be removed and the rate of decay of the orientation examined to produce a value for the rotational diffusion coefficient. Alternatively, a steady state is achieved in which there is a balance in the effect of the orienting and disorienting forces; from an evaluation of the balance, the rotational diffusion coefficient is calculated. There is still another method, not involving the application of any external field to produce preferential orientation of the macromolecules. Instead, attempts are made to measure the angular rotation of the molecules in the brief time interval between the absorption of light of a certain wavelength and the emission of that light as fluorescence. The degree of depolarization of the fluorescent light is a function of the extent of rotation of the macromolecules during the lifetime of the excited state (about  $10^{-8}$  sec.). With polarized incident light, the emitted light also is plane-polarized if the molecules do not rotate during the time period between absorption and emission. If the rotational diffusion coefficient is very large, as with small molecules, the emitted light will be depolarized.

The theory for the polarization fluorescence method permits the calculation of the rotational diffusion coefficient from measurements of the polarization (Perrin, 1926, 1936). Many macromolecules of biological interest are not fluorescent for exciting light with wavelengths which are readily accessible, and fluorescent derivatives are usually employed in which dyes are coupled chemically to the macromolecules. This method has not as yet been applied to viruses although it has been used extensively with proteins.

Each of the methods described above has certain virtues and drawbacks. No single one is likely to be useful for all types of materials. A choice among them depends on the type of problem under investigation and restrictions imposed by the nature of the macromolecule, such as its size, shape, and solubility. Some of the methods are severely limited to solutions of low conductivity, thus precluding experiments at high ionic strength and over a range of pH values.

#### 4. Diffusion

*a. General Considerations.* The spontaneous transfer of material from a region in a solution in which there is a certain concentration of a given species of solute molecules to a neighbouring region of lower concentration is known as diffusion. This mass transfer of solute molecules continues until the concentration everywhere in the solution is uniform. Individual molecules continue to move under the influence of thermal energy after a uniform state is achieved, of course, but this random movement is not directed and no net transport of material results from this Brownian motion. As already indicated in the discussion of rotational diffusion, measurement over a long time period of the individual movements of a particle can provide a satisfactory value for the diffusion coefficient if the material in solution is amenable to direct observation by microscopy. This is rarely the case, of course. Even when such observation is feasible, experimentation is difficult because the movements are very rapid and in all directions. Fortunately, the detailed description of the path of a single particle is not required, since only the net motion over a relatively long period, such as a second, is needed. The net displacement is, of course, much less than the distance represented by the complete path. Thus, a determination of the rate of diffusion by this method consists of the evaluation of the position of a particle every second and the many movements of the particle between observations within the one-second intervals are immaterial. Owing to the lack of techniques for the direct visualization of individual macromolecules in solution, practically all diffusion measurements of interest to biologists are based on the fact that the motions of the particles are directed preferentially when concentration differences are established. Such a gradient of concentration can be formed initially by suitable experimental techniques or it may be the indirect

result of the net movement of particles under the influence of an impressed electric or centrifugal field. The latter two situations are considered separately in later sections. Here the discussion is restricted to experiments in which no external field is imposed.

Measurement of diffusion coefficients requires an apparatus in which a solution of some solute at a given concentration can be brought into contact with another solution that is identical except for a difference in concentration. Generally one of the two solutions contains no solute, i.e., it is pure solvent, although there are occasions when it is necessary to study the diffusion of a solute from one concentration region to another.

Basic to all diffusion measurements, and at the same time providing a definition of the diffusion coefficient, is the statement now known as Fick's first law of diffusion (Fick, 1855). Fick proposed a transport equation which can be written

$$dm = - DA \frac{\partial c}{\partial x} dt \quad (10)$$

where  $dm$  is the mass of material transported in the time,  $dt$ ;  $A$  is the cross-sectional area of the diffusion cell; and  $(\partial c/\partial x)$  is the concentration gradient representing the change in concentration between two levels in the apparatus separated by a very small distance. The negative sign is present in this equation because the transport of solute is in the direction of decreasing concentration. Thus, if we think of a vertical cell in which the bottom solution is more concentrated and the positive direction is downward, mass is transported in the negative direction. This equation provides a definition of the diffusion coefficient,  $D$ , as a measure of the mass of solute transported across a plane of known cross section in a given period of time under the influence of a known driving force. The driving force arises from the difference in chemical potential of the solute in the two solutions. For many macromolecules in dilute solutions the driving force can be related directly to the concentration gradient. Dimensional analysis of the various terms in the flow equation presented above shows that the diffusion coefficient has the units, square centimeters per second. Since the flow of solute is measured relative to the cell walls the return flow of solvent must be considered, but it can be shown that a single diffusion coefficient can be used to describe the transport of either solute or solvent (Gosting, 1956).

Diffusion coefficients can be measured by direct application of Equation (10) to the determination of the amount of material diffusing across a porous membrane separating a pure solvent from a solution containing solute. Such diffusion studies, referred to as *diffusion through a porous disk*, are particularly useful, as shown below, for biological materials for which a specific bioassay is available. Though the method by itself does not give

absolute values for the diffusion coefficient, it is particularly valuable for the virologist who can use the method for the examination of viruses which as yet have not been purified. The second, more popular method of studying diffusion, known as the *free diffusion* method, is based on another equation derived by Fick and known as Fick's second law. This relates the change of concentration with time to the rate at which the concentration gradient is changing with position,  $x$ , in the cell. To derive this equation it is necessary to formulate the so-called continuity equation, which is an expression of the conservation of mass of the diffusing substance. Continuity equations are basic to any theoretical consideration of experiments involving transport of one component. Consider a volume element bounded by the walls of the cell and two hypothetical planes separated by an infinitesimal distance. The accumulation of solute within the volume element can be expressed readily as the difference between the net flow of solute through the first surface and that across the second plane. It is as if observers were stationed at the two planes and each counted the number of molecules crossing the respective planes during a given time period. Clearly any difference recorded by the observers must involve an accumulation or depletion of solute within the volume element, and this would be detected readily by an independent observation, within the selected volume, of the concentration change with time. From a mathematical statement of this principle and Equation (10), Fick's second law emerges

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2} \quad (11)$$

This equation applies only to systems in which the diffusion coefficient is independent of concentration. In performing measurements by this method, a sharp boundary is created between the solvent and the solution, and the change in concentration of the solute as a function both of distance and of time is followed by any one of a variety of optical methods. This method is sensitive in the detection of impurities either much larger or smaller than the main component, or in the determination of the homogeneity with respect to size of the principal diffusing species.

*b. Measurement of Diffusion Coefficients. i. Diffusion through a Porous Disk.* This method, introduced by Northrop and Anson (1929) and applied by them and others to the measurement of the diffusion coefficient of enzymes, viruses, and hormones, is elegantly simple. Despite some limitations, it should find wide application in the study of the size of an infectious agent whether the agent has been purified or not, as long as a sensitive and specific bioassay is available. It should therefore prove invaluable in attempts to establish the identity of an infectious agent as some particular characteristic macromolecule. If purification procedures are not completely successful and

diffusion data are desired for molecular weight determinations, this method is the only one that is satisfactory.

The solution and solvent are placed in separate compartments of a cell containing a porous plate through which the solute diffuses. In the early designs the solution was placed in an inverted funnel across which a sintered glass disk was cemented, and this cell was then brought into contact with the solvent which was in a second, open vessel. The solution was above the solvent, and the diffusing molecules after passing through the porous plate led to stirring of the lower liquid by virtue of the increase of the density of the surrounding liquid to a level above that of the pure solvent beneath.

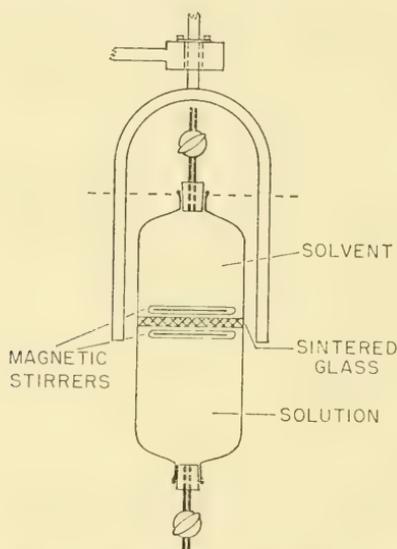


FIG. 1. Diagram of closed porous disk diffusion cell. The glass enclosed magnetic stirrers are so constructed with respect to thickness of glass and length of wire that the magnet in the upper compartment sinks in the liquid while that in the lower one floats. The stirrers are caused to rotate by surrounding the cell with a rotating permanent magnet. The diffusion cell is placed in a constant temperature water bath so that thermal gradients across the cell are avoided.

This system was affected by uncertainties resulting from mass flow of solution across the disk due to temperature gradients across the cell and to convective flow of solution through occasional capillaries of large diameter. The possibility of mass flow through the membrane, with the consequent tremendous errors in the diffusion coefficient, has been reduced substantially in a modified version of the Northrup-Anson cell. This was effected by a change from an open or single cell to one having two closed compartments completely filled with liquid. A sketch of such a cell (Stokes, 1950) which is

free of some of the objections of the older cells is shown in Fig. 1. The solution is placed in the lower compartment and the solvent in the upper to effect gravitational stability, and stirring is accomplished mechanically with rotating magnets. After the cell is assembled, the diffusion is allowed to proceed with periodic removal of the upper liquid and immediate replacement with fresh solvent. This is repeated several times until an assay of the removed liquid reveals that the rate of transport of solute into the upper compartment is constant. This indicates that a steady state condition has been achieved as a result of the formation of the appropriate concentration gradient within the channels of the sintered glass disk. When this condition has been attained (several days may be required for particles as large as viruses), the two solutions are removed, their concentrations determined and the diffusion coefficient is calculated from the following:

$$D = \frac{1}{\beta t} \frac{c_u^o - c_l^o}{c_u^t - c_l^t} \quad (12)$$

In this equation, the superscripts *o* and *t* refer to zero time and to a subsequent time, *t*;  $\beta$  is the cell constant  $(A/L)(1/v_u + 1/v_l)$ , where  $v_u$  and  $v_l$  and  $c_u$  and  $c_l$  are the volumes and concentrations of the upper and lower liquids and *A* and *L* are the effective area and thickness of the porous disk. Evaluation of the cell constant,  $\beta$ , is performed through the use of a substance of known diffusion coefficient. Potassium chloride is the favored substance for this determination, but the use of a virus or protein of known diffusion coefficient is preferable to reduce the difference in size between the calibrating substance and the unknown agent. Equation (12) will be recognized as the product of integration of Equation (10). Thus this technique consists simply of the direct determination of the rate of transport of material,  $dm/dt$ , across a selected plane.

It is obvious that adsorption of any virus particles to the surface of the porous diaphragm will lead to erroneous results, as will the use of a sintered glass disk with pores so small as to be impermeable to the virus particles. In view of the simplicity of the technique, it is advisable to conduct experiments with different cells so that irregularities or deficiencies in an individual cell may be detected.

*ii. Free Diffusion.* In the past ten years major modifications have occurred in the techniques employed for the study of free diffusion, with the result that today such diffusion experiments can be performed routinely and with an accuracy far greater than that expected by the most optimistic research workers of the 1930's and early 1940's. This tremendous advance in experimental precision was due largely to the rediscovery of interferometric optical methods and their adaptation to diffusion studies. As a consequence of these developments, the older, more common procedures used for proteins and

viruses are obsolete. It is fortunate that the newer techniques are neither more difficult nor more time-consuming than the older methods. Versatile equipment, although expensive and complicated, is now available commercially from several sources. No longer is a diffusion measurement relegated to a few select laboratories throughout the world where different investigators pioneered in the design and use of this highly specialized technique.

After many years of experimentation leading to various ingenious designs for diffusion cells, there now seems to be widespread agreement that the Tiselius electrophoresis cell, with only slight modifications, is the ideal cell. This conclusion results in large part from the important proposal of Kahu and Polson (1947) that poorly formed boundaries between the solvent and solution can be made almost ideal by a capillary siphoning procedure. This technique, called boundary sharpening, has permitted the formation of almost infinitely sharp boundaries between the solvent and solution. Moreover, the boundary is located in the cell at a level which permits observation and photography even during the formation of the boundary. Thus the large and somewhat uncertain corrections required because of imperfections in the initial boundary are no longer necessary.

As already indicated, the major innovations were concerned with the rediscovery and adaptation of neglected optical principles and practices. For many years the spreading of boundaries due to diffusion of macromolecules was followed by means of schlieren optical procedures of one type or another. These techniques were convenient, and procedures were developed whereby the photographic patterns were translated into diffusion coefficients by a variety of calculation methods. In effect, the patterns were plots of the concentration gradient versus distance. All of the procedures involve the assumption, which was subject to test, that the plots were Gaussian in shape obeying the following equation:

$$\frac{\partial c}{\partial x} = \frac{c_0}{(4\pi Dt)^{1/2}} e^{-x^2/4Dt} \quad (13)$$

where  $c_0$  is the initial concentration of solute and the other symbols have the meanings already assigned. This equation is the result of integration of Fick's second law (Equation (11)). Most popular of the various methods of treating the data is the so-called height-area method by means of which the diffusion coefficient is calculated from any of the individual photographs. With time, the height of the curve decreases in a prescribed manner while the width increases so as to maintain a constant area under the curve. The shape of the boundary must have a definite form, according to Equation (13), and deviations of the experimental curves from the theoretical shape are an

indication of heretogeneity. However, slight deviations were often undetectable, and homogeneity was ascribed to preparations which are now known to have contained populations of macromolecules of varying size. This can be attributed, no doubt, to limitations in the optical methods then used. Boundaries which did not maintain their symmetry during a diffusion experiment, as was observed in early experiments with tobacco mosaic virus (Neurath and Saum, 1938), were an indication that the diffusion coefficient varied with concentration. For such systems Equation (11) is not applicable, and a more elaborate treatment of the data is necessary.

Despite improvements in the quality of the lenses employed in the schlieren optical systems, further progress was limited for both experimental and theoretical reasons. Therefore developments in the adaptation of interferometric optical techniques were greeted with enthusiasm, and now two different optical methods have been adopted widely. The first is the so-called Gouy interference method (Kegeles and Gosting, 1947) and is the simpler of the two. Instead of a smooth curve of refractive index gradient versus distance, the Gouy system gives a series of horizontal, closely spaced interference fringes from which the concentration gradient curve can be constructed with great precision. The coordinates of the gradient curve are evaluated, in effect, from the positions of the fringes which arise from the interference of pairs of light rays which in traversing the boundary are deviated exactly the same amount and arrive at the same position at the focal plane of the lens system. Since one of each pair of rays experiences a different optical path from the other, the rays may be in or out of phase, giving constructive or destructive interference. This depends on both the refractive index at the two levels within the boundary and the geometrical distances traversed by the light. Early in the diffusion experiment the lines are spaced widely with the lowermost fringe, which corresponds to the maximum concentration gradient, being displaced a large distance from the undeviated light. As diffusion progresses, the fringe spacing diminishes although their number remains constant. From appropriate tables and measurement of the fringe positions, the diffusion coefficient can be calculated. The constancy of the values from different fringes is an indication of the Gaussian nature of the boundary and therefore a valuable criterion of the homogeneity of the diffusing substance. Owing to the fact that the optical system does not contain a lens which images the cell, the location of the boundary within the cell cannot be recorded by this method; the Gouy system is, therefore, not useful for studies of moving boundaries such as are met in electrophoresis or sedimentation velocity experiments. For shapes of boundaries, however, this method is unsurpassed.

The second method, known as the Rayleigh interference method, gives a

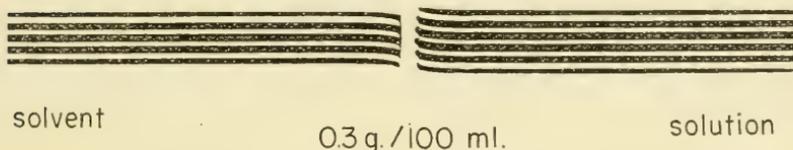
curve of refractive index (or concentration) versus distance, and the conditions for interference arise in a much different way. Rather than exploiting interference conditions from pairs of positions within the boundary as in the Gouy method, here interference is established between each point in the diffusion cell and a conjugate point in a reference cell filled with a homogeneous liquid like water or buffer. The vertical coordinates of the cell are imaged along the length of the vertical fringes. In the absence of a boundary the fringes are straight because the optical path difference for rays passing through the diffusion cell and the comparison channel is a constant. When a boundary is present, the fringes become warped and their course represents the change of refractive index (or concentration) in the diffusing boundary. For optical reasons, a single fringe cannot be traced through the whole boundary. However, the family of fringes, when taken together, gives a direct measure of the change in refractive index (and therefore of concentration) as a function of distance. Measurements are made of the location of each fringe as a function of the vertical distance. From these data and the necessary tables representing the integral of Equation (13), the diffusion coefficient is evaluated (Longworth, 1952). Fringes can be paired in different ways to calculate the diffusion coefficients and the lack of variation in the resultant values is good evidence for the homogeneity of the diffusing substance. Unlike the Gouy method, the Rayleigh method as customarily employed does provide an image of the cell, and it therefore is useful in the examination of moving boundaries. Patterns with this system are likely to find wide application in ultracentrifugation and electrophoresis as well as in diffusion. Representative photographs from a diffusion experiment are given in Fig. 2.

*c. Interpretation of Diffusion Coefficients.* In general, diffusion coefficients are employed in conjunction with the results of sedimentation velocity experiments for the calculation of molecular weights. Without both results such computations cannot be made with certainty unless the shape, degree of hydration, permeability, and flexibility of the macromolecules are known. Rarely, if ever, are these factors available. Despite these uncertainties about the morphology of the macromolecules, no ambiguities accompany the evaluation of molecular weights from the combination of diffusion and sedimentation data. As long as these data are accurate and correspond to infinitely dilute solutions, the molecular weights are reliable. The fact that the diffusion and sedimenting unit in solution contains large quantities of solvent is immaterial. Whether the shape, the permeability, and the flexibility of the kinetic unit conform to some specific model is unimportant. Values of the molecular weight calculated from the sedimentation and diffusion data correspond to the anhydrous particles. In this way they are directly analogous to the results of light-scattering measurements.

Both diffusion and sedimentation theories are based, in large part, on thermodynamic and hydrodynamic considerations which relate the velocity of movement of a particle to the driving force acting on it and the frictional resistance experienced during the particle's migration through a viscous medium. The particles, which are considered to be at rest initially, accelerate rapidly to a limiting velocity at which the frictional force is equal to the driving force.

## DIFFUSION OF BUSHY STUNT VIRUS

### BOUNDARY DURING SHARPENING



### BOUNDARY AFTER DIFFUSION

FOR 14581 MIN.

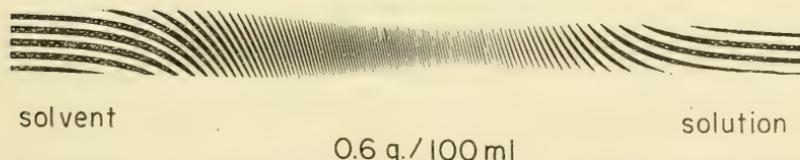


FIG. 2. Representative Rayleigh interference patterns obtained during the diffusion of bushy stunt virus. (From Cheng and Schachman, unpublished.)

The former is written as the product of the frictional coefficient,  $f$ , and the velocity,  $dx/dt$ . For diffusion the driving force is the rate of change of the chemical potential with distance. This relation is occasionally expressed in terms of the osmotic pressure by stating that the force of diffusion is equal and opposite to the force of osmotic pressure tending to drive the solvent that is layered above the solution into the region of the solution. The former is the more rigorous treatment, and the relation between the diffusion coefficient and molecular parameters is derived from it. It is interesting to note that the thermodynamic derivation gives the same result as that obtained earlier by Einstein (1906) from a kinetic analysis of Brownian motion. For solutions sufficiently dilute that concentration effects are negligible, the diffusion coefficient is related to the frictional coefficient as follows:

$$D = \frac{kT}{f} \quad (14)$$

where  $kT$  is the kinetic energy of the molecules,  $k$  is the Boltzmann constant and  $T$  is the absolute temperature. This relation shows how the diffusion coefficient varies with temperature. An additional term is required for solutions at finite concentrations. It is customary, therefore, to perform experiments at several concentrations and to obtain the diffusion coefficient at infinite dilution by extrapolation. The frictional coefficient is a function of the size, shape, permeability, and flexibility of the kinetic unit. According to Stokes (1851), the frictional coefficient for rigid, spherical particles can be written

$$f = 6\pi\eta r \quad (15)$$

where  $\eta$  is the viscosity of the solvent and  $r$  is the radius of the spherical particle. Thus an evaluation of a diffusion coefficient provides a direct measure of the radius of a diffusing sphere. It should be noted that radii calculated in this way correspond to the hydrodynamic unit. If a virus particle imbibes a large amount of water and the swollen particle acts as a rigid unit, with its imbibed water immobilized, the diffusion coefficient yields the radius of the hydrated unit. Comparisons of the radii derived in this manner with the values from electron microscopy are to be discouraged, unless special efforts are made to preserve the size and shape of the kinetic units by special desiccating procedures prior to electron microscopic observation. For bushy stunt virus (Neurath and Cooper, 1940), southern bean mosaic virus (Miller and Price, 1946), and turnip yellow mosaic virus (Markham, 1951), all of which have been shown by electron microscopy to be essentially spherical, the radii calculated from diffusion can best be compared with those evaluated by low-angle X-ray scattering.

A more general treatment of the frictional coefficient must include particles of other shapes as well as spheres. For this purpose it is customary to write

$$f = (f/f_o)f_o \quad (16)$$

where  $(f/f_o)$  is the frictional ratio which expresses the frictional resistance for ellipsoids of revolution relative to that for a sphere of the same volume, and  $f_o$  is the frictional coefficient for a hypothetical spherical particle of the same volume as the real particle. If it is known that the macromolecule acts as a rigid, anhydrous unit in solution and the molecular weight,  $M$ , is available from other data, the frictional ratio can be calculated from combination of Equations 14, 15, and 16 with the equivalent radius,  $r_o$ , being replaced by  $(3M\bar{V}/4\pi N)^{1/3}$ , where  $N$  is the Avogadro number. This procedure assumes that the density of the particle is given by the reciprocal of the partial specific volume. From the value of  $f/f_o$ , the axial ratio of the ellipsoid of revolution is calculated directly from hydrodynamic theories which, in

effect, are extensions of Stokes' law. The results of these elaborate, theoretical investigations are given in tabular form,  $f/f_o$  as a function of axial ratio, for prolate and oblate ellipsoids of revolution (see Table I).

Most macromolecules in solution are solvated to some extent and there is generally some interaction with the solvent; for them, calculation of the equivalent radius from the molecular weight and the partial specific volume is invalid. Some factor must be included so as to account for any swelling which results from imbibing of water into the kinetic unit. Alternately, the effective volume of the kinetic unit is considered as an unknown and written as  $V_e$ . The former method is that suggested and used by Oncley (1941) and others, while the latter is the procedure employed by Scheraga and Mandelkern (1953). According to Oncley (1941), the frictional ratio due to the swelling upon hydration of the macromolecules can be written:

$$\left(\frac{f}{f_o}\right)_{\text{hydration}} = \left(1 + \frac{w}{V\rho}\right)^{1/3} \quad (17)$$

Combination of this with the previous equations relating  $D$  to  $f$  and  $f/f_o$  gives:

$$D = \frac{kT}{6\pi\eta(3\bar{V}M/4\pi N)^{1/3}(1 + w/\bar{V}\rho)^{1/3}(f/f_o)} \quad (18)$$

Scheraga and Mandelkern obtained a similar equation which is written:

$$D = \frac{kT}{6\pi\eta(3V_e/4\pi)^{1/3}(f/f_o)} \quad (18a)$$

Both forms of Equation 18 show that the diffusion coefficient cannot be interpreted in terms of an axial ratio for an ellipsoid unless the hydration (or effective volume of the kinetic unit) is known. Alternatively, knowledge of the shape permits a ready determination of the hydration. This dilemma can be handled, in principle, by coupling diffusion measurements with other hydrodynamic data like the intrinsic viscosity, since this, too, is dependent upon the effective volume and shape of the hydrodynamic unit. If the theoretical expressions for the intrinsic viscosity (in units of deciliters per gram) the diffusion are combined, the following expression results (Scheraga and Mandelkern, 1953):

$$\beta = \frac{D\eta[\eta]^{1/3}M^{1/3}}{kT} \quad (19)$$

The parameter,  $\beta$ , is a function only of the axial ratio for ellipsoids of revolution. Table I shows this relationship as calculated from the Simha equation (1940) for viscosity and the Perrin equation (1936) for the frictional coefficient. Insertion of the measured quantities into Equation 19 gives  $\beta$  and thence the axial ratio. This is the procedure suggested by Scheraga and

Mandelkern (1953), who also made the point that these calculations give a value of the axial ratio of an ellipsoid of revolution that is equivalent to, but not necessarily identical with, the real particle. Distortion of the macromolecule or any motion of liquid through the particle during its migration through the liquid would vitiate the correspondence between the calculated particle and the real one since the hydrodynamic theories assume rigid objects (any water of hydration must be immobilized and move with the particle as a unit). Other types of investigations into the physical-chemical properties of proteins and viruses have led to the widely adopted view that these materials are only slightly hydrated and that the swollen particles do act as rigid particles, as required by the hydrodynamic theories. For such materials, Equation 18 is applicable, and knowledge of the hydration ( $w$ ) permits the calculation of the axial ratio directly from the diffusion coefficient. It is interesting to note that  $\beta$  is not sensitive to axial ratio for particles that are almost spherical. Therefore, Equation 19 can be used for molecular weight calculations if the electron microscope reveals that the particles are not elongated.

### 5. Ultracentrifugation

*a. Introduction.* Ultracentrifuges can be used in either of two ways which differ both experimentally and theoretically. In one, the centrifugal field is so large that the force on the solute molecules causes them to migrate through the solution rapidly, and the velocity of movement of the molecules is measured during the sedimentation. In the other, the centrifugal field is so small that the rate of motion of the particles is not the quantity that is measured. Instead, an equilibrium state is established after a long period of centrifugation and the concentration of the solute, although varying slightly at each level in the cell and being finite everywhere, no longer varies with time. Interpretation of the results of this type of experiment is based on measurements of the concentration of the solute as a function of position within the centrifuge cell. For the theoretical and experimental development of each of these ultracentrifugal methods, we owe much to the pioneer work of Svedberg and his collaborators (Svedberg and Pedersen, 1940).

The former method is known as the sedimentation velocity method and has, to date, been the one more widely used. In a sedimentation velocity experiment, the ultracentrifuge rotor is operated at speeds up to 60,000 r.p.m., corresponding to forces of 250,000 times that of gravity. Molecules which initially were uniformly distributed throughout the solution in the ultracentrifuge cell are caused to settle at appreciable rates toward its periphery. This migration of the solute molecules creates, in effect, three regions within the ultracentrifuge cell. One of these is the zone containing only solvent molecules and is termed the supernatant. Another is the region

## VISUALIZATION OF BOUNDARIES IN THE ULTRACENTRIFUGE

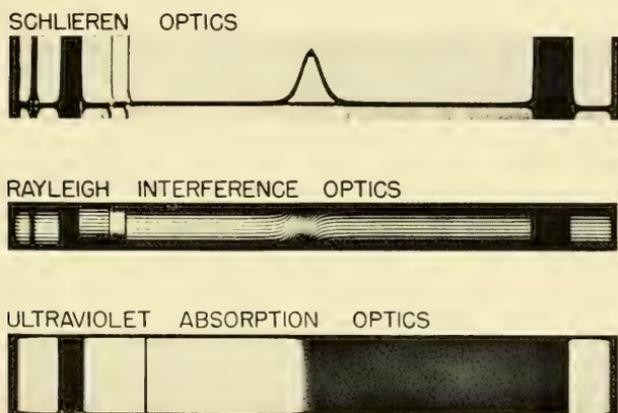


FIG. 3. Visualization of boundaries in the ultracentrifuge. The upper two patterns with schlieren optics and Rayleigh interference optics were obtained with a solution of bushy stunt virus at 0.5 g/100 ml. The lower pattern with ultraviolet absorption optics was obtained with purified MEF-1 poliomyelitis virus at 0.01 g/100 ml. Sedimentation is from left to right.



in which the solute molecules are present in uniform concentration, almost equal to the initial concentration; this zone is called the plateau region. Between these two is a transition zone in which the concentration varies with distance from the axis of rotation. This transition zone is called the boundary; the sedimentation velocity method is based generally on observations, by optical methods, of the rate of movement of such boundaries (see Fig. 3). Although it will not be demonstrated here, it can be shown that the movement of a boundary in such an experiment provides a direct measurement of the net migration of solute molecules in the plateau region. Thus, the directed movement of individual molecules can be measured conveniently and accurately despite the absence of techniques for the visualization of individual molecules. From the rate of movement of a boundary, a quantity termed the sedimentation coefficient is calculated. This is related to the size, shape, and other properties of the solute molecules. In conjunction with other data, particularly the diffusion coefficient, the sedimentation velocity method serves as one of the more popular techniques now available for molecular weight determinations. Moreover, the presence of separate, discrete components is detected easily by the appearance of several boundaries, each representing a different molecular species. Finally, the amount of each component can be evaluated and an approximation can be made of their molecular weights. Owing to diffusion, the boundaries widen continuously in a predictable manner during a sedimentation velocity experiment. For pure materials, this blurring of the boundary can be employed for the calculation of diffusion coefficients. Alternatively, the spreading of the boundary can be used as an extremely sensitive indicator for the study of the homogeneity of the sedimenting material. In this respect, the sedimentation velocity method is unrivaled among existing techniques employed for the examination of macromolecules.

The second type of ultracentrifuge experiment, involving much lower centrifugal fields and known as the sedimentation equilibrium method, implicates diffusion more directly, so that a balance is achieved ultimately between the transport by sedimentation in a centrifugal direction and the countertransport by diffusion in the centripetal direction. During the first stages of a sedimentation equilibrium experiment, the concentration decreases at the meniscus and increases at the cell bottom, owing to sedimentation. As a consequence of back diffusion, however, a region totally devoid of solute is not created near the meniscus as in the sedimentation velocity method. Instead, the concentration remains nonzero and finite everywhere as long as the centrifugal field is not too large. In the center of the cell, during the early stages of a sedimentation equilibrium study, the concentration is independent of position and practically the same as the initial concentration. As the experiment progresses, the plateau region

disappears and there is only one position in the cell at which the concentration is equal to the initial concentration. Finally, after a considerable length of time, an equilibrium state is attained and the concentration distribution becomes independent of time. From measurements of the concentration as a function of distance, the molecular weight is calculated. Except for the partial specific volume, no auxiliary information about the macromolecules is necessary. In contrast, molecular weight determinations by the sedimentation velocity method are dependent upon knowledge of additional physical properties such as the diffusion coefficient. Despite some obvious virtues of a theoretical and experimental nature, the equilibrium method has been employed only infrequently, and there are scarcely any recorded applications of it for the study of viruses. This can be attributed to a variety of factors, principal among them being the lack of apparatus capable of sustained, continuous operation for the long periods (days or even weeks) required before equilibrium is attained. Recent innovations from the standpoint of technique have altered this situation markedly. Although these modifications have not as yet been applied in the study of viruses and some experimental difficulties may be encountered, it is likely that the sedimentation equilibrium method soon will find wide application for molecular weight determinations of viruses and especially of their subunits.

An additional type of ultracentrifuge experiment has achieved wide popularity in the past three years. This, like the sedimentation equilibrium method and in contrast with sedimentation velocity studies, gives molecular weights directly. We refer, here, to the Archibald method (Archibald, 1947) by which molecular weights are determined from data obtained in the transient states during the approach to sedimentation equilibrium. Owing largely to the theoretical studies of Archibald and certain technical improvements especially with regard to optical methods, precise determinations of molecular weights are now feasible in experiments of very short duration (Klainer and Kegeles, 1955; Ginsburg *et al.*, 1956). Although equilibrium is not attained throughout the cell until after many hours or days of centrifugation, the conditions for equilibrium (transport of solute by sedimentation equals transport by diffusion) are fulfilled at the two end surfaces of the cell at all times during an experiment. Therefore, molecular weights can be calculated from the approximate data obtained immediately after the ultracentrifuge rotor attains the desired speed. Numerous technical problems have been solved satisfactorily, and this method appears to be among the best available for molecules of the size of proteins. For macromolecules as large as viruses, difficulties still exist, and the method thus far has been applied to materials no larger than  $4 \times 10^6$  in molecular weight. Despite this, it seems likely that studies now in progress will eliminate some of the

existing complications, thereby permitting the Archibald method to be used routinely with viruses.

*b. Sedimentation Velocity Method. i. Sedimentation Coefficient.* Just as in diffusion, so in sedimentation, a transport equation can be written for the amount of material crossing a given surface in a centrifuge cell which is rotating at some fixed angular velocity. This transport equation relates the mass transported per unit time to the area of the surface, the concentration of the solute, the magnitude of the centrifugal field, and the velocity of the molecules per unit field, this last term usually being described by  $s$ , the sedimentation coefficient. In effect, the transport equation serves as a definition of the sedimentation coefficient which is written:

$$s = \frac{dx/dt}{\omega^2 x} \quad (20)$$

where  $x$  is the distance in centimeters from the axis of rotation,  $t$  is the time in seconds, and  $\omega$  is the angular velocity in radians per second. Dimensional analysis show that the sedimentation coefficient has the units of seconds, but it is more meaningful to consider the units as cm./sec./dyne/gm. Sedimentation coefficients are now reported in terms of svedbergs (S) where  $1 \text{ S} = 10^{-13} \text{ sec.}$

Sedimentation coefficients are evaluated from measurements of the position of the boundary as a function of time. For this calculation it is customary to consider the integral form of Equation 20 and plot  $\log x$  versus  $t$ . Except for unusual materials, this plot is a straight line whose slope gives  $s$ . It is interesting to note that tobacco mosaic virus (Lauffer, 1944b) serves as the most prominent substance for which a plot of  $\log x$  versus  $t$  is not linear. Special treatments which are beyond the scope of the present review are required for these materials. Usually, for convenience in the comparison of results, sedimentation coefficients are reported as  $s_{20, w}$  which is the sedimentation coefficient that would have resulted had the experiment been conducted in a solvent having a viscosity and density equal to those of water at 20°C. Actually, electrolytes must be present in the solution during the ultracentrifuge experiments, if serious errors are to be avoided. Macromolecules of biological interest have ionizable groups and frequently possess a net charge under the conditions of the experiment. As a result of the difference in the sedimentation rate of the macro-ion and its counter-ions in the solution, a potential gradient is established during sedimentation. This potential gradient is fully equivalent to an externally applied electric field, and allowances for it must be made in the force equation for sedimentation. Analysis of this problem (Pedersen, 1958) has shown two separate effects, the primary and secondary charge effects, both of which can be minimized if a neutral electrolyte is present to the extent of about 0.1 molar

and if the ions of the electrolyte are of equal size. The primary charge effect, which always causes a reduction of the sedimentation rate of the macromolecule as compared to an un-ionized molecule of the same size and shape, is attributed to the differential sedimentation of the macro-ions and its counter-ions. In contrast, the secondary charge effect can either enhance or diminish the sedimentation rate of large ions. This depends on the charge of the large ion and the relative sizes of the positive and negative ions of the low molecular weight electrolyte added to the solution. If, for example, the macromolecule is negatively charged and the so-called supporting electrolyte is cesium chloride, there will be an enhancement of the sedimentation rate. Had the supporting electrolyte been lithium iodide, there would be a decrease in sedimentation rate as a consequence of the operation of the secondary charge effect.

Like many physical chemical properties, the sedimentation coefficient frequently exhibits a marked dependence on concentration with the sedimentation becoming more rapid as the solutions are diluted. This can be attributed to three effects which are only partially understood in theoretical terms (Schachman and Kauzmann, 1949). Foremost of these factors is the so-called viscosity effect. As the solutions become more concentrated, the viscosity increases and the frictional resistance experienced by an individual molecule during its migration likewise increases. It is an oversimplification, however, to ascribe the concentration dependence of the sedimentation coefficient solely to the viscosity of the solution. Since the centers of two solute molecules can approach one another to a distance no less than the sum of their radii (or equivalent dimensions for nonspherical particles) there is, in effect, an exclusion of solute molecules from the immediate vicinity of each individual one. Thus each solute molecule sediments in a medium that has a viscosity somewhat less than the bulk viscosity of the solution. How much less than the over-all viscosity is this effective viscosity remains difficult to ascertain. Second in importance in causing sedimentation coefficients to decrease with an increase in concentration is the so-called backward flow effect. As the molecules plus their solvation mantles migrate toward the closed cell bottom, there is return flow of liquid to make room for the sedimenting molecules and to fill the space formerly occupied by the molecules in the region above the moving boundary. This backward flow causes a decrease in the rate of sedimentation, which is measured relative to the cell walls, by an amount which might be expected to increase with an increase in concentration. For particles that are nearly spherical, the backward flow correction is the predominant one. Finally, the sedimentation rates decrease as the solutions become more concentrated because the density of the medium increases and the buoyant force on the sedimenting unit consequently decreases. Generally this is a small effect.

In order to use theories relating sedimentation coefficients to the molecular size and shape of the solute molecules it is necessary to employ the sedimentation coefficient corresponding to infinitely dilute solutions. Therefore, extrapolation procedures are employed. Sedimentation data at different concentrations can be plotted as  $s$  or  $1/s$  versus  $c$ , where  $c$  is the concentration, and the best-fitting curve is extrapolated to provide a value of  $s$  at infinite dilution. For systems showing a marked dependence of  $s$  upon  $c$  the plot of  $1/s$  versus  $c$  is preferred. Lauffer (1944b) has suggested a plot of  $s(\eta/\eta_0)$  versus  $c$  where  $\eta/\eta_0$  is the relative viscosity of the solution. Such plots along with a graph of  $s$  versus  $c$  on the same scale facilitate the extrapolation to infinite dilution since  $(\eta/\eta_0)$  approaches 1.0 as the concentration decreases. This combination plot has been particularly useful for studies of several animal viruses where impurities were still present in the preparations and the viscosities of relatively concentration solutions were ascribed to the contaminants (Lauffer and Stanley, 1944; Schachman, 1951a). With the recently renewed application of light absorption optical systems employing ultraviolet light (Schumaker and Schachman, 1957), reliable data are obtained with dilute solutions of viruses. Because of their content of nucleic acids and the consequent absorption of ultraviolet light, such solutions are now examined at concentrations tenfold lower than those commonly examined with schlieren optics. Difficulties in extrapolating the experimental data are thereby circumvented.

Corrections of the observed sedimentation coefficient,  $s_{\text{obs}}$ , to the standard state (a liquid with the viscosity and density of water at 20°C.) are made according to the equation

$$s_{20,w} = s_{\text{obs}} \left( \frac{\eta_t}{\eta_{20}} \right) \left( \frac{\eta}{\eta_0} \right) \left( \frac{1 - \bar{V}\rho_{20,w}}{1 - \bar{V}\rho_t} \right) \quad (21)$$

where  $(\eta_t/\eta_{20})$  is the principal correction factor corresponding to the viscosity of water at  $t$  relative to that at 20°C.  $(\eta/\eta_0)$  is the relative viscosity of the solvent to that of water, and  $\rho_{20,w}$  and  $\rho_t$  are the densities of water at 20°C. and the solution at  $t^\circ$ , respectively. Actually  $\bar{V}$  in the numerator should correspond to water at 20°C. whereas the value of  $\bar{V}$  in the denominator refers to the solvent under the conditions of the experiment.

*ii. Molecular Size and Shape.* The rate at which solute molecules migrate through a liquid is a function of their molecular weight, the difference in density between the solute and the medium, and the frictional resistance experienced by the molecules during their movement. There are various theoretical treatments describing in quantitative terms the behavior of molecules in a centrifugal field. Most popular of these is the so-called microscopic picture which considers the driving force on a single particle in terms

of its mass and the volume of liquid of known density which is displaced upon the addition of the particle to a large volume of solution. Alternatively this force can be expressed, without resort to a mechanistic point of view, by application of thermodynamics of irreversible processes. In this treatment the force is written as the gradient of the total potential. Under the influence of the centrifugal field the particles quickly attain a limiting velocity at which the frictional force (which is proportional to the velocity) is equal to the driving force. Each of the different theoretical treatments gives the result

$$s = \frac{M(1 - \bar{V}\rho)}{Nf} \quad (22)$$

This equation, it should be noted, is limited to two component systems, i.e., those solutions containing a macromolecule and the solvent. When other components are present, such as buffer salts, an additional term is required. Generally the evaluation of this term is difficult, and it is tacitly assumed that it can be neglected as long as the salt concentration is low. Omission of the salt, though desirable from a theoretical point of view (in terms of Equation 22), would cause a greater error owing to the electrostatic effects discussed previously. When large amounts of a third component like urea, sucrose, or inorganic salts are present, the use of Equation 22 is likely to lead to serious errors (Schachman and Lauffer, 1950).

For dilute solutions of the macromolecules, the frictional coefficient in sedimentation is considered to be the same as that encountered in diffusion; therefore Equations 14 and 22 can be combined to give the familiar Svedberg relation

$$M = \frac{RTs}{D(1 - \bar{V}\rho)} \quad (23)$$

where  $R$  is the gas constant,  $8.314 \times 10^7$  ergs/mole/degree. No assumptions as to the shape or degree of hydration of the sedimenting molecules are involved. Despite the fact that the molecules may be extensively hydrated in solution, correct values of the molecular weight are obtained through the use of Equation 22; moreover, the calculated molecular weight corresponds to the anhydrous molecule. In this respect the sedimentation-diffusion method is analogous to that of light scattering.

Treatments of the frictional coefficient similar to those already presented for diffusion are applicable to sedimentation as well, and the shape or hydration are calculated readily from Equations 22 and 23. In the absence of knowledge of either the shape or hydration, the sedimentation coefficient and molecular weight are combined with the intrinsic viscosity, according

to the equation of Scheraga and Mandelkern (1953), to calculate the parameter,  $\beta$ . From  $\beta$  the shape and volume of the equivalent ellipsoid are evaluated in the manner described earlier.

$$\beta = \frac{N_s[\eta]^{1/3}\eta}{M^{2/3}(1 - \bar{V}\rho)} \quad (24)$$

Use of the procedure suggested by Oncley (1941), who considered the observed frictional ratio,  $ff_o$ , as the product of two terms, one for solvation and one for shape, is facilitated by contour diagrams showing the various combinations of hydration and shape compatible with any single value of  $ff_o$ . These contour diagrams are simultaneous plots of Equation 17 and the Perrin equation. Again it should be noted that  $\beta$  is insensitive to shape for globular materials; therefore molecular weights can be calculated from  $[\eta]$  and  $s$  according to Equation 24.

*iii. Partial Specific Volume.* In all ultracentrifugal methods the term,  $(1 - \bar{V}\rho)$ , appears as one of the important factors. Since the partial specific volume of viruses is about 0.70 ml./gm. (slight variations occur depending upon the composition), errors in the determination of  $\bar{V}$  are effectively doubled in the calculation of molecular weights. It is imperative, therefore, that this quantity should be evaluated with great precision. There are, in effect, three different ways of determining the partial specific volume, defined as the increase in volume of an infinite amount of solution caused by the addition of one gram of solute.

First of these is the classic method which involves a series of density measurements on solutions of varying concentrations and the solvent (Lewis and Randall, 1923). From the density of each solution paired with the value for the solvent, the apparent specific volume is calculated (this assumes additivity of the volumes and weights of the solvent and the solute). Almost invariably for macromolecules of the size of viruses the apparent specific volume is independent of concentration, and the average of the individual values is taken as the partial specific volume. More elaborate treatments are required if the apparent specific volume is dependent on concentration. There are many different methods for the density measurements, and a choice among them is dictated mainly by the availability of material. Often the limited amounts of the substance preclude the use of techniques involving pycnometers, and the density gradient column of Linderström-Lang and Lanz (1938) is recommended. It is important to note that the density difference between the solution and solvent is very small (about 0.003 gm./ml. for a 1% solution). Measurements of high precision are therefore mandatory. The computation of the partial specific volume involves knowledge of the concentration. Because this is invariably determined on the basis of the dry weight, the molecular weight refers to the anhydrous material. If the viruses

formed a stoichiometric complex with water which could be isolated and weighed as such, then the ultracentrifuge would reveal the molecular weight of that material. Seldom, if ever, is this situation realized with macromolecules.

Frequently the amount of virus is so small as to preclude measurements of concentration and density; in this case all versions of the classic density method are of little value. Moreover, the virus preparation may be of such doubtful purity that density measurements on the solutions are not meaningful. In such circumstances an ultracentrifugal method is the one of choice. Examination of Equation 22 reveals that the sedimentation coefficient can become zero only when  $(1 - \bar{V}\rho) = 0$ . Accordingly, the sedimentation rate is measured in a series of solutions of increasing density and the resulting data plotted in a manner to permit extrapolation to the value of  $\rho$  corresponding to zero sedimentation rate. This value for the density of solution is equal to  $1/\bar{V}$ . Since the equation is restricted to two-component systems, it is not valid to employ any material such as sucrose to increase the density of the solution (Schachman and Lauffer, 1950). It appears, however, that mixtures of  $D_2O$  and  $H_2O$  act as a one-component solvent and therefore they can be used for these experiments. Unfortunately,  $D_2O$  solutions are not sufficiently dense to reduce the sedimentation rate of viruses to zero, and a long extrapolation is necessary. With accurate data, however, this can be performed in a satisfactory manner and the method has, therefore, much to offer. It should be noted that there will be exchange of some of the hydrogens on the virus with deuterium from the heavy water and the measured value corresponds to a deuterated macromolecule. The latter has a partial specific volume about 1.5% less than that of the virus in ordinary water. In this respect the method will doubtless be improved by the use of  $H_2O^{18}$  since there is much less exchange of oxygen than hydrogen. This ultracentrifugal method has already found wide application with viruses, including swine influenza virus (Sharp *et al.*, 1950), the virus of avian erythromyeloblastic leukosis (Sharp and Beard, 1954), bushy stunt virus (Cheng and Schachman, unpublished), and poliomyelitis virus (Schwerdt and Schaffer, 1955). In the latter instance only microgram amounts of virus were available for the whole study.

Often the partial specific volume of viruses and protein has been assumed because of the absence of relevant experimental data. Although this has been done frequently with no apparent rationale, a real basis does exist for such guesses. It has been found for many proteins that the volumes are closely equal to the sum of those of the individual amino acid residues (McMeekin and Marshall, 1952). Thus composition data in terms of the amino acids permits the calculation of the specific volume. Similar computations work satisfactorily for viruses when allowances are made for the nucleic acid.

*iv. Demonstration of Homogeneity.* Among the modern tools now employed in the study of large molecules, the ultracentrifuge possesses singular power for investigations of the purity and homogeneity of the sedimenting substance. Despite the directness and sensitivity of the sedimentation velocity method, there have been many ill-founded claims purporting to prove homogeneity. Most of these erroneous conclusions have been based on a cursory examination of ultracentrifuge patterns which reveal a single, sharp, symmetrical boundary. Such superficial observations more often than not are likely to be misleading. Even those claims based on a much more detailed investigation of the shape of the sedimenting boundaries are likely to require revision. This is necessitated by the refinements in the theoretical treatments which have occurred in the past few years. Thus the conclusions from the thorough investigations of bushy stunt virus (Lauffer, 1942) and T2 bacteriophage (Putnam, 1951) no longer can be accepted without question. If, upon reinvestigation with the greatly improved techniques, the diffusion coefficients are found to be slightly less than the values employed in those studies, the conclusions regarding the homogeneity of those viruses are valid. Alternatively, confirmation of the older data would lead to the inference that the preparations contained particles of varying size and shape. Until new data becomes available this matter of the homogeneity of bushy stunt virus and T2 bacteriophage remains unsettled; but all claims regarding ultracentrifugal demonstrations of homogeneity of virus preparations now must be considered incomplete.

The shape of a boundary, i.e., the distribution of concentration of solute as a function of distance, in a sedimentation velocity experiment is controlled by four factors. First of these is the broadening of the boundary due to diffusion, a consequence of the concentration gradient formed in the boundary region by the migration of the solute molecules. Second, broadening of the boundary occurs during the sedimentation of polydisperse material, since the faster moving molecules become separated from the slower components. In principle, the observed boundary can be considered as a composite boundary resulting from the sum of the boundaries of the individual components in the solution. Opposing these two effects which cause the boundary to spread with time is, third, the so-called sharpening effect resulting from the dependence of sedimentation velocity on concentration. At the trailing, or solvent, side of the boundary the concentration is much lower than on the solution side. Those molecules, falling behind as a result of diffusion or a lower sedimentation coefficient, find themselves in an environment of lower concentration. Their sedimentation rate therefore increases until they overtake the boundary. This self-sharpening of the boundary occurs continuously if the sedimentation velocity of the solute molecules is concentration-dependent; and the greater the concentration

dependence the more serious will be the sharpening effect. For tobacco mosaic virus (Schachman, 1951b) and deoxyribonucleic acid (Peacocke and Schachman, 1954) this self-sharpening leads to hypersharp boundaries and to the misleading impression that the material is homogeneous. Finally the boundary is distorted by the existence of the Johnston-Ogston effect (1946). Because a given molecule sediments more slowly in the presence of other components than it does while sedimenting alone, there is an apparent enhancement in concentration of the slower moving species, with a concomitant reduction in the apparent amount of the more rapidly sedimenting components. It is this effect which often leads to marked errors in the analysis of mixtures of two components. In the absence of complications, the area under the curve (with schlieren optics) is a measure of the concentration of the component responsible for the boundary. However, the existence of the Johnston-Ogston effect complicates the analyses and special treatments are necessary.

The demonstration of homogeneity is a laborious task but the theory and techniques for such an experimental investigation are now available. Several factors must be included in a rigorous test. Not only must there be a single, symmetrical boundary throughout the experiment, but also the concentration of the sedimenting substance must vary in accord with the radial dilution equation which accounts for the cell shape and the varying centrifugal field (Trautman and Schumaker, 1954). All of the sedimenting material in the solution must be accounted for by the moving boundary. Depending upon the optical system employed, different procedures are available for this test. The concentration evaluated from the ultracentrifuge patterns should agree within a few per cent with that obtained by chemical analysis. A single boundary should be observed under a variety of experimental conditions. This test, of course, may be limited by the stability range of the virus. As a prerequisite to more detailed investigations of the boundary shape, the sample should be examined at a series of concentrations to ascertain whether corrections are necessary for artificial boundary sharpening (Fujita, 1956). Through the application of absorption optics with ultraviolet light, solutions at sufficiently low concentrations may be employed so that this complication is avoided. If the change in sedimentation coefficient across the boundary proves to be negligible, the boundary spreading can be analyzed directly in terms of the standard deviation of the boundary curve. As a criterion of homogeneity the plot of the standard deviation versus time should be linear. This is equivalent to finding that the apparent diffusion coefficient, evaluated individually from each ultracentrifuge pattern, does not vary with time. Most systems have sufficient change in sedimentation coefficient across the boundary, however, that such a test is illusory and corrections for self-sharpening are mandatory (Schachman, 1951b). For these

materials use is made of a much more elaborate treatment employing equations derived by Fujita (1956). To use this theory, both the concentration dependence of the sedimentation coefficient and some parameters describing the shape of the boundary are required. As a further test of homogeneity, the boundary shape should be measured accurately and compared with a Gaussian curve. Deviations from a Gaussian shape are an indication of inhomogeneity.

It should be recognized that these measurements reveal heterogeneity, or its absence, with respect to sedimentation coefficient only. As such, any observed heterogeneity may be attributable to variations, within the population of solute molecules, of either density or molecular size and shape. In order to accentuate the effect of differences in density among the solute molecules, tests should be made in solutions of higher density so that the buoyancy term,  $(1 - \bar{V}\rho)$ , is made closer to zero (Cheng and Schachman, 1955a). By this means, variations among the virus particles in a given preparation with regard to their nucleic acid content, for example, may be subjected to direct experimental inquiry. This is feasible because of the high density of nucleic acid. If the relative boundary spreading is independent of the density of the medium, there is no heterogeneity with respect to density. Conversely, any observed enhancement of the boundary spreading, as the density of the medium is increased, indicates that the sedimenting material is heterogeneous with respect to density. The increasing emphasis on the study of the structure of incomplete viruses (lacking in nucleic acid) makes this type of investigation important. The reagent used to increase the density must be inert and exhibit no specific interaction with the virus. Evidence of this same type can be provided by sedimentation equilibrium experiments in density gradients (Meselson *et al.*, 1957).

*v. Sedimentation in Multicomponent Systems.* As already indicated, the equations presented above are restricted to two-component systems. However, it is often necessary to investigate viruses or their degradation products in solutions containing large quantities of a third component, such as sucrose or urea (Bechhold and Schlesinger, 1933; Smadel *et al.*, 1938). If it is known that the sedimenting material does not interact preferentially with either of the components of the solvent or, alternatively, there is no interaction (like solvation) at all, the solution then can be considered as a two-component system described by the equations presented earlier. Rarely, if ever, does this situation obtain. Even for those systems containing inert materials like sucrose there is, as Kauzmann (see Schachman and Lauffer, 1949) has pointed out, preferential interaction between the sedimenting material and water because of steric exclusion of sucrose from the immediate vicinity of the sedimenting solute molecules. The existence of this effect complicates those numerous investigations aimed at measuring the hydration of viruses.

For a comprehensive review of these studies the reader should consult the discussion by Lauffer and Bendet (1954). If hydration is defined as the selective solvation of the virus particle, then this method of determining hydration is ideal. Unfortunately, the total amount of liquid associated with the virus particle as a kinetic unit cannot be measured by this technique. Many workers, however, desire the latter information. This can be obtained, in principle, from measurement of the frictional coefficient, or the intrinsic viscosity, and independent knowledge of the shape of the hydrodynamic unit.

*vi. Sedimentation in a Partition Cell.* Frequently the amount of material available is so small as to preclude sedimentation analyses employing optical techniques, and recourse is made to the so-called analytical method based on the determination of the quantity of substance sedimenting across a fixed plane in a centrifuge cell. This method requires a special ultracentrifuge cell called the separation or partition cell which is so constructed as to effect a separation of the contents of the cell into two parts at the conclusion of the experiment. The amount sedimenting across the partition is the difference between the total amount of material originally in the cell and that which is left above (or centripetal to) the separating plate at the conclusion of the experiment. A specific and sensitive analytical method for the substance in question is required also. This may be a bioassay, a chemical analysis, or a physical chemical measurement such as radioactivity. The analysis need be only relative so that measurement, at the conclusion of the run, of the number of units of activity in the upper solution relative to the number in the original solution leads to a value of the sedimentation coefficient.

The separation cell affords still another important advantage to virologists interested in a specific biologically active substance. Frequently, in the isolation and purification of a given substance two or more boundaries are observed optically in the ultracentrifuge, and it is necessary to determine which of these corresponds to the material of interest. Even when only one component is observed optically and the material is thought to be purified, it may be profitable to measure the sedimentation rate by infectivity measurements to see if there is a correlation between the physical properties of the bulk constituent and the component with the biological activity. This method has been used in an impressive series of investigations by Lauffer and his colleagues (see Epstein and Lauffer, 1952). There are now two types of partition cells available (Tiselius *et al.*, 1937; Yphantis and Waugh, 1956). These work in different ways, as illustrated in Fig. 4. Through the use of this technique, in conjunction with the porous disk diffusion cell, the molecular weight of a virus can be determined even prior to its purification.

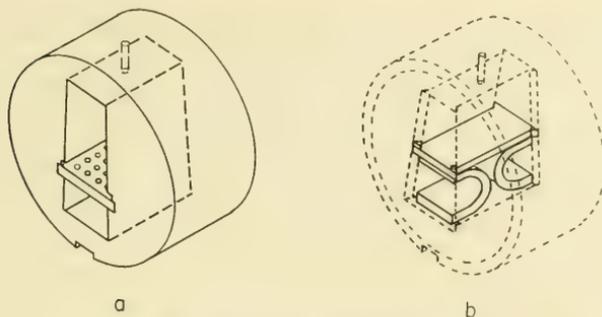


FIG. 4. Diagrams of Separation Cells.

a. Fixed partition cell (Tiselius, Pedersen and Svedberg, 1937). In this cell a fixed, porous, metal plate is built into the cell at a position about  $2/3$  of the distance from the centripetal surface. Filter paper is overlaid on the metal plate, and this unit acts as a barrier against mixing of the contents of the upper and lower compartments at the conclusion of the experiment. The sedimentation of the macromolecules occurs through the filter paper-plate combination.

b. Movable partition cell (Yphantis and Waugh, 1956). The partition in this cell is supported by two synthetic rubber strips which act as a spring. Under the influence of the centrifugal field developed during acceleration of the rotor, the plate, which is solid and plastic, settles to the bottom of the cell. Sedimentation then occurs undisturbed by the presence of the barrier at the bottom of the cell. At the conclusion of the run, during deceleration, the rubber springs cause the plate to rise slowly to its rest position thereby effecting a separation of the contents of the cell into centripetal and centrifugal fractions which can be removed readily without intermixing.

c. *Sedimentation Equilibrium*. As early as 1938 careful sedimentation equilibrium experiments were performed with bushy stunt virus (McFarlane and Kekwick, 1938). Actually some work had been conducted prior to that time with tobacco mosaic virus (Eriksson-Quensel and Svedberg, 1936), but details of these studies were not given. Despite the promising results obtained in the experiments with bushy stunt virus, no other studies appear to have been made in the ensuing twenty years. This work with bushy stunt virus is particularly interesting, even though the results seem to be erroneous, because it illustrates some of the important aspects of experimentation by the sedimentation equilibrium method. Very low centrifugal fields must be employed; otherwise the solute molecules (if they are of the size of most viruses) will be sedimented to the bottom of the cell. Also extremely short columns of solution are necessary if prohibitively long experiments are to be avoided. With a column height of only 2 mm. (as contrasted with the columns of about 15 mm. height that are used for sedimentation velocity experiments) sedimentation equilibrium was attained in about 48 hours. As a result of recent developments in instrumentation, routine operation of the ultracentrifuge for such time periods is now commonplace. Moreover, the risk of inactivation of the virus is minimized by operation at low temperatures.

Improvements are still needed, however, in the construction of damping devices to eliminate vibration and precession of the rotor at speeds as low as 500 r.p.m. When these improvements are realized, sedimentation equilibrium experiments with viruses will become routine.

The equations describing the concentration distribution in an ultracentrifuge cell in which equilibrium is attained can be derived either from thermodynamics or from consideration of the transport of material by sedimentation and diffusion. That such distribution functions can be deduced from thermodynamics constitutes the major appeal of the sedimentation equilibrium method. The shape, permeability, and hydration of the solute are irrelevant to a determination of the molecular weight, for the thermodynamic description of a system at sedimentation equilibrium does not involve these factors. For homogeneous materials in solution sufficiently dilute that concentration effects are negligible, the molecular weight is calculated from a plot of  $\log c$  versus  $x^2$  according to

$$M = \frac{2RT}{(1 - \bar{V}\rho)\omega^2} \frac{d \ln c}{d(x^2)} \quad (25)$$

Equation 25 is useful for ultracentrifuge experiments employing ultraviolet absorption optics or the Rayleigh interferometer. This equation can be used in a different form when schlieren optical systems are used. Alternatively the molecular weight can be calculated from data expressing the concentration gradient as a function of distance:

$$M = \frac{2RT}{(1 - \bar{V}\rho)\omega^2} \frac{d \ln \left( \frac{1}{x} \frac{dc}{dx} \right)}{d(x^2)} \quad (26)$$

It should be noted that these equations are restricted to two-component systems. As in the sedimentation velocity and light-scattering method complications are introduced when large amounts of a third component are present in the solution. Neglect of possible preferential interactions between the solute and one of the components of the solvent can lead to serious errors in molecular weight determinations. This is also the situation with the novel sedimentation equilibrium method involving sedimentation and flotation of macromolecules in a density gradient formed by the distribution of a solute of low molecular weight (Meselson *et al.*, 1957).

Some information regarding heterogeneity is also provided by sedimentation equilibrium experiments, but it should be noted that this method is not nearly as sensitive as the sedimentation velocity technique. If solutions exhibit concentration effects, as is often the case particularly with elongated macromolecules, experiments at different concentrations are necessary. The data are then used for extrapolation to infinite dilution.

*d. The Transient States during the Approach to Sedimentation Equilibrium.*

During the past five years there has been vigorous activity in developing procedures for molecular weight determinations during the approach to sedimentation equilibrium. All of these efforts are based upon the investigations of Archibald (1947) aimed at securing mathematical solutions of the differential equation describing sedimentation in the ultracentrifuge. Although the detailed mathematical solutions are not of much practical value at present, Archibald emphasized that the limiting (or boundary) conditions themselves provided the basis for an ultracentrifugal method for the direct determination of molecular weights. The net transport of solute *across* any given surface in the ultracentrifuge can be expressed as the difference in the fluxes due to sedimentation and diffusion. At equilibrium the net flux is equal to zero everywhere in the cell. To achieve this equilibrium state throughout the cell, as already noted, very long time periods are required. However, at the two end surfaces of the cell the net flux is equal to zero for all times. This is a consequence of the fact that the cell is closed and the macromolecules cannot cross the meniscus from the air bubble nor can they leave the aqueous solution at the bottom. To be sure, the concentration does decrease at the meniscus (and increase at the cell bottom), but the concentration gradients change accordingly and there is no transport of solute *across* the two end surfaces. When these relations are expressed in mathematical terms and the equations rearranged we find

$$M = \frac{RT}{(1 - \bar{V}\rho)\omega^2} \frac{(dc/dx)_m}{x_m c_m} \quad (27)$$

The subscript, *m*, in Equation 27 refers to the meniscus and a corresponding equation can be written for the cell bottom. It should be noted that these relationships are derived by a thermodynamic treatment as well. Like the equations presented earlier, these refer to ideal solutions containing only two components. Extrapolation procedures are required in order to obtain values of the concentration gradient,  $dc/dx$ , at the two ends of the cell. The corresponding concentrations are evaluated by calculation procedures which, though tedious, yield reliable values.

For homogeneous materials the molecular weights calculated for the top and bottom of the cell should be the same. Thus the results secured from the two ends of the cell provide some measure of the homogeneity of the sedimenting material. Evidence of gross heterogeneity is obtained readily by this method, but it does not possess the sensitivity inherent in the sedimentation velocity method. The success and scope of the method have been so great that a vast amount of data has been accumulated already by its application. As yet this method has not been employed successfully with viruses. However, recent technical improvements which permit operation of

the ultracentrifuge at extremely low speeds should soon allow the Archibald method to be applied as routinely to virus particles as it is now applied to virus subunits (Hersh and Schachman, 1958).

### 6. *Electrophoresis*

Electrophoresis, like sedimentation and diffusion, involves the measurement of the migration of molecules (or ions) under the influence of a driving force. In diffusion and sedimentation the driving force stems from a concentration gradient and from a centrifugal field, respectively, while in electrophoresis an electric field is employed to cause the directed movement of the charged molecules. Unlike the other two methods discussed above, the results of electrophoresis experiments cannot be interpreted in an entirely satisfactory manner in terms of the molecular parameters of the migrating particles. For this reason, only a brief discussion of electrophoresis is presented here. The brevity of the remarks should not be taken as implying that electrophoresis is only of moderate usefulness to virologists or protein chemists. Indeed, in the study of the purity of macromolecules, for example, electrophoresis has proved to be a powerful tool comparable in sensitivity to the sedimentation velocity method. Electrophoretic investigations complement the other hydrodynamic methods by virtue of their utilizing not the size and shape of the migrating particles but, rather, the nature and number of the ionizable groups on their surfaces. Whereas a mixture of strains of tobacco mosaic virus may appear homogeneous by the criteria of sedimentation and electron microscopy, electrophoretic examination reveals distinct molecular species (Singer *et al.*, 1951). Also electrophoresis provides important information relevant to the location of nucleic acid within a virus particle.

The application of an electric field to a solution of charged molecules creates a force on the molecules which is directly proportional to their net charge and to the strength of the electric field (the potential gradient). This force causes the molecules to move, rapidly attaining a limiting velocity that is fixed by the driving force and the frictional resistance experienced by the moving particle. It is tempting, therefore, to write equations similar to those employed in the treatment of sedimentation. However, this approach is not fruitful for two reasons. First of all, the effective net charge on the macromolecule is not dictated solely by its own chemical composition. Electrophoretic experiments are conducted in salt solutions. As a result of electrostatic forces, ions from the medium are attracted toward the surface of the charged macromolecules with ions of charge opposite to the macromolecule predominating in the neighbourhood of the material in question. This ionic cloud causes a partial screening of the total charge of the macromolecule. Thus the effective charge is less than that expected from the

chemical composition and the characteristics of the ionizable groups of the macromolecule. Second, the hydrodynamic resistance to motion is complicated by the fact that the layers of liquid immediately surrounding each macromolecule are themselves subject to electric forces. The flow patterns are not governed solely by viscous drag of the type considered by Stokes (1851) [or by Perrin (1936) in the modified treatment for nonspherical particles]. Consequently, the hydrodynamic behavior observed in diffusion and sedimentation is not exhibited in electrophoresis. Owing to the ambiguities introduced by the screening effect and the distortion of the flow patterns, the net charge on a macromolecule is not evaluated directly from electrophoresis studies. Empirical correlations often prove very informative, however.

Electrophoretic investigations are of three general types, each of which possesses special features commending it for use. The so-called microelectrophoresis method (Abramson *et al.*, 1942) is based on the use of an ordinary light microscope for the direct visualization of the migrating particles. Since viruses or proteins are too small to be seen directly, objects capable of optical resolution in the microscope are introduced into the solution. For this purpose glass beads or even droplets of oil are frequently employed. These objects become coated with a film of the protein or other macromolecules, and the electrophoretic migration is then determined by the charged groups of the macromolecules of the film. The method is simple and precise, and data are obtained rapidly. In studies of the dependence of electrophoretic mobility on pH this method proves most useful. The microelectrophoresis technique, to a large extent, became obsolete with the development (Tiselius, 1930) of the moving boundary method. Here the motion of the macromolecules is followed by optical methods which register the position and shape of the boundary between the solvent and the solution. The presence of two types of molecules with different electrophoretic mobilities is clearly demonstrable as two boundaries. Moreover, the amounts of the two components can be evaluated with some accuracy from analyses of the electrophoresis pattern. With the microelectrophoresis apparatus such an analysis is virtually impossible. If each of the two components coats the microscopic particles, an average mobility is obtained which often is not meaningful. The moving boundary method has also found wide application in the isolation and purification of macromolecules since the components in a mixture became separated, depending upon their different migration rates. Such preparative procedures are limited, however, because of the absolute requirement that the system have gravitational stability at all times. This precludes the complete separation of two species into two different zones. Were this to occur, a region would be created which possessed a lower density than the zone above it, i.e., somewhere in the cell there would be liquid

containing solute molecules lying above the pure solvent. Convective stirring would occur immediately. Attempts to circumvent this limitation have led to the development of zone electrophoresis, the third principal type of electrophoresis technique. Here the charged molecules migrate through an aqueous solution which is held partially immobilized in the interstices of paper or in the capillary space between starch granules. Paper and starch electrophoresis are examples of zone electrophoresis. The success of these methods is attributable to the fine capillary spaces which are sufficiently narrow that bulk flow of liquid is effectively prevented. Many ingenious designs of apparatus have been proposed and tested during the past decade, and zone electrophoresis has become one of the more powerful tools available for the detection and isolation of biologically interesting substances. Although zone electrophoresis can be used in studies of mobilities, empirical corrections are required because of the electroosmotic flow of the liquid itself through the supporting medium.

Most quantitative electrophoretic investigations have involved the moving boundary method in a form somewhat like that employed by Tiselius (1930), Longworth (1945), Alberty (1953), and their collaborators. By this method both large and small molecules can be examined with ease. The number of components in a mixture is evaluated readily and fractionation can be effected so as to identify the component responsible for a given biological activity. Results are reported as the electrophoretic mobility, which is the velocity of the charged particles per unit potential gradient, and has the units, cm./sec./volt/cm. By convention the mobility is given a positive or negative sign in accord with the sign of the net charge of the migrating ions. Perhaps the most popular application of electrophoresis is the determination of the isoelectric point, the pH at which the molecules do not migrate in an electric field. In this regard electrophoresis yields valuable information about the interaction of macromolecules with specific ions like phosphate and chloride ions. This is revealed by the dependence of the isoelectric point on the amount and nature of the salts present during the electrophoresis experiments.

Despite inadequacies in the theoretical treatments for the interpretation of electrophoretic mobilities, there has been spectacular success in explaining boundary anomalies by means of the moving boundary theory (Longworth, 1945; Dole, 1945). These anomalies include (1) different mobilities in the ascending and descending limbs of the electrophoresis cell; (2) different shapes of the boundary in the two limbs, with the rising boundary being sharper than the descending one; (3) the presence of additional boundaries, the so-called  $\delta$  and  $\epsilon$  boundaries in the ascending and descending limbs, respectively; and (4) the lack of correspondence of the amount of the migrating material in the two limbs. Any precise electrophoretic studies must

be interpreted in terms of the well-established theory for moving boundaries.

Electrophoresis is likely to prove of greatest value to virologists in studies of the homogeneity of a given preparation. Different tests can be applied. First of all, a single boundary should be observed over as wide a range of pH and ionic strengths as can be employed. Such tests are necessarily limited to conditions under which the virus is stable. The observation of a single boundary at one pH is not a sufficient basis for conclusions about homogeneity. Different materials may have identical mobilities under one set of conditions, and the differences become manifested only when the pH or ionic strength is altered. Secondly, the rate of spreading of the boundary is analyzed quantitatively. In effect such homogeneity tests in electrophoresis involve measurement of an apparent diffusion coefficient. If this is independent of time, homogeneity is indicated. As in sedimentation, there are sharpening effects which make such tests illusory. One simple test is the so-called reversible boundary-sharpening test. After the boundary has migrated a considerable distance, the polarity of the current is reversed. Any sharpening of the boundary upon its return to its original position is conclusive evidence of inhomogeneity. If, instead, the boundary continues to broaden after the current is reversed, the spreading is attributable to diffusion. Most delicate of the various tests is the analysis of boundary spreading during electrophoresis at the average isoelectric point of the material. If all of the molecules are identical the spreading should be governed solely by diffusion. Any heterogeneity, however, leads to a marked increase in the rate of spreading of the boundary. Experiments performed at the isoelectric point obviate many of the anomalies resulting from the movement of boundaries.

## *B. Optical Methods*

### *1. Light Scattering*

Light scattering is one of the phenomena that may be observed when particles, such as viruses, interact with radiation. Without exception, when radiation is incident upon a particle an event generally describable as scattering occurs in which some of the radiant energy is diverted from its incident linear path to follow paths that have the particle itself as the source of the scattered energy. Four kinds of physical methods involving scattering of radiation have been demonstrated to be useful in the study of viruses. Two of these, light scattering (see Stacey, 1956; Edsall, 1953) and low-angle X-ray scattering, are essentially similar in principle and in method, differing only in the wavelengths of the radiation used. In both, the observed radiation originates as wavelets that are scattered from nearly independent particles in a suspension with only moderate coherence among the wavelets. A

third method, involving the examination of crystal structures by X-ray diffraction, utilizes scattered radiation that has come from ordered arrays of particles and in which there is a high degree of coherence, resulting in predominant interference effects. A fourth method, electron microscopy, utilizes electrons that have been scattered, but the optical arrangements are such that these electrons can form a real image of the scattering particles.

*a. Small-Particle (Rayleigh) Scattering in a Gas.* If a single particle, suspended in a medium with an index of refraction different from its own, is illuminated with electromagnetic radiation, an oscillating dipole is established whose strength is a function of the amplitude of the incident wave motion and of the spherical polarizability of the particle. (We shall see later that the polarizability can be related to the relative indices of refraction of the particle and of the medium, but the polarizabilities of molecules are also a function of their volumes. The larger the molecule the more easily is there a separation of charges upon it, hence the greater is its polarizability.) This oscillating dipole acts as a source of radiation, effectively "emitting" light without change of frequency. If the incident radiation is unpolarized the expression for the intensity of the observed scattered light is:

$$i_{\theta} = \frac{8\pi^4 I_o \alpha^2}{r_s^2 \lambda^4} (1 + \cos^2 \theta), \text{ where} \quad (28)$$

$i_{\theta}$  = intensity of light scattered at the angle  $\theta$

$I_o$  = intensity of incident light (proportional to the square of its amplitude)

$\alpha$  = spherical polarizability of the particle

$r_s$  = distance from particle to point of observation

$\lambda$  = wavelength of the incident light in the medium

$\theta$  = angle between the forward direction of the incident light and the line between particle and observer

The ratio  $\left[ \frac{i_{\theta}}{I_o} \right]_{\theta}$  is usually written as  $R_{\theta}$ , Rayleigh's ratio.

The scattered light is plane-polarized at  $\theta = 90^{\circ}$ , with the degree of polarization decreasing symmetrically as  $0^{\circ}$  and  $180^{\circ}$  are approached, at which angles it vanishes. The intensity of scattering is a minimum at  $\theta = 90^{\circ}$  and has a value twice this minimum at  $0^{\circ}$  and  $180^{\circ}$ . The envelope of the intensity of the scattered radiation, in any plane containing the incident beam, has a shape somewhat between that of a dumbbell and an ellipse, with the major axis lying along the incident beam.

A collection of randomly arrayed particles in a medium whose own scattering is negligible will scatter light proportionately to the number of scattering centers per unit volume, allowing a simple addition to be made of the scattering contributions of each particle. To write an expression for

such cumulative scattering effects, we note first that it can be shown from Maxwell's equations that:

$$\alpha = \frac{n^2 - n_o^2}{4\pi\nu}, \quad (29)$$

where  $n$  and  $n_o$  are the refractive indices of the particles and of the medium, and  $\nu$  is the number of particles per unit volume.

Since the value of  $\nu$  is rarely known, it is more convenient to express it in terms of weight concentration, molecular weight, and Avogadro's number

$\nu = \frac{Nc}{M}$ . Then, for unpolarized incident radiation:

$$R_\theta = \frac{\pi^2}{2\lambda^4} \left( \frac{n^2 - n_o^2}{c} \right)^2 \frac{Mc}{N} (1 + \cos^2 \theta), \quad \text{where} \quad (30)$$

$c$  = weight concentration of the scattering particles

$M$  = mass of each particle in grams per mole

$N$  = Avogadro's number

The above expression gives  $R_\theta$  for a gas—a collection of scattering particles in a vacuum. For this case, Equations 29 and 30 can be simplified, since  $n_o = 1$ . The wavelength,  $\lambda$ , of the radiation can be written as  $\lambda_o$ , the wavelength *in vacuo*.

*b. Small-Particle Scattering in Solutions.* When light scattering occurs in a solution, the equation just derived can no longer be conveniently applied. To be sure, in an ideal solution the solute molecules are sufficiently widely dispersed to allow Equation 30 to be applied to scattering from these particles alone. Thus, for an ideal solution the *increment* in scattering due to the solute particles is directly calculable. But if the scattering from the solvent or from the whole solution is considered, it is no longer correct to make a scalar summation of the scattering effects of the individual molecules. The scattering centers are now so close together that interference effects become important, i.e., the wavelets scattered from one center may destructively or constructively interfere with those from another. Actually, destructive interference is overwhelmingly the more effective at measurable angles of scattering, and a straightforward application of Equation 30 to liquids would predict a scattering intensity many-fold greater than is observed. It is possible to develop a theory of scattering for solutions from detailed consideration of interference effects, but the calculations are laborious. A more useful approach, due initially to Einstein (1910), is to treat the scattering as a result of statistical fluctuations in the density of the solvent, and in the concentration of the solute molecules, leading to local fluctuations in the dielectric constant and hence in the refractive index (Partington, 1953). Since we are here interested in the scattering due only to

the solute molecules, the treatment that follows refers to variations of concentrations of only these molecules. This distinction is accomplished experimentally by subtracting from the amount of light scattered by the solution the amount that is scattered by the pure solvent.

By the application of thermodynamic reasoning it can be shown that for a solution we can write:

$$R_{90} = \frac{\pi^2}{2\lambda_0^4} \cdot \frac{RTc}{N(\partial P/\partial c)} \left( \frac{\partial \epsilon}{\partial c} \right)^2, \quad (31)$$

where

$$R_{90} = \frac{i_0 r_s^2}{I_0}, \text{ for } \theta = 90^\circ$$

$R$ ,  $T$ , and  $N$  = gas constant, absolute temperature, and Avogadro's number, respectively

$P$  = osmotic pressure

$\epsilon$  = optical dielectric constant

From this equation it is seen that the amount of scattering is dependent upon the osmotic work required to produce the fluctuations in concentration.  $\partial P/\partial c$  can be written as the differential form of the Van't Hoff equation for nonideal systems:

$$\frac{\partial P}{\partial c} = RT \left( \frac{1}{M} + 2Bc + \dots \right),$$

where  $M$  is the molecular weight.

The optical dielectric constant and the experimentally determinable index of refraction are related thus:

$$\frac{\partial \epsilon}{\partial c} = \frac{\partial n^2}{\partial c} \simeq 2n_o \left( \frac{dn}{dc} \right),$$

where  $n_o$  is the refractive index of the solvent.

Upon making the indicated substitutions we have:

$$\frac{2\pi^2 n_o^2 (dn/dc)^2}{\lambda_0^4 N} \times \frac{c}{R_{90}} = \frac{1}{M} + 2Bc + \dots \quad (32)$$

Grouping the left-hand constants under the term  $K$ :

$$\frac{Kc}{R_{90}} = \frac{1}{M} + 2Bc + \dots \quad (33)$$

If  $\frac{Kc}{R_{90}}$  is plotted against  $c$  a straight line will result, the slope of which is the coefficient of nonideality,  $B$ , and the intercept of which is the reciprocal of the molecular weight.

If the solute molecules are polydisperse the molecular weight term in Equation 32 must be taken to represent some sort of average. It is readily seen that this is the *weight average* molecular weight, previously defined as

$$M_w \equiv \frac{\sum c_i M_i}{\sum c_i}$$

where  $c_i$  and  $M_i$  are respectively the weight concentration and molecular weight of the  $i^{\text{th}}$  component of a polydisperse mixture. We can write for the  $i^{\text{th}}$  component:

$$\frac{K}{R_{90i}} = \frac{1}{c_i M_i}$$

Since  $R_{90 \text{ obs}} = \sum R_{90i}$ , and  $c_{\text{obs}} = \sum c_i$ , we have:

$$\sum \frac{K}{R_{90i}} = \frac{K}{R_{90 \text{ obs}}} = \frac{1}{\sum c_i M_i}$$

Hence,

$$\frac{K \sum c_i}{R_{90 \text{ obs}}} = \frac{K c_{\text{obs}}}{R_{90 \text{ obs}}} = \frac{\sum c_i}{\sum c_i M_i} = \frac{1}{M_w}.$$

*c. Large-Particle Scattering.* It is to be recalled that Equation 33 is valid only for solutions containing solute particles whose largest dimensions are small ( $< 1/10$ ) compared with the wavelength of the scattered light. If the particles are comparable in at least one dimension with the wavelength, a correction to this equation must be introduced, owing to interference effects that are intraparticle in origin (Debye, 1947). If we consider the relative intensities of light scattered in the generally forward and backward directions, it is evident that in the latter case there will be some destructive interference owing to phase differences between scattered wavelets that have originated at different places along the particle. In the forward direction, the phase differences are on the average much nearer zero. The consequence of the interference effects is to warp the shape of the envelope of scattered intensity so as to decrease the magnitude of the backward portion.

The correction factor for dissymmetry in the scattering envelope is generally called the "particle-scattering factor,"  $P(\theta)$ . Its reciprocal,  $P^{-1}(\theta)$ , is used to multiply the observed  $R_\theta$  in order to correct for the interference effects.  $P(\theta)$  is a function of  $\theta$  and of the size of the scattering particles compared to the wavelength of the light employed. For example, in the case of spheres of diameter equal to  $\lambda/2$ , the value of  $P^{-1}(\theta)$  is 2.7, for  $\theta = 90^\circ$ . For spheres of this size, then, the observed  $R_{90}$  must be multiplied by 2.7 before it can be used in Equation 33 for the calculation of  $M$ .

In practice, of course, the value of  $P^{-1}(\theta)$  must be calculated from measurements that do not include knowledge of particle dimensions or shapes. What

may be measured is the ratio of  $R_\theta$  at 45 and 135°, the so-called "dissymmetry ratio." For a given particle shape this ratio is a unique function of the ratio of the characteristic dimension of the particle to the wavelength of light,  $L/\lambda$ . [Characteristic dimensions are: diameter (sphere); length (rod); root-mean-square end-to-end distance (random coil).] If a model shape for a particle is presumed to be known (a sphere, a rod, or a random coil) a value of  $L/\lambda$  is directly obtained from the dissymmetry measurements, allowing the correction factor  $P^{-1}(\theta)$  to be evaluated. Hence, by the dissymmetry method molecular weights can be determined for particles of size comparable with a wavelength of light if a particle shape is assumed. Actually the shape of the particles can be ascertained in various ways from the appropriate light-scattering data.

If many light-scattering measurements can be made at various  $\theta$ 's and concentrations, it is possible to calculate molecular weights without making any assumption as to particle shape (Zimm, 1948). In this method use is made of the fact that at  $\theta = 0^\circ$ , the value of  $P^{-1}(\theta)$  is unity (no interference effects), and at  $c = 0$ , there are no particle interactions. A "Zimm plot" is made, which is a gridlike representation of  $Kc/R_\theta$  as a function of both  $\theta$  and  $c$ . Extrapolations are made both to  $\theta = 0$  and  $c = 0$ ; the two extrapolated lines should meet at the same point. This intercept is then simply  $1/M$ . The slope of the  $\theta = 0$  line near the origin gives the value of  $B$ , the interaction term, while the initial slope of the  $c = 0$  line yields a value for the radius of gyration of the particle. The radius of gyration is defined as that distance from the center of mass of a body such that its moment of inertia remains the same if all the mass is concentrated at that radius. It can be used to calculate the characteristic dimension of a particle, if the shape is known.

We have seen that under favorable conditions the methods of light scattering will yield molecular weights of particles in the range of size of the viruses. They will also provide a determination of a characteristic dimension, although this is the Z-average dimension, and is heavily weighted toward the larger particles in a polydisperse suspension. The beauty of the methods of light scattering is that they can be made quickly, that they do not disturb the particles under measurement, and that they are essentially equilibrium methods not involving any hydrodynamic effects.

It should be emphasized that the value of  $M$  determined by light scattering is a weight-average molecular weight, while the calculated characteristic dimension,  $L$ , is a Z-average value. This means that the determination of both quantities is highly sensitive to the presence of small amounts of foreign material of relatively large size. Contamination by dust particles is particularly to be avoided. If the solute particles under investigation are polydisperse, such as partially aggregated solutions of tobacco mosaic virus, the

value of  $M$  will be distinctly greater than the number-average molecular weight. For example, a solution containing equal numbers of tobacco mosaic virus particles of molecular weights  $1 \times 10^7$  and  $10 \times 10^7$  would yield a value of  $5.5 \times 10^7$  as the number-average molecular weight and a weight-average molecular weight of  $9.2 \times 10^7$ .

While the methods of light scattering yield highly reproducible results under favorable conditions (dilute solutions of spherical particles that are small with respect to the wavelength of light), caution is to be exercised in the acceptance of results for those cases where the *absolute* values of  $M$  of large, elongated particles have been reported. The determination of the value of  $M$  depends upon an absolute determination of  $R_\theta$ , a quantity involving the ratio of scattered to incident light intensities. The direct measurement of this ratio is quite uncertain, and recourse is generally taken in using other calibration methods, i.e., the relation between turbidity and light scattering, as a function of wavelength, for suspensions of small, spherical particles such as polystyrene latex. But the most likely absolute error due to calibration difficulties remains hard to assess.

In deriving values of  $M$  from light scattering the most likely source of error lies in the  $dn/dc$  term (see Equation 32), because its value is squared when used in the equation.

It is now almost universal practice to use Zimm plots in evaluating  $M$  for large, anisometric particles. Here the handling of the data is particularly susceptible to systematic error owing to the nature of the extrapolations. It is necessary to extrapolate the data to  $\theta = 0^\circ$  in order to determine  $M$ . Unfortunately, in the region of  $\theta$  near zero, direct measurements cannot be made, but it is just in this region that effects of contaminating dust and large aggregates of solute particles become the greatest. A large dust particle will scatter much more light in the  $\theta = 0^\circ$  direction than in the  $\theta = 45^\circ$  direction, and an extrapolation that must go from, say,  $\theta = 20^\circ$  to  $\theta = 0^\circ$  is inevitably hazardous.

## 2. Low-Angle X-ray Scattering

It has been seen that the methods of light scattering will provide an evaluation of the molecular weight of particles in suspension whether they be large or small compared with the wavelength of light. However, it is to be noted that the shape of the envelope of scattered intensity is quite insensitive to particle size and shape when the particles are smaller than about  $500\text{\AA}$  in their characteristic dimension. Many virus particles are smaller than this, and if the sizes and shapes are to be determined by radiation scattering it is evident that shorter wave lengths must be employed. For an object of a given size the dissymmetry of the scattering envelope (which is dependent

upon intraparticle interference effects) increases as the wavelength of scattered radiation decreases. By the use of radiation in the X-ray region it is possible to determine sizes, and, to some degree, shapes of particles as small as the smallest viruses, since at very short wavelengths the intensity of scattering from such particles falls off very rapidly with the angle of scattering  $\theta$ . In fact, the scattering dissymmetry is so great for ordinary X-radiation that values of  $\theta$  only extremely close to zero can be measured, hence the term "low-angle scattering". It would be convenient if considerably longer wavelengths could be used, but since aqueous fluids are quite opaque to these the experimental difficulties of measuring at  $\theta \approx 0^\circ$  must be put up with (see review by Edsall, 1953).

*a. Particles of Any Shape.* The derivation of the relevant scattering equations follows the same pattern as that for the light-scattering equations, except that individual electrons are taken as the source of the scattered radiation instead of induced oscillating dipoles within the macromolecules. Also interparticle interference effects are neglected in the derivations, as in the case of light scattered by the molecules of a gas. The general form of the scattering envelope for molecules large compared with the wavelength of the X-rays has a maximum centered at  $\theta = 0^\circ$  with minima and subsidiary maxima as  $\theta$  increases. The central maximum has a shape that is approximately Gaussian for particles with centrosymmetry, and in this angular region the equation for the scattered intensity can be written (Schmidt *et al.*, 1954; Guinier, 1939):

$$\phi^2(h, R) = e^{-\frac{h^2 R^2}{3}} \quad (34)$$

where  $\phi$  is the amplitude of scattering,  $h = \frac{4\pi \sin \theta/2}{\lambda}$ , and  $R$  is the radius of gyration of the centrosymmetrical particles. This equation is valid whatever the shape of the particles. Therefore, if  $\log \phi$  is plotted against  $(\sin \theta/2)^2$  the shape of the straight line will yield a value of  $R$ . To obtain from a knowledge of  $R$  the actual radius of the particle requires either additional information or an assumption regarding its shape. For spherical particles  $R^2 = \frac{3}{5}a^2$ , where  $a$  is the particle radius.

*b. Spherical Particles.* Low-angle X-ray scattering has been used particularly for determining the size of spherical virus particles for which an additional equation (not restricted to small angles of scattering) is applicable:

$$\phi^2(h, a) = \left[ \frac{3 (\sin ha - ha \cos ha)}{(ha)^3} \right]^2, \quad (35)$$

where  $a$  is the particle radius, and  $h = \frac{4\pi \sin \theta/2}{\lambda}$ . This equation has maxima and minima which can be tabulated as functions of  $\theta$  and of  $a$ . Thus, by

measuring the scattering envelope over a range of  $\theta$  and by noting the positions of the maxima and minima, one can calculate the particle radius,  $a$ .

Since there are two equations relating to  $a$ , it is possible to estimate whether or not an assumption of sphericity is valid. From Equation 34 a value of  $R$  is directly obtained, from which  $a$  can be calculated on the assumption that the scattering particles are spherical. If this value of  $a$  agrees with that obtained from the positions of the maxima and minima by use of Equation 35, the assumption is likely to be valid.

The radius of particles calculated from low-angle X-ray scattering is that dimension within which the electron density is, on the average, larger than that of water. If a virus particle contains water of internal hydration its calculated radius will include the resulting enlargement of the particle. A shell of external hydration will not show as an increased  $a$ , since any water bound to the surface of the particle will have the same X-ray scattering power as will the general aqueous environment. An interesting case is encountered when the particle is a spherical shell, believed to be the form of turnip yellow mosaic virus. The shape of the scattering curve is then very nearly like that of a solidly spherical particle of the same diameter, but the maximum and minimum points are shifted in a direction that would correspond to a larger particle. This is in accord with the relation between the outer radius and the radius of gyration of a shell; these radii are more nearly equal for a shell than for the case of a solid sphere.

Low-angle X-ray scattering, like light scattering, can be affected by high concentrations of solute molecules. Since the wavelengths are much shorter, however, considerably higher concentrations can be tolerated before interparticle interference effects become appreciable. But interaction phenomena, such as aggregation and orientation of the particles, raise equally serious problems of interpretation. In these cases it is necessary to plot measured scattering intensities as a function of concentration and extrapolate to zero concentration.

### 3. X-ray Diffraction

Some of the smaller, spherical viruses have been found to be crystallizable, i.e., to form into fully ordered, three-dimensional arrays. Tobacco mosaic virus, which is rod-shaped, has not been fully crystallized *in vitro* but it can be oriented into a paracrystalline array in which all rods are parallel and equidistant. In both kinds of crystals the internal orderly arrangement of the virus particles has made it possible for the methods of X-ray analysis to be used to disclose certain aspects of their structures (see review by Low, 1953).

*a. The Simple Lattice.* A crystal is characterized by having within itself a regular, repeating three-dimensional pattern of particles, such as molecules or atoms. If the crystal is illuminated with X-rays, each atom will act as a

scattering center, and a phenomenon quite analogous to light scattering will be originated. But since there is an ordered array of such centers the effects of interference among the scattered wavelets will be predominant, with the result that there will be constructive interference only in certain, highly restricted directions, and destructive interference in all others.

The existence of repeating, ordered arrangements of atoms within a crystal makes it convenient to think in terms of intracrystalline *planes*. Suppose we have a crystal made of a multitude of just two kinds of atoms, A and B. . . . Planes can be drawn through the A atoms in numerous ways; the most significant for our purposes are those that contain the highest density of atomic population. Suppose one of these planes is designated with respect to its orientation by three direction indices (Miller indices)  $h$ ,  $k$ , and  $l$ . Numerous parallel planes can be drawn through the A atoms, all having the same  $(h k l)$  values. But each B atom in a crystal is spatially related in a particular way to each A atom. Hence, for every  $(h k l)$  plane drawn through the A atoms an equivalent plane can be drawn through the B atoms. In a crystal whose molecules have a complex character it is clear that for each planar orientation of a given  $(h k l)$  designation there will be sets of parallel planes through atoms A, B, C, . . .

Since a crystal being analyzed in an X-ray apparatus is always small in comparison with the distance from the X-ray source to the receiver, all parallel planes are geometrically equivalent. If the conditions (discussed below) are correct for constructive interference to exist along a certain direction for the wavelets scattered by atoms in a given  $(h k l)$  plane, they will be correct for all planes of this Miller index whether the atoms contained therein are of species A, or B, or . . . The X-ray beam is usually small in cross section, and well collimated, so that the trace of a constructively interfered beam on the receiving plane (such as a photographic film) is usually a spot. From the measured coordinates of a "spot" one can ascertain the value of  $(h k l)$  for the parallel planes that gave rise to it.

If a single crystal is held fixed in front of a beam of monochromatic X-rays, very few spots will be found on the receiving plane despite the infinite number of  $(h k l)$  values of the planes within the crystal. This circumstance is due to the very stringent conditions that are imposed upon constructive interference from a three-dimensional lattice. W. L. Bragg showed in 1915 that the conditions can be very simply expressed. Suppose a given set of planes make the grazing angle  $\theta$  with respect to the incident X-ray beam, and suppose further that the distance between the parallel planes is  $d$ . The simultaneous conditions for constructive interference are then:

(1)  $\theta = \theta'$ , where  $\theta'$  is the angle between the planes and the constructively interfered beam of scattered wavelets;

(2)  $m\lambda = 2d \sin \theta$ , where  $m = 1, 2, 3 \dots$  (the spectral order), and  $\lambda =$  the wavelength of the X-rays.

The imposition of simultaneity upon these equations means that, for a given orientation of the crystal, only a few sets of planes indeed will serve to create constructive interference in the scattered X-rays. In order to obtain spots from many sets of planes of different ( $h k l$ ) values it is necessary, in one way or another, to rotate the crystal about different axes during the recording of the spots.

Certain geometrical characteristics of a crystalline lattice may be calculated from relatively simple measurements of the intensities and positions of the diffracted X-ray spots. First of these is the size of the *unit cell*. Since a crystal is composed of repeating units it is convenient to think of it as being composed of a number of identical elementary volume elements. A unit cell is the smallest parallelepiped that can be constructed within the crystal such that the entire crystal can be built up by means of unit displacements of the cell. If the density of the crystal, and its chemical composition (molecular weight of its molecules) are known, a knowledge of the size of the unit cell allows a computation to be made of the number of molecules (or atoms) within it.\* It is also possible to determine fairly readily the *symmetry* of the crystalline array. While the general subject of crystal symmetry is too extensive to discuss here, certain clarifying points can be made. A crystal can be thought of as a regular array of points. A given point, then, will be related to other points by certain operations of symmetry. If a crystal lattice is distinguished by a certain group of symmetry elements, and if a point is placed anywhere (to start with), the operation of the symmetry elements will effectively multiply this point into a collection of points. Translations of this collection will build up an ordered pattern in space. Depending upon the exact location of the original point there will be different patterns built up, but all will have the same symmetry elements. If the initial "point" is an asymmetric collection of  $N$  atoms disposed as would be the case in, say, a protein molecule, the operations of symmetry will build up, for each atom, a space pattern. Each space pattern is identical in its symmetry with those made of the other atoms. The entire set of space patterns would be the actual protein crystal, if it were infinitely extended. The scaffolding of symmetry elements upon which an infinite crystal may be built is known as its *space-group*; there are 230 such space-groups possible. For a detailed analysis of an actual crystal structure it is first necessary to determine the number of molecules per unit cell, and the space-group to which the crystal belongs (see Robertson, 1953).

*b. The Compound Lattice.* As we have seen, a crystal structure with  $N$

\* Alternatively, if the number of molecules in a unit cell is known, the same kind of calculation provides an accurate value of the molecular weight.

atoms in the unit cell may be considered to be the equivalent of  $N$  equal and parallel, interpenetrating space lattices, one for each kind of atom of the unit. Such a lattice is the type usually encountered and is known as a *compound lattice*. The ultimate purpose of X-ray crystal structure analysis is to determine the relative position of each of the  $N$  atoms, or more precisely, of their electron clouds. But the X-ray data furnish direct information only about the positions and the intensities of the X-ray spots. In a compound lattice (suppose  $N = 2$ ) all planes with the same  $(h k l)$  value will be involved in producing the observed intensity of one spot. Depending upon the spacing between the  $h k l$  planes of atom 1 and of atom 2, in terms of the spacing of the planes of atoms 1 or 2 alone, the coherent scattering from the two sets of planes will have differing phase relations. For example, if the planes through atoms 1 are midway between those through atoms 2, the scattering amplitudes from the two sets of planes will be completely out of phase for  $m = 1, 3, \dots$ . In a very complex lattice, where  $N$  might be 1000, the intensity of every X-ray spot will be the square of the vector sum of some 1000 scattering amplitudes all having different phase relations. But these phase relations cannot be calculated precisely until the structure is known—the very problem being investigated.

*c. Calculation of X-ray Intensities.* In order to attempt to understand the methods employed to resolve the dilemma of unknown phases it is useful to approach the inverse problem: that is, to assume that the positions and X-ray scattering powers for each of the  $N$  atoms are known, and to calculate the predicted intensities of the X-ray spots. If in the unit cell there are atoms of kinds A, B, . . . , there is associated with each kind a scattering power,  $f_A, f_B, \dots$ . The value of  $f$  depends upon the radial density distribution of the scattering electrons within the atom, upon the wavelength of the X-rays, and upon the angle of scattering,  $\theta$ . It can be shown that

$$f = \int_0^\infty U(r) \frac{\sin \phi}{\phi} dr, \text{ where } \phi = \frac{4\pi r \sin \theta}{\lambda} \quad (36)$$

$U(r)$  is a function of the radial density distribution of the electron cloud and may be written:  $U(r) = 4\pi r^2 |\psi|^2$ , where  $|\psi|^2$  is a solution of Schrödinger's equation, and where  $|\psi|^2 dv$  is proportional to the chance of finding an electron within a small volume,  $dv$ . Values of  $f$  for many kinds of atoms have been calculated from their wave functions.

The total amplitude scattered from a unit cell and forming a single X-ray spot will depend upon the scattering factors for the atoms within the unit cell and upon their spatial distribution. The spatial distribution will govern the phase relations among the amplitudes of scattering from the parallel planes (of a given  $h k l$ ) that go through all the kinds of the  $N$  atoms. The

problem is solely a geometrical one, and it turns out that the entire structure amplitude,  $F$ , of a unit cell may be written as:

$$\begin{aligned} |F_{hkl}| \cos \alpha &= f_A \cos \phi_A + f_B \cos \phi_B + \dots = P \\ |F_{hkl}| \sin \alpha &= f_A \sin \phi_A + f_B \sin \phi_B + \dots = Q \\ F_{hkl}^2 &= P^2 + Q^2; \tan \alpha = Q/P \end{aligned}$$

where the sine and cosine terms represent the phase relations that result from the relative positions of the  $N$  kind of atoms within the unit cell. The  $\phi$ 's are simple functions of the atomic positions, of the value of  $(h \ k \ l)$  for the planes giving rise to the X-ray spot, and of the order of the X-ray reflection. It might be noted that the  $f$ 's are dimensionless quantities, and consequently so is  $F$ .  $F^2$  represents the ratio of the expected intensity of the X-ray spot to that resulting from the scattering of X-rays by a single classic electron. Since the latter value is calculable, the expected intensities may be calculated and may be compared with the observed ones. This method of comparison is universally employed in checking the reliability of any X-ray analysis.

*d. Fourier Summations.* The practical problem in X-ray crystallography is formally the inverse of the above-described operation: the calculation of the atomic positions from the observed intensities and  $(h \ k \ l)$  designations of the X-ray spots. Since a crystal is a periodic arrangement, in three dimensions, of electron densities (atomic positions) it is possible to represent the electron density at any point by a triple Fourier summation:

$$\rho_{xyz} = \frac{1}{V} \sum \sum \sum |F_{hkl}| \cos [2\pi(hx + ky + lz) - \alpha_{hkl}]$$

$\rho_{xyz}$  is the electron density at the point  $x, y, z$  in the unit cell, and  $V$  is the volume of the cell. The coefficients of the summation are the absolute values of the structure amplitude factors discussed previously. The summation is formally an infinite one over all values of  $h, k$ , and  $l$ ; in practice it would be limited to those values for which corresponding X-ray spots are measured. The evaluation of the triple sum is a formidable task, but, what is worse, for the case of complex molecules it cannot in principle even be attempted. The difficulty is that only quantities proportional to  $F^2$  can be measured, but  $F$  itself is a quantity having both a magnitude and a phase angle. Hence, in the summation the value of  $|F_{hkl}|$  is simply the square root of  $F^2$ , but the value of  $\alpha_{hkl}$  is usually unknown. Another way to express this fact is to say that the Fourier sum represents the integrated information contained in the entire set of X-ray spots from all measured planes, and that this information cannot be evaluated until the phase relations among the X-ray beams forming all spots are known. The  $\alpha_{hkl}$  term represents these phase relations.

*c. Patterson Vector Maps.* One useful approach to the problem of determining the phases of the structure-amplitude terms was suggested by Patterson (1934), who proposed that serious consideration be given to a Fourier summation in which the *squares* of the  $F_{hkl}$  terms would be the coefficients and in which no phase-angle term would be present. Such a summation can be evaluated from the observed intensity data, rather readily so if only a two-dimensional projection is desired. Patterson, and later Harker (1936), showed that this type of summation has a physical significance. A simple example will suffice to show the interpretation of a "Patterson summation". Suppose that there are three atoms: A, B, and C. If we draw lines interconnecting the atoms, there will be three lines, but each line will have two "directions": depending (for example) upon whether the line is directed  $A \rightarrow B$  or  $B \rightarrow A$ . There will thus be six "vectors", and, in general, there will be  $N(N - 1)$  such vectors for the case of  $N$  atoms. If we now select an origin we may draw these six vectors from it, displacing them as necessary but keeping their directions fixed. The end of each vector will be given a weight (a so-called "vector density") corresponding to the product of the number of electrons in the atoms at the two ends of the vector. Thus, an analog of a true electron density map will be drawn. The Patterson summation yields such a "vector density" map. In general it cannot be analyzed to give the positions of the electron density peaks themselves. It is useful in that it gives *general* notions about what are most likely the relative positions of the X-ray scattering centers; a pattern appearing prominently in the Patterson projection sets limits upon the number of guesses that can be taken about the positions of the true electron density peaks. For complex molecules, at least, the most commonly appearing contour maps are Patterson projections, since these can always be secured, and they offer some shreds of information.

*f. Heavy Atom Replacement.* While Patterson summations are useful in attempting to arrive at some useful notions as to electron density distributions, they have proved of only limited use in the case of complex molecules such as the proteins and viruses. What is wanted, of course, is some method whereby the relative phases of the  $F_{hkl}$  terms can be evaluated. Two somewhat similar methods exist whereby this problem can be directly approached: (1) the method of heavy atom introduction, and (2) the method of isomorphous replacement (Green *et al.*, 1954). In the former an atom of great X-ray scattering power is introduced within the molecular structure, while in the latter successive replacements of heavy atoms are attempted, the crystalline structure remaining isomorphous during the replacements. In both cases the hope is that the position of the heavy atom is the same within each molecule of the crystal and that its position in the unit cell can be found. The effect of the heavy atom is to modify the relative intensities of the

X-ray spots; in the extreme case the intensities are due primarily to this atom. The position of the target atom can be estimated from a Patterson summation, since its position will be indicated by especially prominent vector peaks. In favourable cases its position can be inferred from stereochemical considerations. When once the place of the heavy atom in the unit cell is approximately ascertained, its effects upon the intensities of the X-ray spots helps to clarify the determination of the phase relations of all spots. The method of isomorphous replacement is especially powerful in that, if more than two different kinds of heavy atoms can be found for replacement, the estimates of the phase relations of the structure amplitude terms are more strictly constrained by the multiplicity of conditions imposed upon them. The recent success of Kendrew and colleagues (1958) in the use of the method of isomorphous replacement for the analysis of the structure of myoglobin makes it appear that similar methods will prove useful for the spherical viruses.

In actual practice the analysis of complex structures by X-ray diffraction is by no means as mathematically straightforward as the foregoing discussion might imply. The crystallographer leans heavily on all the subsidiary evidence that he can find and apply. Consideration is taken of the spatial requirements of atoms, of their bond distances and angles, of the requirements for structural stability, and of the types of atomic coordination likely to be encountered. Considerable use is made of successive approximations—a Patterson summation will suggest certain spatial parameters for the electron density peaks; these are used to obtain tentative two-dimensional projections of the true electron density; inconsistencies in the  $F_{hkl}^2$  values calculated from these positions, as compared with observed intensities, will suggest their further refinement, etc. But the final test must always be that the proposed model makes stereochemical sense, and that calculated intensities of the X-ray spots agree reasonably well with the measured ones.

It is premature to expect that any virus crystal has been structurally analyzed to a degree that even begins to designate the atomic positions. This may not even be anticipated until considerably more is known about the chemical structure of viruses, because the scattering power of carbon, nitrogen, and oxygen are so similar that X-ray analysis cannot distinguish among them. The complete three-dimensional atomic positioning (except for the hydrogen atoms) has been achieved for vitamin B<sub>12</sub> (Hodgkin *et al.*, 1956), but only with the aid of exhaustive chemical information. As will be seen in more detail later, some evidence of structural detail at the level of large atomic groups has been obtained for tobacco mosaic virus, but for the few spherical viruses examined only intimations of internal structures have been secured. Notions of sizes and molecular weights of some viruses have

been derived, as well as some details of their packing arrangement within crystals, but this is about all at the present.

*g. Diffraction Patterns of Oriented Fibers.* The preceding discussion has been concerned with X-ray diffraction analysis of three-dimensional crystal-line arrays, such as a crystal of rock salt. There is another general type of oriented structure, however, than can be described as a fiber, or paracrystal. It consists of a closely packed array of elongated particles with the fiber axis parallel to the axes of the individual particles. Such paracrystals can be formed, for example, by drawing out fibers of deoxyribonucleic acid (DNA) or by orienting the rods of tobacco mosaic virus by rolling or by controlled evaporation.

An X-ray pattern of a fiber is usually obtained by directing the X-ray beam perpendicular to the fiber axis. Two directions in the X-ray pattern are then distinguishable: the "meridional" direction that is parallel to the fiber axis, and the "equatorial" direction that is perpendicular to the axis. If the fiber contains repeating structures, the X-ray pattern will consist of a set of spots, or short arcs, that has symmetry about its center. In general the spots will be arrayed along "layer lines"; these are linear groupings that run perpendicular to the direction of the fiber axis and represent successive orders of constructive interference of the X-rays scattered from some axial periodicity of structure. From the spacing of the layer lines a determination can be made of the size of this repeating structural unit. The spacing and intensities of the X-ray spots in the equatorial direction give information about the interparticle spacing in the oriented array, and may disclose something about the intraparticle radial distribution of electron density.

X-ray diffraction patterns of paracrystals made up of particles of a *helical* configuration have recently assumed great importance owing to the ubiquitous presence of elongated molecules of this type in biological systems. Examples of helical configurations are the polymers of DNA and the rods of tobacco mosaic virus. The theory of the X-ray analysis of helical structures has been developed (Cochran *et al.*, 1952), and predicts certain distinctive aspects that allow such structures to be recognized and analyzed. The most striking aspect of the diffraction patterns of helices is seen along the meridian, where a region of emptiness prevails. The innermost spots of the layer lines are off-meridian, with the distance from the meridian increasing with the number of the layer line. To a first approximation, for example, the innermost spot of the fifth layer line will be five times as far off-meridian as will the corresponding spot of the first layer line. As a consequence the pattern appears at first glance to have an X-like shape. Meridional spots do appear, however; if the structural unit within the helical particles repeats  $n$  times in  $m$  turns of the helix (where  $n$  and  $m$  are integers) a meridional spot will be found on the  $n^{\text{th}}$  layer line. For example, in DNA there are 10 nucleotides in

one turn of the helix; hence there is a meridional spot on the tenth layer line. The appearance of diffraction patterns of helices are generally so characteristic that there is no difficulty in recognizing such structures.

#### 4. *Electron Microscopy*

*a. Principles of the Formation of an Electron Image.* The electron microscope is a close optical analog of the ordinary light microscope in that it has a source of radiation, a condenser lens, one or more objective and projector lens, and it images the specimen by transmitted radiation. The source of radiation is a hot filament from which electrons are emitted thermionically *in vacuo* and subsequently accelerated through 50 to 100 kilovolts potential. At these velocities the electrons have associated with them a wave of length about  $0.04 \text{ \AA}$ ; it is this extremely short wavelength that makes possible the high resolving power of the electron microscope. Since moving electrons are carriers of charge they are influenced in their motions by electric and magnetic fields. A strong magnetic field that is coaxial with an electron beam and that has both axial and radial variations will serve as a positive lens and will focus a diverging electron beam to an image.

The three principal lenses of an electron microscope are usually wire-wound solenoids in the axis of which is a soft-iron core (the pole-piece) having a central hole. The strength of such a lens is varied by changing the current in the solenoid, and lenses with focal lengths as short as 0.2 cm. have been produced. The function of the first lens, the condenser, is to image the source of electrons near or in the plane of the specimen. The specimen objects, such as viruses, are mounted upon a thin film, almost completely transparent to the electrons, while the objects themselves must be nearly so. The electrons passing through the specimen either emerge unaffected or are scattered through small angles. Those that fall within a sufficiently narrow cone, centered on the specimen, pass through the objective lens and are imaged near the front focal plane of the projector lens. This lens casts a final, real image of the specimen upon either a fluorescent screen for direct visualization, or upon a photographic film where the exposure times are of the order of a few seconds. When a change of focus is required it is accomplished by changing the strength of the objective lens, while changes of magnification are the function of the projector lens. The sole purpose of using an instrument as complicated as the electron microscope is to form images whose resolution is greater than that found in the images formed by a light microscope (see Hall, 1953).

*i. Resolving Power of an Optical System.* Whenever a field of particles is imaged by transmitted light it is convenient to regard the process as one in which the final image is an interference pattern created by the recombination of radiation that has been scattered with that which has passed

through the field unscattered. As a consequence, the degree of structural detail that can be discerned in the final image (or interference pattern) is dependent upon the wavelength of the radiation employed and upon the angular aperture of the imaging system. It has been established for many years, both from theoretical considerations and from experimental tests, that a simple formula can be written to represent the minimal distance by which two opaque points can be separated in the object plane and still be separably imaged (the "minimum resolvable distance"). The formula is based upon diffraction theory and assumes that the effects of spherical aberration are negligible. It may be written:

$$d_{\text{diff}} = \frac{0.61\lambda}{n \sin \theta} \quad (37)$$

where  $\lambda$  is the wavelength of the radiation in air,  $\theta$  is the half-angle subtended by the imaging lens at the object point, and  $n$  is the refractive index of the medium between the object space and the lens.

It is evident from Equation 37 that  $d_{\text{diff}}$  cannot be less than about one-half the wavelength employed, since  $n \sin \theta$  cannot be made much greater than 1.25. Consequently it cannot be expected, when visible light ( $\lambda = 0.5\mu$ ) is employed in ordinary microscopy, that any but the largest viruses can be resolved one from the other nor can a single virus particle be discerned against its background. Under dark-field conditions, where only scattered light is used for image formation, and where a particle-free nonscattering background will appear dark, the image of a particle can be *discerned* no matter how small it is. Resolution is not improved in dark-field microscopy, of course, but the visibility of single, small particles is distinctly enhanced.

*ii. Resolving Power of an Electron Microscope.* In an electron image Equation 37 is still valid in setting a lower limit upon the minimum resolvable distance, but unfortunately it does not represent the only consideration. If only Equation 37 were the relevant one (and if  $n \sin \theta$  could be made as large as unity) the  $d_{\text{diff}}$  would be about  $0.02\text{\AA}$ , corresponding to an electron wavelength of  $0.04\text{\AA}$ . In the derivation of Equation 37 it is assumed that the lens systems are perfect, i.e., that the geometrical aberrations of the lens system are negligible. In the case of electron imagery there is a severe restriction upon the degree to which aberrations can be reduced. Focusing of the electrons is accomplished by means of either magnetic or electric fields which universally act as positive lenses, thus preventing the correction of aberrations by combinations of positive and negative lenses, a universal procedure in ordinary microscope lens systems. The only aberration of magnetic lenses (or electrostatic ones) that is important for paraxial electrons, but which has so far proven uncorrectable, is spherical aberration. Owing to

this aberration alone there is a certain minimum resolvable distance that can be written:

$$d_{\text{sph}} = C\theta^3 \quad (38)$$

where  $C$  is a constant depending upon the focal power of the lens and upon the variation of the magnetic field along its axis, and  $\theta$  is the aperture angle as used in Equation 37.

It is evident that a compromise must be effected in the design and use of electron lenses, inasmuch as changing the value of  $\theta$  affects the value of the minimum resolvable distance in opposite senses when the effects of both diffraction (Equation 37) and of spherical aberration (Equation 38) are considered. It can be shown that the sum of the two effects is minimized when they are made equal to each other, i.e., when  $d_{\text{diff}} = d_{\text{sph}}$ . It turns out that  $\theta$  has a value of only about 1 degree under these conditions. Hence the resolving power of an electron microscope is only about 1/100 as great as it would be if lenses could be made having the numerical apertures of objective lenses commonly met with in light microscopes. It is generally agreed that an electron microscope can have a minimum resolvable distance as small as 6Å when it is used to photograph ideal objects: small, discrete particles of high electron opacity.

*iii. Effects of Specimen Thickness on Resolution and Contrast.* In practice it is unusual for a specimen to have ideal properties for electron imagery, and in the case of viruses, at any rate, the effective resolving power of the electron microscope is notably inferior to the 6Å mentioned above. There are two important factors affecting this additional limitation upon resolving power: thickness of the specimen, and contrast between specimen object and the background. When electrons penetrate an ordinary specimen they are scattered, most of them elastically but some inelastically. The inelastically scattered electrons will suffer a decrease in velocity, and hence will not be focused in the same plane as the main electron beam. The effect is to degrade the image sharpness in a manner analogous to the effects of chromatic aberration in a glass lens system when white light is employed. The consequences of chromatic aberration in electron lenses are serious when the specimen thickness is greater than a few hundred Angstrom units.

The source of contrast in an electron microscope is different from that in a light microscope. In the latter the contrast is due to differential opacity of the specimen objects; light of certain wavelengths is actually absorbed here and there in the specimen. But all the light leaving the specimen is brought to a focus where the image is observed. In phase-contrast and interference microscopy the specimen need not absorb light to exhibit contrast, but rather it need only have variations of thickness and/or refractive index from point to point. But here, too, all the light leaving the object plane is imaged by the objective lens. In an electron microscope (if we neglect inelastic scattering)

all the electrons entering the specimen leave it with their velocities unchanged in magnitude and changed only in direction—the effects of elastic scattering. An electron lens that would image all electrons leaving the specimen, scattered or not, would deliver an image devoid of information, since it would have no contrast from point to point. Actually, an electron lens operates at a numerical aperture of only about 0.01 (as shown above), the aperture limitation being usually effected by the insertion of a metal disk with a very small central hole in the back focal plane of the lens. This is the so-called “objective aperture.” Its effect is to block all electrons scattered through an angle greater than about  $1^\circ$ . Thus, a relatively thick specimen that scatters electrons widely will be imaged less brightly than one that scatters electrons throughout only a narrow cone. Contrast phenomena are the direct result of scattering effects, the more highly scattering objects appearing relatively dark in the image.

In the observation of particulate materials, such as suspensions of virus particles, the limitations imposed upon the effective resolving power of the electron microscope are largely those brought about by deficiencies in contrast. Even when a very small objective aperture is used it is still a fact that a virus like tobacco mosaic, with a diameter of  $150\text{\AA}$ , appears with very low contrast indeed, as ordinarily photographed. Although it is difficult to set even an approximate figure, it might be estimated that with materials of the chemical composition of viruses the effect of low contrast in electron images changes the useful resolution of the electron microscope from its theoretical value of about  $6\text{\AA}$  to a figure more like  $50\text{\AA}$ .

Fortunately, the contrast exhibited by small objects can be artificially enhanced. One way is to employ shadowing (Williams and Wyckoff, 1946), whereby a thin film of a heavy metal is cast obliquely upon the specimen surface. The result is that the condensed metal is unevenly distributed, owing to topographical variations, affording contrast through the great electron-scattering power of even a thin film of a heavy metal (Fig 5). By application of this technique the effective resolving power of the electron microscope approaches  $15\text{\AA}$ , where it is apparently limited by the structure of the shadowing film. Another method of enhancing contrast is to impregnate the specimen materials with a stain of high electron-scattering power—a volume stain, as distinct from the surface-staining effects of shadowing. Osmic acid (Porter and Kallman, 1953) and phosphotungstic acid (Hall *et al.*, 1945) have been the compounds of choice in such staining procedures, but it appears that neither is particularly effective when applied to virus particles under reasonably normal pH conditions (Hall, 1955).

Not only is the contrast in the images of small particles dependent upon their electron-scattering power per unit thickness, but it is influenced from one micrograph to another by the exactness of focus. This influence is

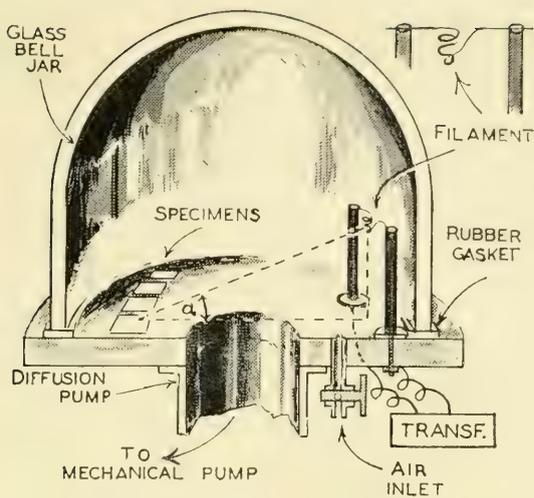


FIG. 5. Schematic representation of apparatus used in shadowing for electron microscopy. The filament is normally charged with a small amount of a heavy metal such as uranium or platinum. The "shadowing angle" is defined as the reciprocal of the tangent of the angle  $\alpha$ . (From *The Anatomical Record* **96**, 27, 1946)



FIG. 6. (a) Particles of tobacco mosaic virus, not shadowed; (b) shadowed with uranium. Both at  $\times 150,000$ . Although the shadowed particle does not appear smooth on its surface, there is no evidence of periodic structure.



especially unfortunate whenever attempts are made to assess the effectiveness of staining procedures on very small particles. If the image of a specimen object is formed further from the lens than its proper position it is said to be "underfocused." An underfocused image of a circular object is characterized by having a bright interference halo around its periphery, lending to the image an enhanced contrast. Resolving power is decreased by underfocusing, but in the interests of contrast it is common practice to underfocus slightly. An overfocused image has both a loss of resolving power and of contrast.

*b. Techniques of Electron Microscopy.* With many biophysical methods the theoretical complexities and uncertainties underlying the interpretation of the experimental data outweigh in importance a consideration of the techniques used to secure the data. In some cases the techniques are well-established and have not changed fundamentally for years. In electron microscopy there is no great body of theory that has to be considered in interpretation of micrographs, and in those cases where interpretation is obscure the most relevant factor is usually a consideration of the technique employed. Methods assume a relatively large importance in electron microscopy, also, because it is a new field in which the full usefulness of the instrument has yet to be explored.

*i. Virus Suspensions, Morphology.* The electron microscopy of suspensions of virus particles, either intact or disintegrated, can be conveniently divided into two types of investigation. One of these is morphological in character, where the shape, size, and structural arrangement of virus particles or their subunits are being examined. The other is essentially quantitative in nature, where the purpose is to count the numbers of various kinds of particles in a known volume of suspension. The development of techniques for the former type of investigation has tended toward enhancement of the effective resolving power of the microscope and toward the preservation of structure of the virus particles. In the latter type of work considerations of morphology are secondary. Rather, experimental advances have been directed toward securing specimen fields that are qualitatively and quantitatively representative of the entire suspension being investigated.

As has been indicated above it is necessary to enhance the contrast exhibited in the images of small virus particles before anything useful can be told about their structural details, either interior or exterior. Unfortunately, electron stains for virus particles are so poorly developed that little can be said about enhancement of interior detail. The exterior form and surface detail, however, can be greatly accentuated by the application of shadowing methods (Fig. 6). The principal objectives in the development of these methods are to secure a substrate surface that is devoid of apparent irregularities, and to obtain shadowing films that show no evidence of granularity. While conditions have existed for some time by which detail of the

general order of 30 Å can be reliably observed, it is only recently that this level of detail has been considerably reduced. Hall (1956) has developed a method involving preshadowed replication (Williams and Wyckoff, 1945), wherein a surface of freshly cleaved mica is used as a structureless substrate surface upon which the specimen objects are dispersed. Shadowing is done with platinum, over which is deposited a film of silicon monoxide or carbon. Subsequently the shadowing film and its overlay are stripped from the mica with the aid of a supporting collodion film. In principle the method is quite old; the excellence of Hall's results seems to reside in the use of mica and in the application of a film of great stability (such as silicon monoxide) over the shadowing one.

The preservation of the details of virus structure has always been a problem in electron microscopy, brought about by the necessity of observing the virus particles after they have dried from their aqueous suspending medium. Two problems are involved here: one is the elimination of non-volatile salts that are frequently found in a suspending medium, while the other is the prevention of distortion of the virus particles as they dry. Viruses are frequently suspended in solutions of appreciable ionic strength, and if the ions are those of nonvolatile salts, such as NaCl, a virus suspension when dry will exhibit far more salt than virus. A way out of this difficulty has been found by the use of suspending media whose ionic constituents are completely volatile (Backus and Williams, 1950). At the present time it has been found that ammonium acetate, bicarbonate, and benzoate are particularly useful salts to use in those cases where pure water is not an adequate suspending medium. Another method that has proved effective in the elimination of nonvolatile salts in a dried virus preparation is one in which a suspension is allowed to dry on a film of collodion that is in contact with a block of semisolid agar (Kellenberger and Kellenberger, 1955). The effect of the agar is to imbibe the salt ions along with the water molecules, leaving the larger virus particles on the surface of the collodion.

It is readily seen that when a particle as small and as nonrigid as a virus dries out of a water suspension it is subject to considerable pressure brought about by surface tension forces. The effect of this pressure is to flatten the particle and to distort its structure (Fig. 7). The alleviation of this artifact has taken two directions, by Anderson (1951), and by Williams (1953a). In the method developed by Anderson the virus particles are first seen exposed to osmic acid and are then brought through various miscible solvents into carbon dioxide at a pressure and temperature such that it is liquid. When the temperature is raised it passes through its critical point, and the virus particles now find themselves in a gaseous environment. After the CO<sub>2</sub> gas is allowed to escape the virus particles are ready for observation, having been dried without exposure to the forces of aqueous surface tension. The method developed

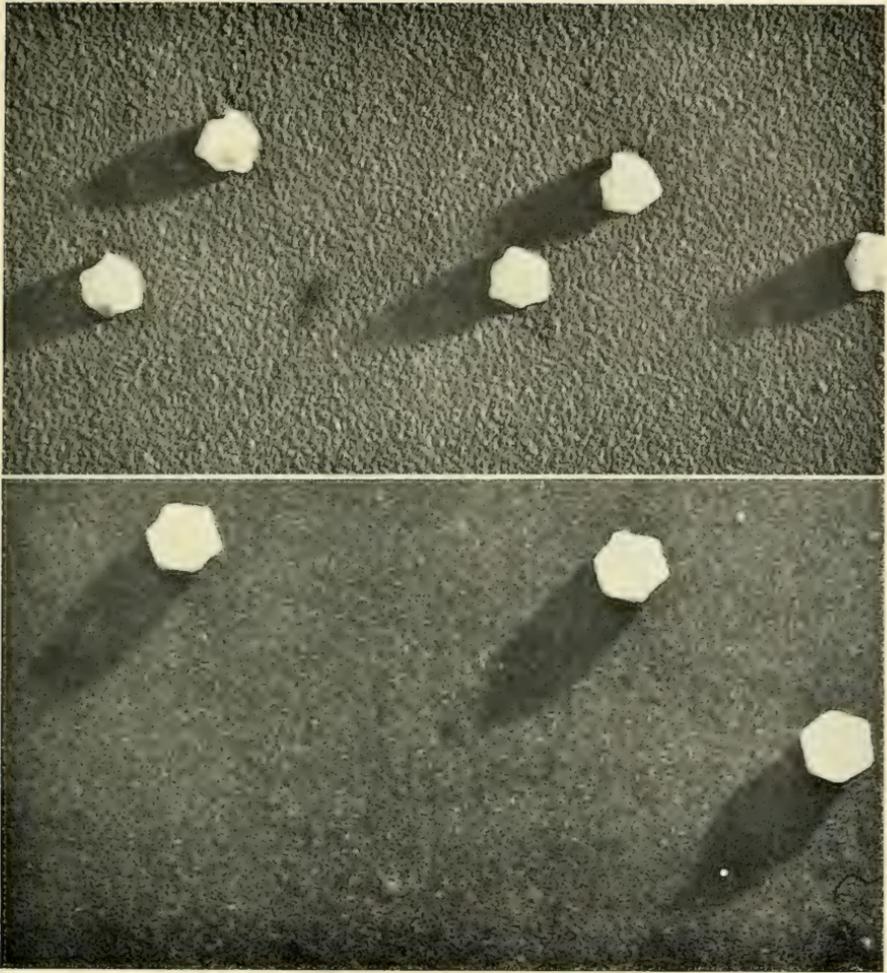


FIG. 7. (a) *Tipula iridescent* virus dried from a water suspension and showing distortion due to drying; (b) frozen-dried. Both at  $\times 65,000$ . Note the sharp contours of the frozen-dried particles and the geometrical regularity of their shadows. Only an icosahedron can cast shadows of these shapes.

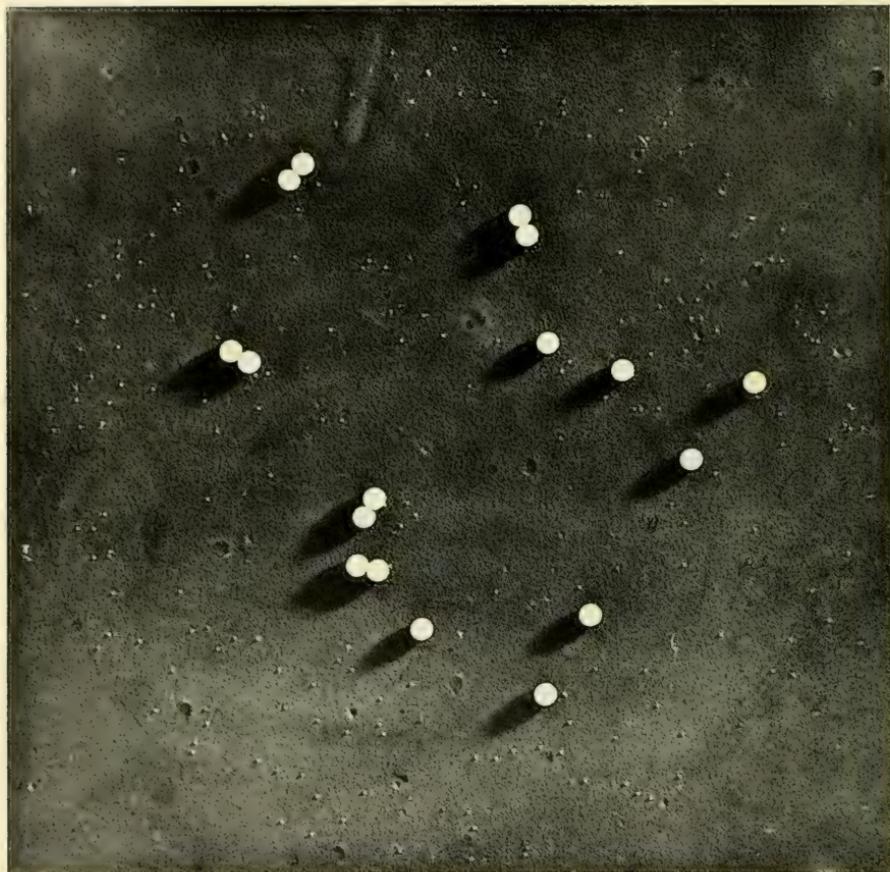


FIG. 8. A drop-pattern of poliomyelitis virus.  $\times 12,000$ . The large spheres are polystyrene latex particles that have been mixed with the virus at a concentration of  $3.2 \times 10^{10}$  particles/ml. From the number of these particles and of the poliovirus particles in this drop pattern it can be concluded that the virus was sprayed at a concentration of  $3.8 \times 10^{11}$  particles/ml.

for electron microscopy by Williams is simply an application of freeze-drying in which particularly rapid freezing rates and low sublimation temperatures are employed. It is gratifying to note that these two methods, so widely different in principle, have been found to yield essentially identical results when applied to the same kinds of biological objects. The application of these methods has revised our notions of the morphology of viruses by revealing, primarily, that several of the "spherical" ones are actually polyhedral.

*ii. Virus Suspensions, Quantitative.* It is frequently useful to observe samples of suspensions of virus particles in a quantitative manner, where the observation of the details of their structures is not important. This type of investigation arises when one is interested in morphological identifications of viruses, where quantitative assay of specific viral infectivity is sought, and where particle weights of viruses are being determined. The criteria to be met in preparations made for this kind of work are: (1) that the specimen field is representative of the entire suspension, and (2) that the volume of suspension giving rise to a particular specimen field is known.

Three methods of satisfying the above criteria are now in common use. In order to have a representative specimen field it is necessary either to have a large field, any portion of which is statistically representative of the suspension, or a very small field that is representative and that can be micrographed in its entirety. It turns out that in the method of agar-inhibition the virus particles remaining on the collodion film are spread out at random with no sign of clumping (Kellenberger and Kellenberger, 1955). The volume of suspension corresponding to a given field of virus particles can be calculated by mixing with the suspension some indicator particles, such as polystyrene latex in known number concentrations. A second method (Sharp *et al.*, 1952) is to deposit upon some sort of substrate surface, by high-speed centrifugation, all the virus particles in a known volume of suspension. The particles apparently stick where they impinge upon the surface and a random array is secured. The method in principle is an absolute one, requiring no indicator particles. The third method is the one now most commonly employed and involves the deposition of suspended material, mixed with indicator particles, in the form of minute droplets (Backus and Williams, 1950). The droplets are representative of the suspension and each dried pattern is small enough to allow it to be recorded on one micrograph (Fig. 8). It is evident that by any of the methods one may count the numbers of particles of any distinct morphological class. If previously identified, virus particles may be counted, and if an aliquot concentration is biologically assayed, a measurement of specific viral infectivity can be secured. Also, if the suspension is reasonably monodisperse, and if contaminant material is known to be absent, the particle weight of a virus may be obtained by

counting its numbers and by weighing a dried, aliquot volume of the suspension. The spray-drop method is demonstrably the most accurate of the three as far as counting is concerned, but it requires that the virus suspension contain at least  $5 \times 10^9$  particles/milliliter. The other two methods allow concentrations to be used that are some 100- to 1000-fold less.

*iii. Sections of Infected Cells.* In exploring the morphological aspects of viruses within their host cells it is necessary to use thin sections cut through such infected cells. Except in their extreme peripheral regions, even tissue-cultured cells are far too thick for direct electron microscopic examination. The purely technical aspects of fixation, embedding, and microtomy of cells for electron microscopy are now well worked out. [See Gelber (1957) for a summary of techniques.] Serial sections as thin as 200–400Å are obtained without great difficulty, allowing the electron microscope to be used at its full power.

The fixative and stain almost universally employed in tissues prepared for electron microscopy is osmium tetroxide, and its use reveals an impressively great wealth of fine structures within cells. Unfortunately, it seems not to have any highly selective staining properties as far as viruses are concerned; in fact, its mechanism of reaction with viruses is poorly understood. Nor are the possible leaching effects of osmic acid, of the dehydrating alcohols, and of the monomeric form of the embedding material (butyl methacrylate) adequately investigated. About all that can be said at present is that, if cells known to be infected with a large virus are sectioned, the sections exhibit particles that are not unlike what one would anticipate to be the appearance of the virus. In a very few cases sections of pellets of purified, large viruses confirm this anticipation. It is clear, however, that there is great need for more stains that would be effective in enhancing the contrast of the electron image, and that would be more specific for virus particles.

## 5. Radiation Inactivation

*a. The Effects of High-Energy Radiation.* The effect of the interaction of high-energy radiation with matter is to create ionization and excitation of the atoms. If the radiation is electromagnetic in character, such as X-rays and  $\gamma$ -rays, ionization is produced by the ejection of photoelectrons and Compton-recoil electrons. When particles of high velocity are used for bombardment, such as electrons, protons,  $\alpha$ -particles, and neutrons, the ejection of electrons from the atoms of the bombarded material comes about by direct or near collision. In either type of production of ionization the ejected electrons from the *primary ionization* have considerable velocity and, as a consequence, are capable of producing further, or *secondary*, ionizations along their tracks. Atoms that have been ionized by the ejection

of an electron are, of course, positive ions. The free electron will, in the course of time, be slowed in its motion to the extent that it can join a neutral atom to form a negative ion. But inasmuch as the energy change involved in such attachment is very low (of the order of the excitation energy of the atom) it is believed that the formation of negative ions has far less significance in disrupting chemical arrangements in molecules than does the formation of positive ions (Lea, 1947).

The density of ionization along the tracks of high-speed particles is strongly dependent upon the type of particle, and this fact is of importance in assessing inactivation of small biological objects such as viruses. Electrons, protons, and  $\alpha$ -particles, in that order, have an increasingly greater density of ion production along their tracks, and their range of path is in the inverse order. For example, 1 Mev electrons have a range of over 4 millimeters in tissue and produce less than 2 primary ionizations per  $\mu$ , while  $\alpha$ -particles of the same electron-volt energy have a range of only about 5  $\mu$ , but produce over 5000 ionizations per  $\mu$ . It would be a rare occurrence for an electron to produce more than one ionization within a virus particle, while an  $\alpha$ -particle would be sure to do so.

Particles such as viruses may be irradiated either in suspension or in the dried state. While the former type of preparation is easier to assay for radiation effects than is the latter (which must be re-wet), there are fundamental ambiguities inherent in interpreting the results. Dense ionization in aqueous media results in the production of free radicals whose biological effect is incompletely understood, and if a dilute suspension of virus particles is irradiated with high-energy photons or particles, almost all of the biological effects will be the indirect ones caused by chemical modifications in the solvent. For this reason it is customary to irradiate viruses in the dried, or frozen-dried state, or in a suspension containing a great excess of some protective material, such as gelatin. In these preparations the assumption is probably valid that the biological effects of radiation are direct ones, i.e., caused directly by ionization of atoms within the biologically active molecules.

*b. The Target Theory.* When viruses are irradiated in a way such as to produce direct effects of ionization it is customary to assess the results in terms of loss of infectivity. By making assumptions as to the nature of the relation between the number of ionizations ("hits") within a sensitive structure ("target") and its loss of activity, one can calculate the target size and predict the variation of ionic efficiencies of different radiations. The general outline of this method of calculation is called the "target theory." As applied to viruses the target theory presumes that inactivation of a virus occurs when a single ionization takes place within a certain sensitive volume contained within it. This postulate is probably valid if it is observed that:

(a) The survival curve of irradiated viruses is exponential with dose of radiation (a single-hit curve).

(b) The effect of a given dose is independent of the intensity at which the dose is given.

(c) The same degree of effect is produced by increasing radiation doses as one proceeds from  $\gamma$ -rays, through soft X-rays, to  $\alpha$ -particles.

In general, the cross section or the volume of the target within a virus can be calculated on the target theory by making observations as to the inactivation effects of various doses of differing types of radiation. When the inactivation is of the single-hit type a plot of the logarithm of the surviving virus activity against dose is linear, demonstrating an equation that can be written:

$$n = n_0 e^{-D/D_0} \quad (39)$$

where  $n_0$  = initial infective titer,  $n$  = infective titer of survivors,  $D$  = dose, and  $D_0$  = dose required to score an average of one hit per target. When  $n/n_0 = 0.368$ , then  $D = D_0$ , and this value of  $D$  is called the "37 % dose." Its evaluation is the first step in establishing the target size.

The experimental data on radiation inactivation may be initially handled in forms depending upon the type of radiation employed. If high-energy electrons or X-rays are used, the ionizations produced are sparse and at random, and they are unlikely to produce more than one hit per target. The chances of a given target being hit then depend simply upon its volume and upon the number of ionizations per unit volume produced by the radiation dose. If the dose given equals  $D_0$  (for  $n/n_0 = 0.37$ ), and if such a dose produced  $L$  ionizations per  $\text{cm}^3$ , then the target volume is  $1/L$   $\text{cm}^3$ .

If efficiently ionizing  $\alpha$ -particles are used, only the cross-sectional area of the target can be calculated, since it is now anticipated that every ionizing particle going through a target will create an ionization within it. If, then, the dose equals  $D_0$ , and if such a dose amounts to  $M$   $\alpha$ -particles per  $\text{cm}^2$ , then the target area is  $1/M$   $\text{cm}^2$ . It is to be noted that if the target is large, the volume calculated as  $1/L$  will be an underestimation, while if the target is small, the calculation of its cross-sectional area as  $1/M$  will also be an underestimation. As the targets become smaller the calculation of a target volume should become more nearly correct, and similarly with the area calculations as the target becomes larger. Lea (1947) has developed a method of calculation, called the "associated volume" method, that is applicable in the intermediate case where the target is neither large nor small compared to the separation of ionizing events along the particle track.

It is seen that in principle it is possible to obtain a unique type of information about viruses from radiation experiments: a size (the target) that refers to the infective activity of the virus, on the assumption that only direct hits are responsible for loss of activity. Information about the size of

that portion of a virus particle that is essential for infectivity, combined with determinations of the size of the whole particle, can be expected to elucidate some details of virus structure.

### III. APPLICATION OF PHYSICAL METHODS TO VIRUS STUDIES

#### *A. Homogeneity and Purity as Related to Virus Identification*

When one prepares a suspension of particles from cells known to be infected with a virus disease, a question of paramount interest is whether or not there are virus particles in the suspension. It is in the nature of the word "virus" that this question can be unequivocally answered only in the positive sense, and then only after a biological assay has been made. A negative answer does not prove the absence of virus particles; it may only show that the host-cell system used for assay or the conditions of inoculation were not adequate to demonstrate infectivity. If the preparation proves to be infective, a reasonable conclusion is that it contains virus particles which may be experimentally "identified." Virus identification is a process that means different things to different people, but for one interested in the physical properties of viruses it means the establishment of a certain class of particles, distinguished on physical grounds, as being associated with infectivity. What is looked for, then, in establishing the identification of a class of particles as viruses is a number of physical parameters that the suspected particles are found uniquely to have. These may be size, shape, particle weight, and electrophoretic mobility, for example. An attempt is then made to correlate the existence of infectivity with those particles that have one or more of these parameters. The degree to which a class of particles may be said to include virus particles, and hence "identified," depends upon the number of agreements between physical properties and infectivity, upon the sensitivity of the biological assay, and upon the precision of the determinations of physical characteristics. As we shall see later, ultracentrifugation, diffusion, and electron microscopy are particularly apt methods for the identification of virus particles.

At some stage in the purification of a virus suspension it is usually possible to pick out a certain class of particles, physically defined, and know with some certainty that virus particles are within this class. Before making determinations of the physical properties of the virus particles, however, it is necessary to have some indication as to the homogeneity of the suspension. Some would prefer to use the word "purity" in this context, and the advisability of this choice will be mentioned below. The word "homogeneity" itself, however, is not without ambiguity. It denotes a degree of uniformity, but only in regard to the criteria used for detecting lack of uniformity.

Suspensions of particles of a macromolecular size can be investigated for homogeneity in at least a half-dozen ways: (1) Size, shape, particle weight—these are physical characteristics appropriate to examination by the electron microscope, and by ultracentrifugal methods; (2) density—a parameter observable by centrifugation; (3) infectivity—discoverable only biologically; (4) antigenicity—subject to examination by serological methods (5) surface potential—a physicochemical parameter measured by electrophoretic methods; (6) internal structure—amenable to some degree of determination by X-ray crystallography. It is entirely possible for a virus-containing preparation to be homogeneous in one or more respects and inhomogeneous in others.

Some samples of preparations containing virus particles may be offered in order to show more clearly the ways in which the word "homogeneous" may be interpreted. These will be suspensions of:

1. A T-even bacterial virus.
2. Bushy stunt virus.
3. Tobacco mosaic virus (TMV).
4. A hypothetical mixture of two plant viruses which are identical in shape, size, and particle weight.

1. T-even bacteriophage particles are probably uniform with respect to shape, size, density, and electrophoretic mobility, though these properties are only inexactly known for these viruses. They are antigenically uniform, while little is known of their internal structure. They are uniform in infectivity, in the sense that there is a one-to-one ratio between particle numbers and infective units.

2. Bushy stunt virus particles are found to be highly uniform with respect to all physical parameters, including some X-ray evidence on internal structure. They are probably serologically uniform. But a suspension of bushy stunt virus may be quite inhomogeneous with respect to infectivity, since it is found that over  $10^5$  particles must be contained in an inoculum that will produce only one leaf lesion.

3. Most, but not all, preparations of TMV are inhomogeneous with respect to the lengths of the particles, although they are uniform in width. They are apparently homogeneous with respect to particle density. A preparation of a single strain of virus is serologically and electrophoretically homogeneous. The specific infectivity is low, as with bushy stunt virus, and so the uniformity of particles with respect to infectivity is unknown. X-ray analysis of paracrystalline arrays indicates a high degree of uniformity of internal structure from particle to particle. If the TMV suspension is of particles of different strains, it will have demonstrable inhomogeneity with respect to antigenicity, electrophoretic mobility, and infectivity.

4. The point of adding this hypothetical mixture to the list of virus preparations is to show that it would be homogeneous with respect to morphological and hydrodynamic properties, but would probably be inhomogeneous with respect to other properties. In respect to infectivity, however, it might appear homogeneous, because the presence of one type of virus might completely suppress the expression of infectivity of the other type.

It is abundantly clear from the foregoing that the characterization of a virus suspension in terms of homogeneity is significant only if the terms of reference of the characterization are specified. It is safe to say that no virus exists whose particles are *known* to be uniform, one to the other, in terms of all the tests that can be applied to detect uniformity. The T-even bacteriophages are possibly so, within fairly wide limits of uncertainty in their hydrodynamic properties. Bushy stunt virus, poliovirus, and tobacco ringspot virus are examples of those whose particles may be completely uniform; it is only because of their low specific infectivity that we cannot be fairly certain.

In the light of the above discussion another glance should be given the word "pure." The difficulty with this word is that it is a catchall one, implying too much in too general a sense. It might be argued that a virus suspension can be called pure only if it contains particles that are identical in every measurable and conceivable respect. But short of this extreme point of view the words "purified" and "purification" are useful ones, and even necessary ones. The process of increasing the over-all homogeneity of a suspension of virus particles is conveniently and properly called "purification." We are also faced with the problem of how to describe simply a virus preparation from which all known contaminants are removed, and which demonstrates homogeneity in several respects. To call it "homogeneous" is to require that its types of homogeneity be described. Like most preparations of TMV, it may not even be physically homogeneous, or it may be that some aspects of homogeneity have not been ascertained. What are we to call such a preparation, to distinguish it from one that is known to contain many kinds of particles besides virus? The latter kind of preparation is unhesitatingly called "impure." It would seem that the most satisfactory way to describe a generally homogeneous preparation is to call it "purified," in accord with common practice.

### *B. Identification of Physical Particles as Infective Agents*

Physical measurements made on objects as small as viruses must involve great numbers of particles; consequently one can hope to identify physically only a particular *class* of particles with an infective entity. The process of identification consists in the separation of a virus-containing suspension

into classes that are physically distinguishable and in discovering which class is associated with infectivity. The identification is considered good if (1) the methods of separation into classes are precise and the infectivity assays are reliable, and (2) identifications secured by more than one method are in accord in their implied physical descriptions of the particle class. Separation may be achieved in several ways. One method is to disperse an aliquot of the particles for direct, but destructive, observation (electron microscopy); the separation is then made mentally by recording numbers of objects in different morphological classes. The other methods require the virus suspension to be handled in a nondestructive manner, since the material as separated must be assayed in that condition. Separation is accomplished in terms of shape, size, and density (ultracentrifugation); shape, size, and possibly electrical charge (filtration); surface potentials and, perhaps, shape and size (electrophoresis). Only the first of these three latter methods will be discussed here, since it is the most commonly used one.

### 1. *Electron Microscopy*

Since virus particles are noninfectious subsequent to electron microscopy, one must relate morphologies with infective assay by use of aliquot samples. Some form of representative-field technique is employed in which relative numbers of particles in various classes of shapes and sizes can be counted. The spray-drop method, utilizing polystyrene latex spheres in a known number concentration, is particularly convenient. The relative concentration of particles in different classes is determined and an infectivity assay is made. This yields what may be called the apparent specific infectivity of each class. Another preparation must then be made in a manner such that there is a considerable change in the relative concentrations of the particles of different classes. New counts and infectivity measurements provide a new set of apparent specific infectivities. If there is a class of particle for which the apparent specific infectivity remains constant, while it changes for the others, the virus most probably resides in this class.

While in principle the foregoing account is a prescription for the identification of virus particles by electron microscopy, in practice there are circumstances that render identification both simpler and more complex. In the case of several viruses, particularly the bacterial and some of the plant ones, a preparation is likely to appear quite homogeneous in the electron microscope. Unless there is reason to believe from other evidence that the infective units are of an altogether different order of size (as with some of the larger animal viruses), and unless the infectivity assay is negative, the presumption is valid that the monodisperse particles seen are the virus. Examples of this readily achieved state of purity are found in the T-bacteriophages, TMV, bushy stunt virus (BSV), tobacco ringspot virus, and turnip

yellow mosaic virus. At the other extreme is the problem of identifying virus particles in suspensions where the heterogeneity of particle sizes is so great that division into classes is not realistically achievable. For example, the identification of the mammary tumor agent of mice has proven frustratingly uncertain for this reason (Howatson, 1953), and for the additional one that the infectivity assay is tedious and imprecise.

Perhaps the most clear-cut instance of the use of the electron microscope in the identification of particle class with infectivity is found in the investigations on poliovirus (Bachrach and Schwerdt, 1954). Before the investigations by Bachrach and Schwerdt that led to the identification of the virus particle it had been established by methods of filtration and sedimentation that the infective agent was in the size range 10–50  $m\mu$ . Electron microscopy of partially purified suspensions from infected central nervous system tissue of cotton rats showed, fortunately, that only two classes of particles were present: spheres of about 10  $m\mu$  and of about 35  $m\mu$  in diameter. The smaller particle was present also in control material, presumptively pointing to the larger particle as the virus. This could not be assumed, however, since the possibility existed that the smaller-sized particle was the virus and that similar-sized particles also existed in the control material. Separations of the two particle classes were attempted by means of centrifugation in a special separation cell; these experiments were successful in drastically altering the relative numbers of particles in the two size classes. Correlation of particle counts with infectivity showed that the apparent specific infectivity remained constant for the larger particle, before and after the separation, and that it changed greatly for the smaller particle. On this numerical evidence it was concluded that the class of particle with a diameter of about 35  $m\mu$  contained the virus, and subsequent work has abundantly confirmed this. The great sensitivity of the electron microscope in identification studies is indicated by the fact that none of the purified preparations contained more than  $10^{-5}$  gm. of virus protein; actually  $10^{-7}$  gm. would have been enough for the spray-drop examination.

## 2. *Sedimentation*

The ultracentrifuge can be used to identify the particle class containing an infective agent with those physical parameters for a particle that are amenable to sedimentation analysis, namely, the ratio of the molecular weight to the shape factor, and the effective density. In assessing the identifications so obtained it is convenient to consider two cases: where the suspension is nearly physically homogeneous, and where the suspension is quite inhomogeneous. In the former case one can speak of a "characteristic" particle for which the problem is to see whether or not the infective agent has the physical attributes of this particle class. In the latter case, the virus

particles are, at best, present as only minor constituents of the suspension and the problem is to determine their sedimenting characteristics in the presence of large amounts of particulate impurities.

The methods of identifying virus particles with the aid of the ultracentrifuge have been developed and refined particularly by Lauffer and his colleagues (Epstein and Lauffer, 1952), and the work done by them on Southern bean mosaic virus (SBMV) can be used as an example to illustrate the present status of the techniques. SBMV can be purified sufficiently to form a homogeneous suspension. Upon sedimentation its particles form a concentration gradient, or boundary, and the sedimentation coefficient of this boundary has been determined to be 115 S under standard conditions. This gives a measure of the ratio of the molecular weight of the particle to its shape factor. To determine something about its effective density it is necessary to sediment it in solvents of different densities, such as sucrose solutions. To see if the particle class so characterized by these two types of experiments carries infectivity, it is necessary in principle to ascertain that the infectious entity sediments in the same manner.

To reduce the hazards of convective disturbances it is desirable to use a partition cell, although this may not eliminate them entirely. The SBMV suspension is first sedimented, while observing the position of its boundary by optical means, to a degree that it is known that the boundary has passed completely through the partition. The contents above the cell are assayed for a "base infectivity." This is usually very low. A series of centrifugations and assays is performed, varying the time and, consequently, the degree to which the boundary approaches and passes through the partition. In this way it can be told with some precision whether or not the disappearance of infectivity from the upper portion of the cell coincides with the disappearance of the boundary from this portion. A refinement of this technique that will effectively cancel out the effects of residual convection is possible, at least in principle. This is to use indicator particles in concentrations such that their boundaries are discernible. The particles should have free sedimentation coefficients that are known, at least relative to that of SBMV, and two lots of them should be available; one with a sedimentation coefficient greater than the virus under study and one with a smaller coefficient. (In the case of SBMV two such types were not available, but two were used: TMV with  $s = 185S$ , and bushy stunt virus [BSV] with  $s = 132S$ .) The procedure is then to set upper and lower limits upon the sedimentation coefficient of the infective entity by use of the two types of indicator particles.

By sedimenting the virus-containing suspension, either with or without the refinement of indicator particles, in solvents of differing sucrose concentrations additional information is found about the coincidence of sedimentation behavior between the characteristic particles and the infective one.

The similarity (if it exists) is then in terms of effective density. Epstein and Lauffer found that, by using indicator particles and sucrose solutions, the infectivity of SBMV was surely associated with a class of particle of sedimentation coefficient between 115 and 132 S, and that their results were wholly consistent with the conclusion that the characteristic particle of SBMV ( $s = 115\text{S}$ ) was the infective one.

By use of indicator particles it is possible to determine precisely the sedimentation coefficient of an infective agent even in impure suspensions where no "characteristic" particle can be found that will form a boundary. The indicator particles are added in concentrations such as to form boundaries and their passage through the partition noted. By assay methods the passage of the virus under study is also noted. If satisfactory indicator particles are available, the sedimenting characteristics of the virus can be closely bracketed.

Identification of virus particles by electron microscopy has these particular merits: the shapes and sizes of the particles can be determined quite directly; extremely small quantities of material are sufficient for examination; since particle counts are made, the specific infectivity of a virus preparation can be measured; a rapid qualitative and quantitative assessment of impurity particles can be made. The advantages of the sedimentation method are: the same aliquot of virus-containing material is both physically characterized and biologically assayed; it is feasible to make measurements upon a quite impure suspension, particularly with the aid of indicator particles; an identification of virus with particle can be made on considerations of effective density as well as on an indirect estimation of shape and size. It is obvious that by employing both methods with at least partially purified material there is little cause to doubt but what reliable physical identifications can now be made of many types of virus particles.

### *C. Physical Properties of Well-Studied Viruses*

#### *1. Tobacco Mosaic Virus*

*a. Introduction.* Ever since its isolation by Stanley (1935), tobacco mosaic virus (TMV) has been an object of interest to physical chemists exploring the application of thermodynamic, hydrodynamic, and optical principles to the study of macromolecules. Coupled with these investigations has been the vigorous search into the structure and mode of action of TMV, perhaps the best known of all of the viruses. As a result of the varied interests of the many investigators, TMV has been subjected to more extensive research, involving very diverse techniques, than has been focused on any other macromolecule, naturally occurring or synthetic.

A great variety of data has now been amassed on the physical properties of TMV. Most of the data are obsolete either because they were gathered before the techniques had attained a degree of reliability worthy of consideration today or because the virus preparations worked with were of doubtful quality. The assessment of the quality of a preparation requires the subjective judgment of the investigator, and different attitudes are to be expected. For the detailed presentation of a view considered extreme by most workers in the field, the reader should consult Pirie (1957). Other positions are expressed in reviews by Lauffer *et al.* (1949), Bawden (1950), Markham (1953), Markham and Smith (1954), and Schramm (1954a,b).

*b. Molecular Weight.* As long ago as 1938 Lauffer (1938a) combined data from sedimentation velocity and viscosity measurements to make a comprehensive analysis of the size, shape, and molecular weight of TMV. He concluded that the virus particles were rodlike with an axial ratio of about 30 : 1 and that the molecular weight was  $43 \times 10^6$ . His calculated values of the molecular weight, length, and thickness have suffered many changes in the ensuing twenty years, as more precise physical chemical parameters have been obtained and the quality of the virus preparations improved. These changes, although by no means negligible, do not detract from the original contribution which represents one of the important triumphs of physical chemistry. For it was not until a few years after Lauffer's prediction of the size and shape of the virus particles that their direct visualization was achieved with the development of the electron microscope. To review in an integrated fashion at this time the existing data that have been obtained in many laboratories is difficult because only limited studies were made in most cases and most preparations doubtless contained some particles that were partially degraded and others that were aggregated. Despite the recent combined efforts of Boedtker and Simmons (1958) and of Hall (1958), a definitive study of the physical properties of TMV still remains to be done, one in which a single preparation is examined critically by ultracentrifugation, diffusion, viscometry, streaming and electric birefringence, light scattering, electron microscopy, and X-ray analysis.

Of the various sedimentation velocity studies that have been made in the past twenty years, none have been performed with a degree of precision that can be considered by present standards to be adequate. The various sedimentation coefficients reported, for infinitely dilute solutions, range from 185 (Lauffer, 1944a) to 198 S (Schramm and Bergold, 1947). This spread is much larger than would be expected with current techniques, but there is no evidence that the most recent values, as a matter of fact, have been obtained with any greater precision than was attained in those earlier determinations. Owing to the variation of sedimentation coefficient with concentration each experiment must be evaluated in a way which accounts for

this effect (see review by Schachman (1959) for details). Seldom has this been done for any protein and apparently never for TMV. It is tempting to attribute the spread in values to variations among the preparations, but doing this tends to obscure the limitations in the precision of the measurements themselves. To be sure, preparations frequently contain a fraction of the virus in aggregated form, as dimers or higher aggregates, but this circumstance need not interfere with the accurate determination of the sedimentation coefficient of the bulk of the material under investigation. Similarly the presence of partially degraded material in the preparation need not complicate the accurate determination of the sedimentation coefficient of the principal component and its extrapolation to infinite dilution. As indicated in Section II, A, 5, the sedimentation coefficient should be accompanied by a diffusion coefficient if molecular weights are to be evaluated. On theoretical grounds this is the procedure of choice, since no conceptions about the shape or hydration need be invoked. Unfortunately, the diffusion data for TMV cannot be considered reliable. Among the various determinations, the values,  $0.53 \times 10^{-7}$  cm.<sup>2</sup>/sec. (Lauffer, 1944a),  $0.45 \times 10^{-7}$  (Schramm and Bergold, 1947), and  $0.4 \times 10^{-7}$  (Watanabe and Kawade, 1953) are probably the most dependable. None of them, however, can be accepted if present standards are applied. Consequently the molecular weight calculated from these data must be considered as only approximate. It is ironic to note that the partial specific volume, which usually is the least precisely determined among the parameters required for molecular weight calculations, is known with greater accuracy than either the sedimentation or diffusion coefficient. The value 0.73 cc./gm. has been obtained by different workers (Bawden and Pirie, 1937; Lauffer, 1944a; Schramm and Bergold, 1947). From the data enumerated above we can calculate that the molecular weight lies between  $31 \times 10^6$  and  $45 \times 10^6$ .

In view of the foregoing remarks, and especially because of the lack of critical diffusion studies, none of the values mentioned above should be considered as confirmatory of molecular weights obtained in other ways. Advances in knowledge of the structure of TMV have been extremely rapid in the past few years, but further progress is dependent in part upon accurate information of the molecular weight. Although the means are now available, the sedimentation-diffusion method has yet to be exploited to its fullest capacity.

The molecular weight of TMV has frequently been computed by combining the sedimentation coefficient with the intrinsic viscosity. This calculation involves assumptions about the rigidity and hydration of the particles and the absence of permeation of solvent through the particles during their movement through the liquid. However, it can be shown theoretically that, if both the viscosity and the sedimentation velocity determinations are

precise, molecular weights of TMV calculated from these data will not be much in error even if the virus particles are swollen to have a shape markedly different from that characteristically seen in the electron microscope. Even in the worst case the error in the molecular weight might be expected to be no more than 20 %. But if a degree of precision around 1 % is desired, then it is necessary that the assumed model of the particle in solution conform to the real particle.

The intrinsic viscosity provides a sensitive measure of the degree of aggregation of the virus particles. This was demonstrated by Lauffer (1944a) when he compared, for different preparations, the intrinsic viscosity with the length-distribution curve evaluated from electron micrographs. In this way he was able to provide good experimental evidence for the validity of the Simha equation (Simha, 1940). Because of the tendency of the virus particles to aggregate end-to-end to form dimers and even higher aggregates, it is not surprising that the intrinsic viscosities reported in the literature exhibit wide variation. Even for preparations which seem to contain predominantly unaggregated material values between  $0.28 \text{ (gm./100 ml.)}^{-1}$  (Lauffer, 1944a; Schachman and Kauzmann, 1949) and  $0.37 \text{ (gm./100 ml.)}^{-1}$  (Watanabe and Kawade, 1953; Boedtker and Simmons, 1958) have been obtained. These values, in conjunction with the sedimentation coefficients given above, yield  $35 \times 10^6$  to  $44 \times 10^6$  for the molecular weight. For this calculation a value of  $\beta = 2.5 \times 10^6$  has been used in Equation 24. The reader may find it profitable to repeat this calculation for particles of different assumed axial ratios.

Molecular weights for TMV have also been obtained by light scattering. The most recent determination (Boedtker and Simmons, 1958) gave the value,  $39.0 \pm 1.2 \times 10^6$ , which seems to be in excellent agreement with the value obtained ten years earlier by Oster *et al.* (1947). In comparing these values, however, it is important to note that the newer measurement of the specific refractive increment ( $dn/dc$ ) gave a value 15 % greater than the older value. It is general custom to correct older results from light-scattering investigations by inserting the more recent value of  $dn/dc$ , since the determination of  $dn/dc$  is much more accurate now than it used to be. Doing this would cause a decrease of 24 % in the molecular weight reported in the earlier investigation and would destroy the apparent agreement mentioned above. Also the results of Doty and Steiner (1950) would require revision, leading to values below  $40 \times 10^6$ . In all three investigations it was claimed that aggregation of the virus had not occurred to a significant extent. It is clear that these results, like those from sedimentation velocity studies, differ by amounts much larger than the presumed experimental errors. Again it is tempting to attribute this to differences among the samples, but a careful reappraisal of the experimental aspects of light scattering by TMV

seems necessary as well. Since light-scattering techniques have improved in recent years, the most reliable value is that obtained by Boedtker and Simmons (1958), particularly since they provided other data helping to characterize their preparations.

The molecular weight of TMV has been obtained by analytical electron microscopy by Williams *et al.* (1951). This technique involves direct particle counting of the virus particles in a microdrop of known volume. From the measured number of particles per unit volume and the dry weight concentration the molecular weight is evaluated directly. Since some breakage of the particles occurs during the preparation of the specimen for electron microscopy, Williams *et al.* (1951) measured the total length of all the particles in the drop pattern and then calculated the mass per unit length. This figure multiplied by the length of the virus particles, which was determined independently, gave the molecular weight,  $49 \times 10^6$ . No adequate explanation for the discrepancy between this value and those given above has yet been presented. Errors in the determination of the size of the polystyrene latex particles (used in determining the droplet volume) may be responsible. Evidence against this explanation is provided by the results of the length measurements (discussed later). Alternatively, the virus sample may not have been dried completely; this, too, seems an unlikely explanation since the method used in this work is similar to that employed by others. Errors in the determination of the dry weight would affect the accuracy of light-scattering determinations as well as those of partial specific volume. A third possible error, which would result in a high molecular weight by the counting procedure, would be that impurities were present and not recorded as countable particles. However, examination of many fields containing thousands of virus particles did not reveal such impurities, and it is unlikely that they account for any appreciable increase in the apparent dry weights.

It is also possible to determine the molecular weight of TMV by a combination of X-ray and chemical analysis. The former has shown (Franklin, 1956a,b) that the virus rod is built of a number of crystallographically identical substructures. If these be identified with the chemical subunits of the virus (Knight, 1954; Harris and Knight, 1955; Ramachandran, 1958), and if a length of 3000 Å is taken for the intact particle, a value of  $40 \times 10^6$  can be calculated for the molecular weight. It should be pointed out that this calculation is based on the unconfirmed assumption that the crystallographic and the chemical subunits are identical.

It is hoped that reinvestigation of the molecular weight of TMV by all of the methods described above will produce individual results characterized by higher precision and leading to a unique value that is accurate to a few per cent. For the present, the tentative value of  $4 \times 10^7$  gm./mole is proposed.

*c. Homogeneity and Length.* There has been considerable disagreement over the question of the "length" of the particles of TMV, particularly as measured by electron microscopy. Part of the source of this disagreement lies in the fact that the word "length" has more than one connotation when applied to TMV. It may mean: (1) the length distribution that is found by electron microscopy of virus suspensions that have been prepared by any one of several purification methods. It may mean: (2) the length distribution that is found when the preparative procedure, from infected plant to the electron microscope, has been performed in ways such as to minimize (or account for) the effects of breakage and aggregation of the TMV particles. But the word "length" may also mean: (3) the minimal length of the particle which is infectious; (4) the length distribution of the particles that exist in the crude juice from infected plants immediately after extraction; and (5) the length distribution of the virus particles as they exist within infected cells. Failure to distinguish which type of length is being investigated or discussed is an obvious source of confusion.

Most TMV preparations that have been examined by electron microscopy have exhibited a rather broad length distribution (Bawden, 1950), with the mode usually falling in the vicinity of 3000 Å. The breadth of the distribution curve, the position of its mode, and the type and severity of its skewness can be influenced by the extraction procedures, the method of purification, the pH conditions of storage, and the preparation of the suspension for electron microscopy (Pirie, 1957). In two published cases, however, a high degree of uniformity of length has been reported. In the earlier one (Williams and Steere, 1951), the virus was prepared for observation by extracting the plant juice by homogenization, followed only by heating to 50°C. for 5 minutes and clarification by low-speed centrifugation. Samples of the entire virus suspension were sprayed upon the electron microscope grids at dilutions such that the lengths of all the particles in each droplet pattern could be measured, and the data handled in a way such that the effects of breakage and aggregation due to drying could be corrected for. The results showed that: (1) before correction of measured lengths, about 70 % of the particles had lengths between 2900 and 3100 Å; (2) after correction, about 95 % of the particles had lengths within this range, or had lengths exactly twice this. Recently, a measurement of length distribution has been made of TMV particles that had been purified by gentle centrifugal methods (Hall, 1958). The distribution found was practically identical with that found by Williams and Steere from their measurements, before corrections for breakage were applied.

The experimental results just described could have conceivably arisen from one or more of the following situations: (1) the virus in the crude juice was polydisperse, consisting of numerous short particles as well as long ones,

but the preparative conditions resulted in aggregation to particles of uniform length; (2) the virus was polydisperse, but the preparative methods preserved a selection of monodisperse particles at the expense of the shorter ones; (3) the virus in the crude juice was essentially monodisperse. If one is unwilling to accept the third assumption one would have to explain the results of Williams and Steere on the basis of the first assumption, unlikely as it is, since there was no chance of the method having resulted in a selection of monodisperse particles. The second assumption cannot be definitely ruled out as an explanation of Hall's results unless it can be shown that the purification method retains most of the viral starting material. However, as is well known, a centrifuge discriminates poorly between rod-shaped particles of differing length when a length of about 1200 Å, for TMV, is reached or exceeded. It appears that the most likely explanation of the agreement between the results of Hall and of Williams and Steere is that their experiments sampled without bias the contents of the suspensions and, inasmuch as the preparative conditions were quite different, that the suspensions were representative of the material in the crude juice. Furthermore, since it is hard to imagine that the extraction process alone would introduce uniformity of length where there was none before, the most reasonable supposition to be made at this time is that the particles of TMV *in vivo* are essentially monodisperse and are 3000 Å long.

Other methods also have given information about the length of the virus particles. These methods, such as viscometry and streaming birefringence, are not direct like electron microscopy and some ambiguity is involved in the interpretation of the data. Basic to the determination of the length of TMV particles by the hydrodynamic techniques is the view that the particles imbibe little or no water. This contention arises largely from the X-ray diffraction studies of Bernal and Fankuchen (1941), who showed that some of the intramolecular spacings of completely dried virus were the same as those in wet gels of TMV. This view gained support from the results of ultracentrifugal studies of TMV in solutions of different densities (Schachman and Lauffer, 1949). If the absence of hydration is taken for granted, the physical chemical data can be interpreted directly in terms of the shape of the particles. For this purpose ellipsoidal models are considered, since most hydrodynamic theories deal with particles of that type rather than with cylinders. Thus perfect agreement with the electron microscopic evidence cannot be expected.

From the viscosity data enumerated above an axial ratio between 20 and 24 is calculated. In conjunction with the thickness, 152Å, evaluated from X-ray diffraction, various workers have inferred values between 3000 to 3600Å for the length of the particles. Length measurements have been obtained directly from rotational diffusion studies without any requirement

for information about the thickness. O'Konski and Haltner (1956), and Norman and Field (1957), using electric birefringence, obtained a length of about 3400Å, and Rowen and Ginoza (1956) and Boedtker and Simmons (1958) obtained 3300 and 3500Å, respectively, from flow birefringence measurements. It should be noted that rotational diffusion measurements are particularly suited for studies of the length of elongated particles because the rotational diffusion coefficient varies inversely with the third power of the length. Thus both flow and electric birefringence techniques have given valuable information about the inhomogeneity of the particles with regard to length. Axial ratios and the dimensions of TMV have also been obtained by a combination of sedimentation and diffusion data. These results are not given here, however, because of the wide disparity among the reported diffusion coefficients.

Evidence as to the shape of TMV was also derived from measurements of the angular dependence of the intensity of light scattered by a solution of the virus. The dissymmetry of the scattering envelope showed clearly that the particles were rodlike in solution and not coils or spheres. The earlier results of Oster *et al.* (1947) gave 2800Å for the length, while the more recent investigations (Boedtker and Simmons, 1958) yielded 3200Å.

Despite the insensitivity of the sedimentation coefficient to length for long rodlike particles, considerable information as to the homogeneity of TMV can be derived from ultracentrifugal studies. For some years there appeared to be a paradox between the results of the workers employing the ultracentrifuge, on the one hand, and those investigating TMV with the electron microscope, on the other. Whereas the latter workers invariably observed many small particles in their micrographs, the ultracentrifugal studies frequently yielded sharp, symmetrical boundaries with no obvious evidence for the presence of the smaller particles. It should be noted here that the optical system in the ultracentrifuge gives weight fractions, whereas electron micrographs are almost invariably interpreted in terms of number fractions. When this difference is taken into account some of the discrepancy disappears immediately because a particle having one-third the most common length (3000Å) makes only one-third the contribution to the ultracentrifuge pattern that the same particle makes to the histogram prepared from electron micrographs. Further evidence aimed at clarifying this apparent discrepancy between the results of the two techniques was provided by the ultracentrifugal studies of Schachman (1951b). By exploiting a boundary anomaly, the Johnston-Ogston effect (Johnston and Ogston, 1946), Schachman found that particles about two-thirds as long as the most common particle were readily detectable even if these smaller particles were present to the extent of only 2% of the total weight. By inference this work supported the conclusions of Williams and Steere (1951) to the effect that most

of the short particles observed in the micrographs were the result of breakage of the longer particles. The ultracentrifugal studies have since been extended, and the results show clearly that the ultracentrifuge, if used in this way, can detect very small amounts of particles shorter than the usual length. Through this type of study and the more conventional boundary spreading analyses a great deal of information regarding homogeneity can be derived from the ultracentrifuge patterns. It is to be deplored that workers are still apparently content with the presentation of an ultracentrifuge pattern which by itself gives no assessment of the homogeneity of the sample.

There is some direct evidence as to the length of the particles of TMV as they exist within the inclusion crystals commonly found in the hair-cells of infected tobacco plants (Steere, 1957). By the use of a technique that provides a replica of a frozen, lightly sublimed surface that has been cut through a crystal, it has been found that the virus particles are arrayed in palisades, and that all the parallel particles in each array are about  $3000\text{\AA}$  long. It is possible, of course, that only the rods of uniform length are able to be formed into the crystals. The observed monodispersity within the crystals does not necessarily imply monodispersity of the particles as they exist throughout the cell. Unfortunately, particles of TMV are difficult to discern in sections of plant tissue fixed and embedded in the usual manner for electron microscopy, and consequently nothing is known about their lengths when observed in this way.

Electron microscopy, combined with biological assay, has given information about the relation of infectivity to the length of TMV particles. Many experiments have consistently shown that preparations containing a large proportion of rods distinctly shorter than  $3000\text{\AA}$  are less infectious than those that are essentially monodisperse at  $3000\text{\AA}$ . It is known, further, that aggregation of short particles to long ones will not enhance infectivity. The experiments relating infectivity with length are not sufficiently precise, however, to demonstrate whether there is a critical minimal length for infectivity or whether, as some would prefer to say (Pirie, 1957), the shorter rods have a lower "probability" of being infectious.

Through the use of the separation cell in the ultracentrifuge, Lauffer (1943a) obtained important information relating the infectivity to the particle size. He showed that the sedimentation coefficient of the infectious agent was similar to that of the principal component in the preparation, and he was able to exclude an hypothesis that infectivity was restricted to particles having a weight, for example, less than one-half the predominant molecular species.

*d. Diameter and Cross-Sectional Shape ; Surface Structure.* Both electron microscopy and X-ray analysis are capable of yielding precise information about the diameter of the TMV rods, and less exactly, information about

their cross-sectional shape and their surface structure. There is little disagreement about the diameter of the particles of TMV. When the rods are in a packed array their center-to-center distance is  $152\text{\AA}$ , a figure which is found by both electron microscopy and by X-ray analysis of dried virus paracrystals. However, when single particles are measured, it is found that their diameters are about  $180\text{\AA}$  (Williams, unpublished; Kaesberg, personal communication), although there is no evidence that the virus particles have flattened upon drying (Williams, 1952). A possible explanation of the discrepancy between the widths of packed and isolated particles is found in the discussion below of the X-ray analysis of TMV structure. Information about the diameter of TMV can also be derived from sedimentation studies. The sedimentation coefficient of elongated particles is relatively independent of length but markedly sensitive to mass per unit length and, therefore, to thickness. The diameter can be calculated directly in the manner suggested by Peacocke and Schachman (1954) for deoxyribonucleic acid. The resultant value,  $150$  to  $160\text{\AA}$ , is in fair agreement with the other measurements. Again it should be noted that this calculation is based on ellipsoidal models. As Peacocke and Schachman (1954) pointed out, this diameter corresponds to the dry particle, and swelling in solution, if isotropic, would not complicate this calculation of the diameter of the equivalent, anhydrous particle.

The surface structure and the cross-sectional shape of the particles of TMV have been investigated by electron microscopy of the intact virus rods and of short segments of the rods seen end-on. If the shape and surface structure of the intact particles are to be examined it is necessary to shadow them and derive inferences from their over-all appearance. Results secured are somewhat uncertain and conflicting, owing probably to the shadowing anomalies discussed previously. The most likely statement that can be made is that the over-all shape of intact particles appears more like a cylindrical rod than anything else, and that there is no clear indication of periodicities in a generally pebbly-appearing surface. These observations appear to hold for either air-dried or frozen-dried material.

Electron microscopists occasionally see short segments of TMV in an end-on orientation. Such segments frequently appear polygonal in contour, with a hexagonal shape the most common. Williams (1952) has concluded from his observations of the segment that the most likely cross-sectional shape of the intact virus is that of an hexagonal prism (like a lead pencil). There can be little doubt but what the segments frequently appear hexagonal, but in the light of the X-ray evidence on the structure of the intact virus (see below) the relation of this appearance to the virus shape is obscure. Two electron microscopic observations of virus shape are unquestioned: particles of all lengths have blunt ends which appear to be perpendicular to the axis of the rod; when short rods are aggregated to form long ones, by lowering the

pH, for example, the junctions cannot be discerned. The two observations suggest that the virus has a structural anisotropy that provides an easy direction for breakage and that all broken ends are geometrically equivalent. This conclusion is not out of accord with the X-ray finding that the virus particle is formed as a helix, since its pitch is so small that a break along the helical grooves could not be distinguished from a break truly perpendicular to the particle axis.

X-ray analysis has provided details concerning the surface structure of TMV that so far have been unconfirmed by electron microscopy owing, perhaps, to inadequacies of technique in the latter method. The major structural feature of the rods has been shown to be (Watson, 1954; Franklin, 1956a,b; Franklin and Klug, 1956) the existence of a helical structure for the protein portion of the virus, in which there are a nonintegral number of crystallographic substructures in one turn of the helix: 49 substructures in three turns. This result casts doubt upon the likelihood that the cross-sectional shape of the rod is hexagonal. In addition, the X-ray results indicate that the surface of the particle is deeply grooved, with the grooves following the pitch of the helix (1 turn per 23Å). This finding implies that the electron microscope should show a periodic structure along the virus surface; a prediction yet to be visually confirmed.

*e. Internal Structure.* The internal structure of TMV has been investigated by electron microscopy, X-ray diffraction, and by sedimentation techniques. Electron microscopy of partially disintegrated TMV particles has helped to reveal the localization of the ribonucleic acid (RNA) within the virus and the form taken by its protein substructures. The first intimations of the localization of the RNA were obtained from the segmented appearance of occasional frozen-dried rods, the segments being linearly arrayed and connected with a fine, axial strand that was thought to be RNA (Rice *et al.*, 1953). Later, it was found that electron micrographs of alkali-degraded TMV showed fine fibers, occasionally connected axially with undegraded bits of virus, which were of the right order of size to be strands of RNA (Schramm *et al.*, 1955). It remained for Hart (1955) to make a positive identification of these fibers in experiments in which the TMV was degraded in a controlled way with detergent, and subsequently suspended in distilled water. Each undegraded bit of TMV was found to have a coaxially localized fiber protruding from it, usually from one end only, which fibers were dissolved away following the application of RNAase. The observations were not exact enough to distinguish between an *axial* and a *coaxial* localization of the RNA fibers. Quite recently Hart (1958) has found that the appearance of the RNA fibers depends upon whether or not the partially degraded material is suspended in distilled water or in an ionic solvent subsequent to degradation. When ammonium acetate was used the RNA was apparently uncoiled,

revealing fibers that occasionally extended as far as  $3\ \mu$  from the degraded end of the virus (Fig. 9). A rough proportionality was found between the maximum lengths of RNA fibres and the amount of virus rod that was degraded, based upon the assumption that most of the rods were initially  $3000\text{\AA}$  long. The value of the proportionality constant was such as to lead Hart to conclude that the uncoiled RNA fibres are probably single polynucleotide strands, possibly contaminated with small amounts of residual protein. No decision could be reached as to whether a single RNA strand follows the pitch of the helix, or whether there is a single strand which is folded back and forth parallel to the axis of the virus rod.

As has been mentioned previously, tobacco mosaic virus may be degraded by several methods to provide a separation of its protein and RNA constituents (Bawden and Pirie, 1940a,b; Lauffer and Price, 1940; Lauffer and Stanley, 1943; Lauffer, 1943b; Schramm, 1947). Degradation of the virus in mildly alkaline solutions (pH about 9.5) produces a relatively homogeneous protein fragment, the "A" protein of Schramm (1947). This protein, having a molecular weight of about  $10^5$ , tends to aggregate to form long rodlike particles morphologically similar to those of TMV (except for a much broader length distribution), and it may be "coaggregated" with purified RNA from TMV to form reconstituted, infective virus (Fraenkel-Conrat and Williams, 1955). During the degradation of the virus several discrete, sharp boundaries were observed in the ultracentrifuge, leading Schramm (1947) to propose that the boundaries corresponded to different subunits of the virus. Continuation of this work by Harrington and Schachman (1956) showed that many of the presumed degradation products were, in fact, the result of aggregation of smaller products which formed and then quickly aggregated. From a kinetic study of the formation and disappearance of the different components these workers concluded that the degradation process involved the "stripping-off" or unraveling of protein units from only one end of the particles. This stripping off is rapid until about two-thirds of the protein is removed, leading to a fragment having one-third the length of intact virus. Additional evidence for a structural polarity in TMV rods is provided by the micrographs of partially degraded virus (Hart, 1956). Only very rarely was the central core detectable at both ends of the same particle. Instead, the core was exposed at one end only. Both the protein (A-protein) and the one-third fragment aggregate to larger particles. The former gives inhomogeneous material of molecular weight about  $10^6$ ; they are almost certainly the short disklike particles seen in electron micrographs of partially degraded virus (Fig. 10). They appear to have a height about  $30\text{--}50\text{\AA}$  and a diameter of  $150\text{\AA}$  with a central hole of  $60\text{\AA}$  diameter. This detection in electron micrographs leads to the impression that these doughnut-like objects are removed as such from around the RNA core, but this interpretation is eliminated by the

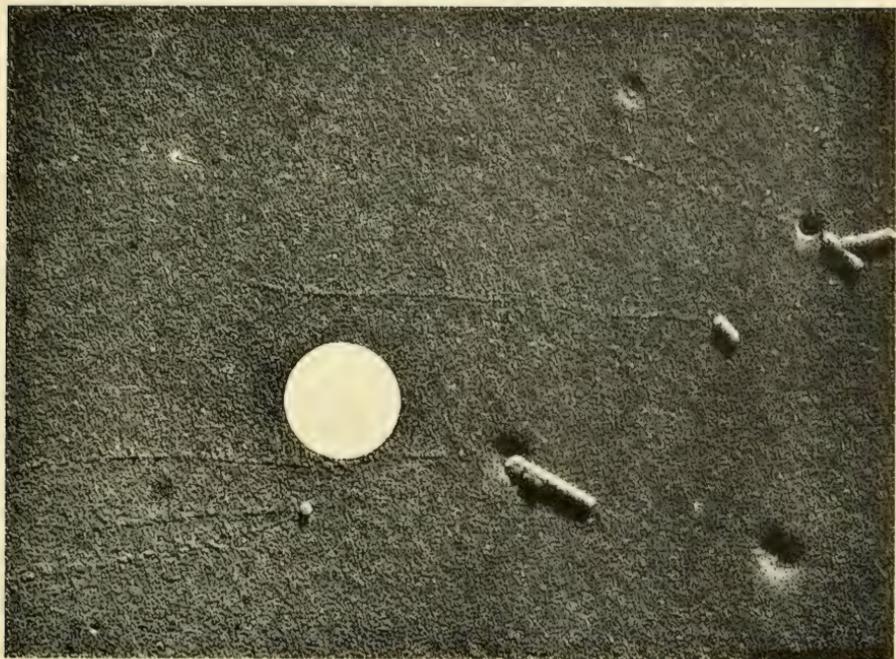


FIG. 9. Partially degraded particles of tobacco mosaic virus. The ribonucleic acid of the particles is seen as fine, linear filaments that are several times the length of the virus rods from which they have come.  $\times 58,000$ . (Courtesy of R. G. Hart, 1958.)



FIG. 10. Aggregates of the A-protein obtained from the degradation of tobacco mosaic by detergent treatment. They are seen to have the same diameter as the virus and to possess a central hole.  $\times 120,000$ .



FIG. 11. Frozen-dried particles of tomato bushy stunt virus. Note that the contours of the particles are not circular, but exhibit some degree of angularity. This virus is most likely polyhedral in shape, although the demonstration of this shape is not as unequivocal as in the case of the *Tipula* iridescent virus.  $\times 110,000$ .

ultracentrifugal studies which showed that these objects likely were aggregation products of the A-protein. Why the degradation seems to give a fragment one-third the size of TMV is still unknown, but these results would suggest that chemical studies may reveal heterogeneity of structure along the rodlike particles. The one-third fragment was shown to form dimers which were resistant to alkaline degradation, as though the resistant end of each of the one-third particles was on the outside in the dimer (Harrington and Schachman, 1956). Recently Lauffer *et al.* (1958) found that the aggregation of the A-protein at neutral pH was affected markedly by temperature, with the unaggregated form predominant at 5°C and the aggregates being formed at 25°C. It is important to note that the studies of the aggregation of small units to form either doughnut-like objects or rod-like particles are confined to the sub-units of molecular weight about  $10^5$ . The chemical sub-unit is much smaller than this, with a molecular weight of about  $18 \times 10^3$  (see Ramachandran, 1958), but very little physical chemical work has been done with this material. The ribonucleic acid isolated from TMV has been the object of numerous investigations (e.g. Cohen and Stanley, 1942; Hopkins and Sinsheimer, 1955; Gierer, 1958). The physical properties depend upon the ionic environment during the measurements and upon the method employed for the degradation of the virus. The more recent studies are consistent with the view that there is one RNA molecule with a molecular weight of about  $2 \times 10^6$  in each virus particle. These molecules may themselves be composed of sub-units held together by weak covalent bonds or by secondary forces. Information on this subject is currently being sought, and it is likely that soon the older data which give molecular weights of about  $10^5$  will be combined with the results of more recent investigations thereby furnishing the basis for a model which accounts satisfactorily for all of the data. The results of the X-ray inactivation of TMV (Buzzell *et al.*, 1956) have yielded a target volume about 1/12 the volume of the anhydrous particle. These workers suggested that the sensitive target is the nucleic acid.

It has been possible to localize the RNA within TMV by the application of X-ray analysis. From a comparison of native TMV with polymerized A-protein on a radial distribution plot the position of the phosphorus of the RNA is found to be 40Å from the axis (Caspar, 1956b). The same kind of plot also shows that there is a central tube of 40Å diameter, and that the extreme diameter of the particle is 190Å. The reconciliation of this diameter with the 150Å figure for packed arrays is accomplished by assuming that in the packed configuration the rods fit together, groove to ridge, as would a number of aligned, packed machine screws of the same pitch. There is certainly X-ray-dense material within the region of the RNA. The grooves are now believed to be so deep that, if it were not for the RNAase resistance of the virus, it would be tempting to conclude that the virus is assembled by wrapping the RNA

at the bottom of the grooves that are formed by the polymerization of the protein subunits. The X-ray results, however, give no direct clue as to the geometry of the association of the RNA polynucleotides with the protein helix.

The general structural features described above appear to be common to all forms of TMV-like material: native virus, polymerized A-protein, polymerized X-protein (Takahashi and Ishii, 1952), reconstituted virus, and different strains of native virus. The particles of pure protein, of course, have only suspending fluid in the space otherwise occupied by RNA. The reconstituted virus has an X-ray pattern identical with that of intact virus except for a reduced degree of distinctness at large scattering angles. All strains of native virus examined appear essentially identical except for differences in the heights of the scattering peaks in the radial distribution plots (Franklin, 1956b; Holmes and Franklin, 1958). These differences are presumably due to differences in the numbers, or in the packing, of the amino acid residues in the protein subunits.

## 2. *Tomato Bushy Stunt Virus*

Among the viruses of spherical shape, tomato bushy stunt virus (BSV), first isolated by Bawden and Pirie (1938), has been the most carefully examined for its physical chemical properties. Most of these studies have been made on preparations of virus which have been purified from the extracted juice of infected plants by a series of cycles of alternate high- and low-speed centrifugation (Stanley, 1940).

Various molecular weights have been reported for BSV. From sedimentation equilibrium experiments, McFarlane and Kekwick (1938) obtained the value,  $7.6 \times 10^6$ . The combination of the sedimentation coefficient, 132 S (Lauffer and Stanley, 1940), and the diffusion coefficient,  $1.15 \times 10^{-7}$  cm.<sup>2</sup>/sec. (Neurath and Cooper, 1940), gave  $10.6 \times 10^6$  for the molecular weight. The intrinsic viscosity has also been combined with the sedimentation coefficient to give  $9.9 \times 10^6$  (Markham, 1953). In all of these determinations, measured values between 0.73 and 0.74 cc./gm. were used for the partial specific volume. Because of the relatively large content of ribonucleic acid (17 %) in the virus, such values of the partial specific volume seem high. A recent reinvestigation (Cheng and Schachman, unpublished) gave 0.712 cc./gm., a value in excellent agreement with that inferred from its composition of amino acids and RNA (see Section II, A, 5, b, *iii*). Reexamination of the diffusion behavior in an instrument equipped with Rayleigh interference optics gave  $D_{20, w} = 1.26 \times 10^{-7}$  cm.<sup>2</sup>/sec. Thus the molecular weight from sedimentation and diffusion becomes  $8.9 \times 10^6$  (Cheng and Schachman, unpublished). This compares

favorably with the value,  $9.0 \times 10^6$  (Oster, 1946), inferred from light-scattering studies. It should be noted that this light-scattering determination was one of the earliest applications of this technique to the study of macromolecules. The measurements were made before photomultiplier tubes of high sensitivity were used in light-scattering instruments. Instead of detecting the laterally scattered light, as is customary now, Oster measured the decrease in light transmission (or the turbidity). The molecular weight of BSV was also determined by direct particle counting in the electron microscope yielding the value,  $9.4 \times 10^6$  (Williams and Backus, 1949). Although higher values have been calculated from early X-ray diffraction data (Bernal *et al.*, 1938; Carlisle and Dornberger, 1948; see Markham *et al.*, 1942, for a discussion of these data) it seems likely that the molecular weight is close to  $9 \times 10^6$ .

Both electron microscopy (Williams, 1953b) and low-angle X-ray scattering (Leonard *et al.*, 1953) indicate that the virus particles are essentially spherical. This was inferred also from the early crystallographic studies which showed that the unit cell was body-centered cubic in structure. Early electron micrographs yielded 260Å as the diameter of the particles (Price *et al.* 1946) but more recent examination of air-dried specimens gave a diameter of 300Å for particles in crystal-like arrays (Williams, 1953b). Individual particles, when the sample is air-dried, became somewhat flattened and their volume is estimated to be about 20% less than that of frozen-dried particles. The latter particles are certainly not spherical but bear, instead, the appearance of polyhedra (Fig. 11). Assuming them to be spherical, the mean diameter of individual particles is 300Å (Williams, 1953b). It can be inferred from the careful examination and comparison of the isolated particles in the frozen-dried specimen with both the individual particles and those arrayed in microcrystals in the air-dried preparations that the gross architecture of the particles in solution is preserved in the frozen-dried sample. Comparison with the value from low-angle X-ray scattering is then justified, since this technique measures the size of the particle in solution. The value, 309Å, obtained in this way (Leonard *et al.*, 1953) is in excellent agreement with the results of electron microscopy. From careful study of the maxima and minima in the curve of the intensity of scattered X-rays as a function of angle, Leonard *et al.* (1953) concluded that the virus cannot be described by a sphere of uniform electron density.

The various types of measurements taken together indicate clearly that the virus particles contain appreciable amounts of water. This was suggested by some of the early crystallographic studies which revealed shrinkage of the crystals upon drying. Also the hydrodynamic behavior is incompatible with a model of the virus as a compact, uniform sphere of density equal to the reciprocal of the partial specific volume and molecular weight of  $9 \times 10^6$ .

The data can be reconciled with a sphere containing large amounts of water within it or an anhydrous, elongated particle but the latter is precluded as a result of the results of X-ray diffraction, low-angle X-ray scattering, and electron microscopy. Also the difference in volume between air-dried and frozen-dried particles is evidence for internal hydration. Finally the molecular weight, partial specific volume, and diameter of the particles in solution cannot be reconciled without invoking a spongelike model which contains water in its interstices. The exact amount of water is still uncertain. However, reliable estimates can be expected as the data from individual lines of evidence become more accurate.

As yet there is only scant physical chemical information regarding the internal structure of the virus particles. X-ray diffraction studies show, of course, a high degree of order within the macromolecules. Recently the diffraction patterns have been interpreted in terms of subunits of protein surrounding a core of RNA (Crick and Watson, 1956; Caspar, 1956a). Caspar suggested that the number of subunits is a multiple of 60. Experimental evidence pertinent to this question is provided by experiments of Hersh and Schachman (1958) who showed that the virus can be degraded into much smaller units by the action of sodium dodecyl sulfate at low temperature and mildly alkaline pH. Since this treatment is not likely to cause the rupture of covalent bonds, the isolated fragments can be considered as real structural subunits. Separation of the protein from the RNA followed by examination of the protein by the Archibald method gave  $6 \times 10^4$  for its molecular weight. This value is compatible with the chemical evidence based on end-group analysis (Niu *et al.*, 1958) and amino-acid composition (deFremercy and Knight, 1955). No reports have appeared, as yet, regarding the size of the RNA.

In conclusion, bushy stunt virus appears to be a very highly organized particle composed of a central core of RNA surrounded by protein molecules which are held together by specific, secondary forces. The packing of these molecules provides for interstitial spaces, creating a rigid spongelike structure within which water is immobilized during the movement of the particles in solution. Apparently the spongelike structure possesses considerable rigidity, for removal of the water by freeze-drying does not cause the collapse of the structure.

### 3. Turnip Yellow Mosaic Virus

Although research with turnip yellow mosaic virus (TYMV) has not been as extensive as that with BSV, the limited investigations provide a fascinating tale. These studies, conducted principally by Markham and K. M. Smith (1946, 1949) who first isolated this virus, have been extremely rewarding. As a result TYMV serves as the principal model on which are based

current concepts of small viruses as organized structures composed of a nucleic acid core surrounded by a shell of specifically aggregated protein subunits.

Like other plant viruses, TYMV has been purified by a variety of methods. First of these was the fractionation procedure employing ethanol and ammonium sulfate (Markham and K. M. Smith, 1949). Later, centrifugal methods were substituted for salting-out techniques, and finally treatment with butanol and chloroform replaced the initial step that employed ethanol (Cosentino *et al.*, 1956).

The first preparation of TYMV that was examined in the electron microscope appeared to contain particles of only a single type with a diameter of 220Å (Markham and K. M. Smith, 1946). Further, electrophoretic examination revealed only a single boundary, and analysis of the diffusion behavior did not show evidence of inhomogeneity. These observations, plus the success in crystallizing the purified material, served to heighten the surprise evoked when two boundaries were observed in the ultracentrifuge (Markham, 1951). The slower of the two boundaries represented about 20 % of the material and it was tempting to view this as a dissociation product of the main component. However, centrifugal fractionation of the mixture, effected by the separation cell (Tiselius *et al.*, 1937), quickly eliminated this hypothesis, since the slowly sedimenting component was found to be free of nucleic acid. Moreover, this material was not infectious. It did, however, have a serological specificity identical to the faster migrating component which had been shown to be the viral agent. These results of Markham and K. M. Smith (1949) have since been confirmed, and purification of the so-called "top" and "bottom" components has by now been accomplished in different ways. The unusual physical-chemical data seem consistent with only one interpretation, that given by Markham (1951) and discussed in detail below. Subsequent work in other directions has substantiated in all important details his hypothesis.

Diffusion studies of the individual components gave almost identical values, about  $1.5 \times 10^{-7}$  cm.<sup>2</sup>/sec., showing clearly that the frictional coefficients for the two materials were essentially equal (Markham, 1951). Since electron micrographs of isolated particles and microcrystals of the mixture of both components showed that the particles were essentially spherical (Cosslett and Markham, 1948), the diffusion data were used to calculate the radius of the hydrated particles, giving the value 280Å. To explain the differing sedimentation velocities, 49 and 106 S for the "top" and "bottom" components, Markham (1951) examined the partial specific volumes. For the "top" component he found 0.74 cc./gm., a value expected for proteins; the "bottom" component had a partial specific volume equal to 0.666 cc./gm. On the basis of the additivity of the volumes of protein and

RNA (see Section II, 5, *b*, *iii*), this latter value would correspond to a macromolecule containing 61 % protein and 39 % RNA. Recent analytical studies have shown that the pure virus contains 35 % RNA (Markham and J. D. Smith, 1954). Combination of the partial specific volumes with the diffusion and sedimentation coefficients gives  $3.0 \times 10^6$  and  $5.0 \times 10^6$  for the molecular weights of the two components. This difference in molecular weight is due to the presence of RNA in one component and not in the other. The data indicate, therefore, that one component is pure protein while the second contains, in addition to that protein, a large amount of RNA. Since the particle size is the same, the assumption of an empty protein shell for one and a shell filled with RNA for the other seemed plausible. Evidence for this was provided by the electrophoretic studies. Apparently the charged groups of the nucleic acid were sufficiently buried within the structure that the electrophoretic mobility was not influenced by the presence of RNA. Finally, the immunological reactions seemed to preclude any contribution of RNA in the antigen-antibody reaction commensurate with the RNA content.

Low-angle X-ray scattering measurements (Schmidt *et al.*, 1954) have been particularly valuable in corroborating the conclusions from the hydrodynamic data of Markham. The diameter of the nucleoprotein particles as determined by X-ray scattering was in excellent agreement with that calculated from the diffusion data. Like the situation described for bushy stunt virus, this means that the virus particles must have a spongelike structure. Drying of the particles does not cause them to shrink to the volume calculated for a uniform, compact solid with a molecular weight of  $5 \times 10^6$  and a density equal to (1/0.666) gm./cc. From the scattering data for the "top" component, Schmidt *et al.* (1954) concluded that the protein particles were not uniform spheres. A satisfactory fit of the data was achieved for a spherical model having a large central cavity of lower electron density. With this "hollow sphere model" and a ratio of inner to outer diameter (for the hollow core and periphery of the particle, respectively) of 0.75, Schmidt *et al.* (1954) obtained a diameter for the protein particle which was in agreement with that calculated from the diffusion data. Further support for Markham's view of the structure of the two particles comes from the recent electron microscopic studies of Cosentino *et al.* (1956). They noted that the nucleoprotein particles were rigid and nearly spherical in shape, having a diameter of 260  $m\mu$ , even in air-dried specimens. The particles representing the "top" or protein component, however, were flattened when viewed singly, having diameters as large as 360 $\text{\AA}$ . In clumps, these particles did not flatten, retaining their diameter of 260 $\text{\AA}$ , but they became perceptibly dimpled in appearance, thus showing their hollow nature. Other structural details whose significance is not yet fully evaluated are provided by the electron microscopic investigations of Kaesberg (1956) and Steere (1957).

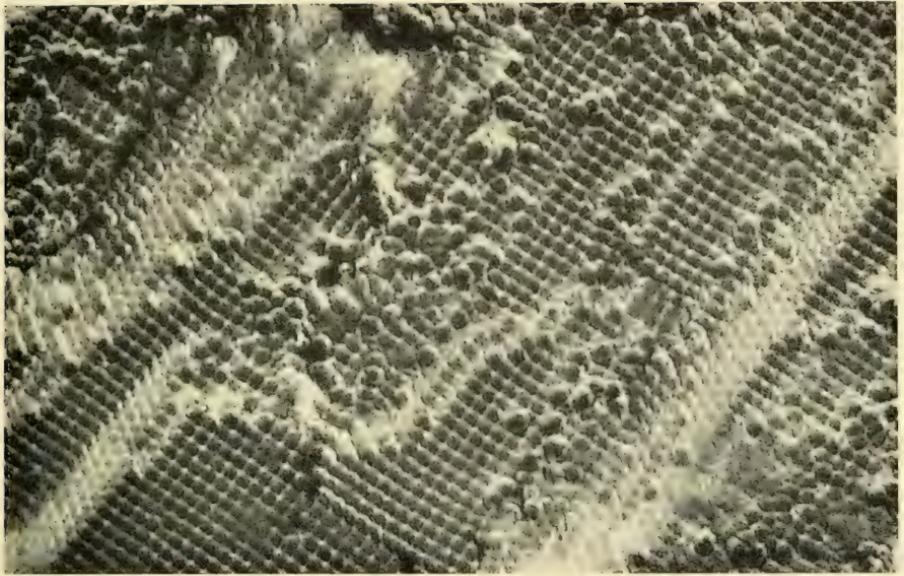


FIG. 12. A replica of a frozen, fractured crystal of poliomyelitis virus showing various planes within the crystal. The most prominent plane in this electron micrograph is the (100) plane.  $\times 75,000$ . (Courtesy of R. L. Steere and F. L. Schaffer, 1958.)



Recent X-ray diffraction studies (Klug *et al.*, 1957a,b) have led to certain revisions in the interpretation of older crystallographic investigations. These studies have also furnished evidence for the existence of protein subunits within the virus particles. As yet the isolation of protein subunits of this virus has not been reported. The RNA has been isolated and characterized by the Archibald method (Cohen and Schachman, 1957) and the molecular weight,  $0.5-1 \times 10^5$ , was found to be 3 to 5 times larger than that inferred from enzymatic determination of the end groups of the polynucleotide chains (Markham and J. D. Smith, 1952). This value for the molecular weight indicates that there are between 25 and 50 RNA molecules per virus particle. However, this number likely will be revised downward, judging from the behavior of other ribonucleic acids, as even milder methods are discovered for the isolation of RNA from the virus. Recently Kaper and Steere (1959) isolated turnip yellow mosaic virus RNA which was infectious, but no physical properties have been reported thus far.

#### 4. Poliovirus

Although several animal viruses have been purified sufficiently to allow significant investigation of their physical properties, only the smaller ones appear to be homogeneous with respect to size. [An exception to this generalization would be the tipula iridescent virus (Williams and Smith, 1957) if insect viruses were to be included in the category of "animal" viruses.] Poliovirus, while not studied nearly as extensively as some of the plant viruses, has been the most precisely characterized of the animal viruses since its isolation and identification in 1954.

All evidence indicates that the particles of poliovirus are uniform in size. They appear so in the electron microscope, when they are examined as single, air-dried or frozen-dried particles, and in two-dimensional packed arrays (Schwerdt *et al.*, 1954). The diameter of single, air-dried particles is about 310Å, but when they are frozen-dried, or when their center-to-center distance is measured in arrays, the diameter is 270Å. This difference of diameter may properly be attributed to flattening upon drying of the single particle. The external form of the frozen-dried particle appears to be that of a sphere (Schwerdt *et al.*, 1954), in contrast to some of the small plant viruses (Kaesberg, 1956).

Poliovirus has been crystallized in fairly large, optically isotropic crystals which generally have a cube octahedron habit (Schaffer and Schwerdt, 1955; Steere and Schaffer, 1958). By use of the frozen replica technique (Steere, 1957) it has been possible to examine the type of packing and the shape and size of the virus particles when within the crystal (Fig. 12). The packing has been found to be face-centered cubic, with the particles appearing spherical and having a diameter of 270Å (Steere and Schaffer, 1958). The possibility

that poliovirus is polyhedral in shape is not entirely ruled out, however, because of the uncertainties associated with trying to distinguish between a polyhedron and a sphere with a particle so small.

Some hydrodynamic study of poliovirus has been made with highly purified material and in dilutions sufficiently great (0.005 %) as to approximate infinite dilution. Sedimentation shows a single, sharp boundary with an  $s_{20, w} = 158$  S for all three strains (Schwerdt, 1957). An attempt has been made to determine the partial specific volume by sedimentation in varying mixtures of  $H_2O - D_2O$  and extrapolation to zero sedimentation velocity. The reciprocal of the partial specific volume found is 1.58 gm./cc. (Schwerdt, 1957).

In the absence of diffusion data the molecular weight of poliovirus cannot be evaluated unambiguously from its sedimentation coefficient. An approximate value can be obtained, however, through the use of certain assumptions. If the sedimenting unit is assumed to be a uniform, solid sphere, with no water of imbibition, the molecular weight that is calculable from the sedimentation coefficient and particle specific volume is  $5.4 \times 10^6$ , and the particle diameter is 220Å. Such a model is implausible if one generalizes from other viruses, such as tomato bushy stunt and turnip yellow mosaic. Some imbibition of water is most likely when the poliovirus is in solution, and this would cause the frictional ratio ( $f/f_0$ ) to be greater than 1.0. If the value of  $f/f_0 = 1.15$  generally found for proteins and viruses is used in the calculations, the molecular weight turns out to be  $6.7 \times 10^6$ .

It is also possible to calculate the molecular weight of poliovirus from its measured partial specific volume and its diameter determined electron microscopically. This amounts to assuming that the 270Å particle has an average density of 1.58 gm./cc. The molecular weight is then calculated to be  $10 \times 10^6$ .

It is obvious that the first and last calculations are likely to yield too low and too high a molecular weight, respectively. It is unlikely that the virus sediments as a solid sphere, completely dry both inside and out. It is also unlikely that the 270Å frozen-dried particle seen by electron microscopy is solid throughout and contains no empty spaces. A compromise way of calculating the molecular weight, in which both the sedimentation and the electron microscope data are used, is to assume that the virus in solution has a hydrated diameter corresponding to the electron microscopic diameter of 270Å diameter. This calculation yields a molecular weight of  $6.8 \times 10^6$ . It is evident that all values of the molecular weight of this virus are on shaky ground, since the partial specific volume is known only inexactly, and the diameter of the solvated particle is uncertainly estimated. The hydrodynamic data for poliovirus are at present limited in precision by the availability of only small amounts of highly purified material.

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## Chapter IV

### Quantitative Relationships between Virus Particles and Their Functional Activity

CARLTON E. SCHWERDT

*Department of Medical Microbiology, Stanford University  
School of Medicine, Stanford, California*

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#### I. INTRODUCTION

An understanding of the nature of viruses progresses only as effective methods of measuring virus concentration are developed. At first, relative virus concentration was expressed in terms of units of infectivity. In time, procedures were devised to enumerate total virus particles by indirect and direct means, thus permitting studies on the quantitative relationship between the physical particles and their biological activities.

There are two basic reasons for wanting to know this relationship. An investigation of the physical and particularly the chemical nature of a virus in its extracellular state requires assurance of the identity of the infectious particle and of the purity of its preparation. Confidence in these areas depends in part upon a knowledge of the ratio of infective to total virus particles. Second, an appreciation of this ratio is essential to an understanding of the mechanisms of virus replication, beginning with the question of the minimum amount of virus necessary to initiate infection.

This chapter is concerned with the method of determining total virus particle count and virus infectivity as well as an interpretation of the ratio of these measurements for certain representative viruses. This field has been most effectively reviewed by Isaacs (1957) for animal viruses. It is inevitable that much of this material will be incorporated here, for which this writer offers his grateful acknowledgment.

## II. TITRATION OF VIRUS INFECTIVITY

The titration of a virus is essentially a determination of the smallest amount of a virus suspension which will produce some manifestation of disease in a susceptible host. Two types of manifestations may be observed, namely, a generalized or systemic infection usually referred to as an all-or-none response, or the production of local lesions. Assays based on the all-or-none response are carried out by inoculating a measured volume of serial (logarithmic) dilutions of the virus suspension into groups of a suitable host, and estimates are made of the minimal volume capable of producing infection. This volume is called an infectious unit, and the titer of the original virus suspension is expressed as the number of infectious units per milliliter. Such titers represent the relative infectivity of virus preparations without indicating the number of virus particles per dose. For the comparison of titers, therefore, it is necessary to define the conditions of titration with care.

In assay systems where a virus produces local lesions, such as pocks or plaques, serial dilutions are also employed but the titer is expressed as the number of pocks or plaque-forming units per milliliter of original virus suspension. Since each lesion is initiated by a single virus particle (Section IV, A), an estimate can be made of the absolute number of virus particles in the original suspension if the efficiency of lesion production is known.

Occasionally a virus will fail to produce local lesions or will exhibit a poor correlation between the proportion of positive responses elicited and dose inoculated. End-point titrations are impractical for such a system. The virus may, however, reveal a regular relationship between the incubation period of the disease produced and log dilution inoculated, thus making possible the determination of the relative potency of the virus in question by comparison with a standard or control virus preparation.

*A. Assay Based on All-or-None Response*

The end-point dilution method of assay based upon an all-or-none or quantal response is used most commonly for the titration of animal viruses. Usually a standard volume of serial tenfold or fivefold dilutions of a virus preparation is inoculated into test groups of five or six susceptible animal or tissue culture hosts, and the number of positive and negative responses are scored for each dilution. A positive response may be death as the result of infection or some gross manifestation of infection, such as paralysis. In the case of infected tissue culture, where each culture is analogous to an individual animal host, readily observable cytopathic effects, such as complete cellular degeneration or giant cell formation, may represent a positive score. A plot of the per cent incidence of positive scores as a function of the log dilution of virus suspension will yield an S-shaped dose-response curve. By interpolation, one can estimate the dilution at which 50 % positive and 50 % negative responses occur. This is called the 50 % infectivity end-point dilution ( $ID_{50}$ ) and represents that point on the curve where the slope is steepest, i.e., where the smallest change in virus concentration will produce the greatest difference in response. The  $ID_{50}$  is therefore the most precisely measured point on the curve. Relative virus concentrations are calculated from the inoculum volume and dilution and expressed as the number of  $ID_{50}$  per milliliter of original virus suspension.

Too often a dose response curve is quite irregular because of variations in host susceptibility at any given dilution of virus inoculated. The  $ID_{50}$  is most frequently calculated, therefore, not from actual incidence values but from their accumulated sums, a procedure devised by Reed and Muench (1938). Here the assumption is made that a host which gave a positive response at a certain dilution of virus would also be positive at the next lower dilution, and vice versa for negative responses. This method is only valid if the variation in response reflects variation in host susceptibility. If, on the contrary, the chance absence of an infective particle in the inoculum is responsible for the negative response, the use of the cumulative procedure is not justifiable (Luria, 1953). This procedure, nevertheless, is almost universally used in estimating virus titers by the end-point dilution method.

An  $ID_{50}$  is a statistic which does not lend itself readily to an estimate of its variability. A distribution of individual end points can be determined experimentally by repeated titrations of the same virus sample and a standard deviation calculated therefrom. Such estimates have been made, for example, on the variability of infectivity titers of influenza virus in mice (Lauffer and Miller, 1944) and chick embryos (Knight, 1944); from these data levels of probabilities for significance in the differences between end points were determined. Five to ten replicate titrations are impractical, of course, for the usual experimental work where some estimate of the reliability of an end

point is desired. De Beer (1945) has published a method for the graphic estimation of the standard error (SE) of individual  $ID_{50}$  values from dose-response curves where the per cent incidence of positive responses is plotted on a probit scale versus log dilution of virus. A simpler method requiring no elaborate scales and nomographs has been devised by Pizzi (1950), who developed a simple formula for the determination of the SE of an individual  $ID_{50}$  estimated by the Reed and Muench method. Estimates of  $SE_{ID_{50}}$  by this formula have shown excellent agreement with estimates of standard deviations from replicate titrations (Schwerdt and Merrell, 1952). From the SE of two individual titration end points, the SE of the difference between the titers can be computed and thus a test of significance applied to this difference.

In a titration based upon an all-or-none response where average host susceptibility varies significantly and the incidence of positive responses is highly irregular, particularly in the neighborhood of the  $ID_{50}$ , some measurement other than frequency of response must be made and correlated with dose in order to quantitate the relative infectivity of a virus preparation. The most useful relationship has usually been found to be the incubation period and log dilution inoculated, i.e., a correlation of the length of time necessary for the detection of symptoms with log dose. This method has not found general application but has proved successful for the assay of mouse encephalomyelitis viruses (Gard, 1940), various tumor viruses such as rabbit papilloma virus (Bryan and Beard, 1939), avian erythromyeloblastic leukosis (Eckert *et al.*, 1954), and Rous sarcoma virus (Bryan, 1956), and several viruses of the psittacosis-lymphogranuloma venereum group (Golub, 1948; Gogolak, 1953; Crocker, 1954). Some of these viruses exhibit a linear relationship between incubation period and log dilution of virus. With others, a linear correlation is found between log virus concentration and the reciprocal of the incubation period.

It is sometimes possible by a great expenditure of time and animals to determine an  $ID_{50}$  for such viruses. Then, if a plot of the log  $ID_{50}$  inoculated against incubation period is linear, one can estimate an  $ID_{50}$  value for a virus preparation from the average incubation period observed after inoculation of a single dilution (Golub, 1948). Generally, however, the incubation period or its reciprocal is plotted as a function of log dilution of virus for both the unknown and a standard sample and a relative potency estimated from the ratio, at a given response level, of the log dilution unknown/log dilution standard. The antilog of this ratio, then, expresses relative potency in arithmetic terms. Relative potencies are most readily estimated if the response (incubation period or its reciprocal) is a linear function of log dose and the slopes of the two curves are the same. The application of this indirect method of bioassay of viruses has been presented with numerous examples in an excellent review by Bryan (1957).

## B. Assay by Local Lesion Count

### 1. Plant Viruses

The counting of local lesions as a means of plant virus assay was first done by Holmes (1929), who observed the development of discrete necrotic spots on leaves of *Nicotiana glutinosa* rubbed with tobacco mosaic virus. The method has been extended to many other plant viruses and, wherever applicable, has completely replaced the end-point dilution assay based upon systemic infection of plants. The local lesion assay is simple in principle. Serial dilutions of a virus suspension are rubbed with some suitable applicator on the surfaces of leaves of susceptible plants. Lesions appear in numbers related to the virus concentration. The phenomenon has been compared to the colony count method of assaying viable bacteria. The two procedures are not strictly analogous, however, since a plot of the number of lesions as a function of virus concentration is not linear for plant viruses over a wide dilution range in contrast to the bacterial colony count assay. The necrotic lesion count falls off with increasing log virus concentration and this is explained in part by the fact that the number of susceptible sites on any one leaf is limited. It is necessary, therefore, to determine the relative potencies by comparing counts obtained with two virus preparations applied to the host plant leaves in as reproducible a manner as possible. For maximum sensitivity the assays should be carried out over a range in which the curve representing lesion count dependency upon virus concentration has its steepest slope.

Improvements in experimental design of local lesion assay of plant viruses have greatly reduced the errors due to inhomogeneity of the test plants. In particular, the method of inoculating one-half of a leaf with the unknown sample of virus and the other half with the standard sample has been employed for more meaningful comparisons of virus samples (Samuel and Bald, 1933). Variation in comparative counts have been further minimized by the systematic distribution of inocula of several virus samples according to some prearranged scheme such as the Latin square (Youden and Beale, 1934). By this method, inocula of the various samples to be compared are distributed equally often on each plant and at each leaf position. It allows the comparison of as many virus samples as there are inoculable leaves on a test plant and permits a more meaningful analysis of the significance of differences between lesion counts of the various preparations tested.

Whatever the design of the assay experiment, it is important that the dilutions of virus preparations to be compared are such that they produce approximately the same number of lesions per section of leaf inoculated. This is made clear from an examination of the curve relating lesion counts to log virus concentration. The flattening of this curve at higher virus concentrations necessitates that comparisons be made in the lower concentration range where the slope is steepest.

The reproducibility of plant virus assay by local lesion counts will vary with the experimental situation (Section II, C). Differences in virus activity of from 50 % (Beale, 1934) to as low as 10 % (Loring, 1937; Spencer and Price, 1943) have been detected for tobacco mosaic virus preparations under carefully controlled conditions. The accuracy and general statistical analyses of the local lesion method for measuring comparative infectivities have been reviewed in detail by Lauffer and Price (1945), Price (1946), and Bald (1950).

The relationship of total virus mass or numbers to lesion count will be considered later (Section IV, B). It might be well to point out at this time, however, that, in general, it is difficult if not impossible to translate differences in counts to differences in plant virus mass.

## 2. *Bacterial Viruses*

The classic example of a local lesion type of virus assay is the highly accurate plaque count of bacterial viruses first described by Gratia (1936). The procedure as it is used currently is presented in detail by Adams (1950) and is carried out as follows: Dilutions of virus are mixed with a bacterial suspension in nutrient agar and poured over the surface of ordinary agar plates. Clear areas or plaques are produced by the virus in the confluent growth of bacteria in the agar overlay after a suitable incubation period at 37°C. The plaque count per plate divided by the volume and dilution of the virus inoculum gives the titer in plaque-forming units per milliliter of original suspension.

A direct proportionality is observed between plaque count and relative virus concentration when both variates are plotted on the same scale (usually logarithmic); thus the curve is linear with a slope of 1. Because of this linear relationship, each plaque is considered an infective center initiated by a single virus particle infecting a susceptible bacterium (Luria, 1940). The plaque count does not necessarily represent the total virus particle count but only that fraction capable of adsorbing to and infecting viable bacterial cells. This fraction is called the "efficiency of plating" and can be estimated in a relative sense by comparing the plaque count with the infectivity of the virus for bacterial cells in broth suspension where conditions are usually more favorable for virus adsorption. For example, a bacterial virus preparation in high dilution is added to a suspension of susceptible bacteria. Small aliquots of the mixture, designed to yield on the average less than one infected bacterium per sample, are incubated until the infected bacteria have burst, liberating virus. Each aliquot is plated and the number of plates yielding no plaques as well as those yielding large numbers of plaques is noted. From the proportion of plates with no plaques one can estimate the average number of virus particles,  $n_1$ , per aliquot from the Poisson formula:

$$P_0 = e^{-n_1}$$

where  $P_0$  is the fraction of samples containing no infective virus particles and  $e$  is the base of natural logarithms. A parallel titration of the stock bacterial virus preparation by the usual plaque assay technique will also yield an average value,  $n_2$ , for the number of infective virus particles per sample. The ratio,  $n_2/n_1$ , the "efficiency of plating" coefficient, is usually less than 1 and indicates that fraction of the infected bacteria in suspension which will go on to produce plaques after plating (Ellis and Delbrück, 1939).

Absolute efficiency of plating can only be estimated after some independent measurement of the concentration of characteristic physical particles has been made (Luria, 1953). Methods of counting particles will be considered later in Section III.

The precision of assay by the plaque technique is readily estimated, since the variance of a Poisson distribution equals the mean (Isaacs, 1957). For an average plate count of  $n$ , the standard deviation equals  $\sqrt{n}$  which, divided by the average count,  $n$ , times 100, yields a value for the coefficient of variation. For example, an average plaque count of 100 per plate has a coefficient of variation of 10%, while 25 plaques per plate yields a standard deviation which is 20% of the count. The higher the count per plate, the greater the precision of assay within the limits, of course, imposed by the size of the agar plate.

### 3. *Animal Viruses*

The first extensive use of the local lesion count as an assay method for animal viruses began with the development of a procedure by Woodruff and Goodpasture (1931) for counting pocks or lesions on chick embryo chorioallantoic membranes infected with fowlpox virus. The method has since been applied to the assay of many animal viruses including the various poxviruses (vaccinia, ectromelia, myxoma, fowlpox), viruses of the herpes group (herpes simplex, pseudorabies, B virus), the myxo group (some strains of influenza virus, Newcastle disease virus) and the psittacosis-lymphogranuloma group, as well as certain arthropod-borne encephalitis viruses, Rous sarcoma virus, and infectious laryngotracheitis (Isaacs, 1957).

The technique of inoculation of the dropped chorioallantoic membrane of developing chick embryos is described in detail in the monograph of Beveridge and Burnet (1946). Serial dilutions of virus preparation are inoculated on the chorioallantoic membranes of groups of prepared chick embryos and the average number of proliferative lesions formed per membrane is found to bear an approximately linear relationship to the relative concentration of the virus inocula. The above authors felt that with viruses producing well-defined pocks in the range of 5-20 per membrane, assays using 4-6 membranes per dilution will give results accurate to  $\pm 50\%$  under standard conditions.

While this type of assay certainly exceeds the precision of end-point assays, it has been recognized that the scatter of counts around the mean for a

particular virus dilution is greater than would be expected if the pock count truly reflected the random distribution of infective virus particles suspended in the inoculum (Burnet and Faris, 1942; Fenner and McIntyre, 1956; Kaplan and Belyavin, 1957; Armitage, 1957). One possible reason for a variability greater than expected on a Poissonian distribution of lesions is the variability of susceptibility of individual chorioallantoic membranes (Kaplan and Belyavin, 1957). Other considerations which may contribute to increased variability of pock counts are: (1) nonspecific lesions resulting from injury to the membrane, with a concurrent decrease in its susceptibility to pock formation in the region of damage (Overman and Tamm, 1956a) and (2) the appearance of secondary or satellite lesions, particularly on membranes that remain moist after the initiation of a lesion. Improvements in the technique of inoculation have succeeded in reducing the count scatter, although it still remains in excess of that expected on a Poisson distribution (Westwood *et al.*, 1957).

The frequent observation that animal viruses propagating in monolayer cultures of mammalian cells produced a grossly visible cytopathic effect was quickly exploited by Dulbecco (1952) for the development of a plaque-count assay. This opened the door to quantitative studies with animal viruses equivalent in precision to those made with bacterial viruses. Bacterial and animal virus plaque-count assays are analogous in principle. The procedure in general is less susceptible to the errors and uncertainties inherent in the pock count assay on chorioallantoic membranes of chick embryos.

Monolayer cultures for plaque assay are generally prepared by seeding trypsinized cells from freshly excised tissues (Dulbecco and Vogt, 1954a; Youngner, 1954) or from cultures of established cell lines (Scherer *et al.*, 1953) in petri dishes or small flat bottles under an appropriate medium. The cells attach to the glass surface and produce a confluent growth after incubation at 37°C. for a varying number of days. Virus inocula are then introduced and the infected cultures layered with agar containing a maintenance medium. The necrotic plaques which ultimately appear in the sheet of healthy cells represent primary foci of infection. Secondary plaques fail to arise since virus dissemination is prevented by the agar overlay.

The plaque assay has been applied to an increasing number of animal viruses. These include western equine encephalomyelitis and Newcastle disease viruses (Dulbecco, 1952; Dulbecco and Vogt, 1954b); vaccinia (Noyes, 1953); the human polioviruses (Dulbecco and Vogt, 1954a); fowl plague (Hotchin, 1955); certain strains of influenza virus (Ledinko, 1955; Granoff, 1955); foot-and-mouth disease (Sellers, 1955; Bachrach *et al.*, 1957); Rift Valley fever (Takemori *et al.*, 1955); as well as Coxsackie and ECHO viruses (Hsiung and Melnick, 1955).

The sensitivity of the plaque assay of animal viruses varies greatly depending upon the virus and cell system used. For example, at one extreme, Granoff (1955) found that plaque titers of PR8 and MEL strains of influenza A assayed on epithelial cell cultures from chick embryo lungs were 800-fold less than the 50 % egg infectious titer. In contrast, Dulbecco and Vogt (1954a) found that titers of poliovirus, type 1, obtained by plaque assay on monkey kidney cultures were slightly higher than the infectivity titer obtained by monkey titration. In studies relating physical particle to infective particle counts, it is desirable, obviously, to work with assay systems of the highest possible efficiency. Such needs have stimulated the search for more susceptible cell lines which can be rewarding, as exemplified by Fogh and Lund's (1955) finding that plaque titers of poliovirus types 1 and 2 were 3 to 6 times higher on monolayer cultures of human amnion cells than on monkey kidney cell cultures.

As has been found with bacterial virus plaque assays, a linear relationship exists between plaque count and relative virus concentration for most of the animal viruses assayed by this method thus far. On the basis of this proportionality, Dulbecco and Vogt (1954a) have established on statistical grounds that a single virus particle is sufficient to produce a plaque (Section IV, A). Thus, we have available to us not only an accurate method of measuring infectivity but also a means of isolating pure lines of virus from single clones.

The reproducibility of plaque titers is similar to that noted earlier for bacterial virus plaque assays (Section II, B, 2). For a plaque count of 100 on a single plate, the estimated standard deviation is 10, assuming that the number of plaques reflects a Poissonian distribution of the virus particles in suspension in the inoculum. Thus, upon repeated titrations, the highest count should not exceed the lowest by more than 50 % for a deviation from the mean of not more than 2 standard errors (Dulbecco, 1955). In contrast, a 50 % end-point assay (e.g., poliovirus in cotton rats), in which serial 3-fold dilutions are inoculated into groups of 6 animals per dilution, will yield a SE of approximately 0.2 log units with an estimated variation of 600 % between highest and lowest  $ID_{50}$  expected within the two SE deviations about the measured  $ID_{50}$  (Schwerdt and Merrell, 1952).

### *C. Factors Affecting Virus Infectivity Titrations*

In most cases, every virus particle in suspension is not capable of infecting a susceptible host; hence an infectivity titer is a relative measure of virus concentration and usually represents some unknown fraction of the total number of virus particles present. Various environmental and host factors may have a marked effect upon the efficiency and reproducibility of assay. It is necessary, therefore, to control the conditions of assay well if meaningful

comparisons are to be made among titers of different preparations of the same virus.

The effects of the external environment on the virus inoculum are well known. Viruses are, after all, subject to the same denaturing activity of chemical and physical agents as any protein or living protoplasm. They exhibit varying degrees of resistance to inactivation by heat, unfavorable ionic environment, and irradiation. Even exposure of inocula to daylight or artificial illumination of ordinary intensity may inactivate some viruses. Skinner and Bradish (1954) investigated such effects as possible sources of error in the titration of the viruses of vesicular stomatitis, influenza, Newcastle disease, and fowlplague and in some cases found after 4 hours of exposure titers as much as 3 to 5 log units lower than those of the dark controls.

The selection of host species for assay as well as route of inoculation is well known to influence the virus titer measured. To cite one example of many for animal viruses, monkey, cotton rat, and white mouse yield different titers when used as hosts for parallel assays of Lansing poliovirus. Titers obtained in the first two species may exceed those obtained in the white mouse by a factor as great as 2 log units (Bodian *et al.*, 1950). Habel and Li (1951) have compared titers of this same virus by intraspinal and intracerebral inoculation into the white mouse and found titers not only consistently higher (0.7 log unit) but also more reproducible by the former route. The method of inoculation may affect titers, as illustrated by the increased lesion counts obtained with plant virus inoculated with the aid of a fine abrasive (Kalmus and Kassanis, 1945). Steere (1955) has enumerated and reviewed the many host factors which can influence plant virus titers, such as physiological condition, age, and nutrition of the host plants, as well as genetic differences among plants of the same species. Similar host factors are also important in the assay of animal virus. For example, Fulton and Armitage (1951) found the chorioallantoic membrane of the intact chick embryo more sensitive to infection with influenza virus than surviving sections of membrane *in vitro*.

Finally, the volume of the inoculum may have a marked effect upon virus titer. Sprunt (1941) made comparative titrations of vaccinia virus by intradermal inoculation into rabbits using 1.0- and 0.05-ml. inocula and observed that the larger inocula yielded only a 3-fold higher 50 % end point than the smaller, instead of the 20-fold difference expected. Similarly, Bachrach *et al.* (1957) found that plaque counts of foot-and-mouth disease virus on monolayer cultures of bovine kidney cells varied inversely with volume of inoculum. Identical amounts of virus in 0.1- and 6.4-ml. volumes yielded plaque counts in the relationship of 6 : 1, respectively. Sprunt believed that the smaller intradermal inocula spread more readily than the larger and exposed more

cells to infective virus particles, thereby increasing the titration efficiency. The greater plating efficiency of smaller-volume inocula of foot-and-mouth disease can probably be attributed to the increased probability of effective collisions between virus particles and cells.

### III. METHODS OF DETERMINING TOTAL VIRUS PARTICLE CONCENTRATIONS

Estimates of virus concentrations by infectivity measurements are based upon readily observable manifestations of local or systemic infection. While such estimates may vary in accuracy, depending upon the virus-host system employed, and will yield little if any information regarding the actual number of virus particles present in suspension, they can, nevertheless, be made objectively. Methods of estimating total virus particle concentration, on the other hand, may be subject to greater degrees of uncertainty.

Perhaps the most satisfactory method of enumerating physical particles depends upon their direct visualization in the electron microscope. The obvious problem encountered in this approach is the identification of the virus particle. When particles of unique morphology are isolable only from infected but not from analogous normal tissues there may be little question of their viral identity. If the virus is small and spherical, however, distinguishing it from the other, nonviral particulate matter of the cell protoplasm can be difficult. It may help to purify and concentrate such viruses partially and then test the suspected particles for possible association with infective as well as noninfective activities of the disease agent, e.g., agglutination by convalescent serum or adsorption and elution from red blood cells if it is a hemagglutinating virus. The several criteria applicable to the identification of a virus with particles seen by electron microscopy are reviewed by Bang (1955).

Indirect methods have also been used for estimating virus particle concentration. Here again the results are uncertain because assumptions must necessarily be made which are difficult to verify. One procedure depends upon estimates made of the virus particle's volume, density, and mass and requires preparations of pure virus. Virus purity is a state for which there are no completely satisfactory criteria. Another procedure is based upon an estimate of the number of hemagglutinating particles present in suspension. It is applicable to the myxoviruses and does not require purified preparations. The assumptions which must be made, however, about the mechanics of agglutination may not be justifiable.

#### *A. Direct Methods: Electron Microscopy*

##### *1. Sedimentation*

A method for enumerating virus particles by sedimentation on a collodion membrane and subsequent examination in the electron microscope was developed by Sharp in 1949. The procedure is simple in principle although

somewhat tedious and complex in execution. A glass coverslip coated with a film of collodion is placed on the bottom of a specially designed analytical ultracentrifuge cell which is filled with the virus suspension. The virus particles are sedimented onto the film, which is then prepared for examination in the electron microscope. The number of particles counted per unit area of collodion membrane is related to their concentration in suspension and to the volume from which they were sedimented. The counts obtained in this way appear to be closely correlated with the dilution factor of the original suspension and with the particle concentration estimated indirectly from physical data (Section III, B).

This technique can be applied to the counting of virus particles from suspensions of purified virus or even from crude suspension, provided the virus particle has been previously identified and possesses a readily recognizable size and shape distinguishing it from the nonviral particulate matter released by disrupted cells. It offers the advantage of permitting counts of particles suspended in relatively low concentrations ( $10^7$ /ml.), but suffers the disadvantage of requiring distilled water rinses after sedimentation to remove salts which, when dried, may interfere with the examination of the specimen in the electron microscope. Such rinsings may remove particles as well as salts if conditions are not carefully controlled.

A modification of this basic method has been described by Sharp and Beard (1952) which entails the sedimentation of the virus particles upon an agar block instead of a collodion film. The sedimented particles are fixed with osmic acid, removed quantitatively from the agar by means of a collodion pseudoreplica, and subsequently counted in the electron microscope. This improvement in design permits the sedimentation of virus particles from salt solutions, since the salts diffuse into the agar and away from the virus on its surface. Thus, high local concentrations of salts are avoided in the vicinity of the virus particles during drying, minimizing possible distortion of particle shape and obscuration of particle image in the electron microscope. Excellent agreement was found by Sharp and Beard (1952) among the results obtained by the two sedimentation methods and the spraying technique of particle counting described below.

## 2. *Spraying*

The technique of electron microscopic counting of virus particles in sprayed microdrops was developed in 1950 by Backus and Williams. It is easily applied to virus suspensions of high concentration ( $10^9$  to  $10^{10}$  particles/ml.) in water or volatile salt solutions, such as ammonium acetate and ammonium bicarbonate. The same requirement holds here as for any electron microscopic method, namely, ready identification of the virus particle on the basis of size and morphology.

The virus concentrate is mixed with a suspension of polystyrene latex reference particles of known concentration. Since the latter are spheres of uniform diameter and density, their mass can be calculated and their concentration in suspension estimated with a fair degree of precision by gravimetric means. The mixture is sprayed from a spray gun of simple construction or from a commercially available nebulizer upon electron microscope specimen grids covered with collodion. Individual droplets can be examined in their entirety in the electron microscope and counts made of both reference and virus particles per droplet pattern. The ratio of the number of latex to the number of virus particles provides an absolute assay of the latter per unit volume of the mixed suspension. Bovine albumin added to such a mixture prior to spraying ensures the uniform dispersion of particles and delineates the droplet pattern on the collodion film. Backus and Williams (1950) found that the estimated standard deviation of the mean ratio of virus to reference particle numbers counted in 20 or more droplet patterns generally lay close to the statistically anticipated value, suggesting that all particles are randomly mixed in suspension and that each droplet is a representative sample of the entire mixture.

The method has been most effective in establishing the ratio of particle numbers to infective units for several viruses of distinctive and characteristic morphology, in particular the T series of coliphages (Luria *et al.*, 1951), meningopneumonitis virus (Crocker, 1954), the myxoviruses (Isaacs and Donald, 1955), and several poxviruses (Dumbell *et al.*, 1957). It has also been used to identify the poliovirus particle as a sphere 28  $m\mu$  in diameter (Bachrach and Schwerdt, 1954) and to establish the ratio of the number of such spheres to plaque-forming units in tissue culture (Schwerdt and Fogh, 1957).

### 3. Agar Filtration

Kellenberger and Arber (1957) have devised a method for the quantitative analysis of particle suspensions which embodies features of both the sedimentation and spraying techniques. In this procedure a suspension of virus is mixed with a known concentration of polystyrene latex spheres. An aliquot of this mixture is spread over a collodion film formed on agar which contains exactly the same medium as that used to suspend the virus and reference particles. The liquid medium (with its dissolved salts) filters through the collodion membrane and diffuses into the agar gel. After such filtration, the film is fixed over formol and sections are floated off the agar and collected on specimen grids for electron microscopy. When a 0.1-ml. aliquot of a suspension containing  $10^{10}$  particles per ml. is spread over a surface area equivalent to that of an ordinary petri dish (50 cm.<sup>2</sup>), the number of particles observed per field at 4600  $\times$  magnification averages 48. In this concentration range, the

coefficient of variation of virus to reference particle ratios is found to be about 15 % when counts are computed from 5 micrographs.

This procedure permits counts to be made of particles suspended in solutions of nonvolatile salts without using the more elaborate equipment required in the sedimentation method. The principal source of error in this as well as the spraying technique for the determination of *absolute* titers of particles is the estimation of reference particle concentration which, in turn, is calculated from the weight of a dried sample of this suspension and the mass of a single latex sphere as computed from measurements of density and diameter.

#### 4. Adsorption

Another method for the quantitative assay of virus particles by electron microscopy depends upon the specific adsorption of the particles to susceptible cells (e.g., phage particles to susceptible bacterial cells) or to hemolyzed red blood cells in the case of the myxoviruses (mumps-Newcastle disease-influenza group). From micrographs of shadowed preparations of such adsorption mixtures the average number of virus particles per cell can be counted and the absolute virus particle concentration computed from the known cell concentration. Luria *et al.* (1943) counted phage particles in this way. Various members of the myxovirus group were similarly counted by Dawson and Elford (1949) after adsorption to hemolyzed chick red blood cells. Donald and Isaacs (1954a) also made particle counts of adsorbed influenza virus, as well as of various other myxoviruses (Isaacs and Donald, 1955) and found them to compare favorably with parallel counts by the spray droplet technique. Investigations employing this method of enumerating hemagglutinating virus particles have been reviewed by Isaacs (1957).

It must be remembered that the adsorption technique only accounts for those particles which have adsorbed and tells us nothing about the numbers which failed to attach. Furthermore, the assumption must be made that the adsorbed particles are randomly distributed over the entire surface of the cell and, thus, from average counts per unit area, estimates are made of the number of adsorbed particles on the underside of the cell and also overlying the nucleus. In general, however, the assumptions and allowances appear to be justified, since the results correspond to those obtained by the more direct spray droplet technique (Isaacs and Donald, 1955).

### B. Indirect Methods

#### 1. Based on Physical Properties

The earliest attempts to determine the particle concentration of a virus suspension were based upon an estimate of the mass of a dehydrated virus

particle. This value divided into the dry weight of an aliquot of purified virus suspension yields a figure for the number of particles per unit volume. Such an approach requires (1) objective criteria for judging virus purity, (2) the assumption that the particle is spherical in shape, and (3) reasonably accurate methods of estimating virus particle diameter and density values which are used to calculate particle mass.

Purity as used in this context is analogous to physical homogeneity, i.e., all particles are similar in size, shape, density, and surface charge, as judged by their behavior upon ultracentrifugal and electrophoretic analysis. That these criteria may be inadequate has been pointed out by Smadel *et al.* (1940), who demonstrated that a mixture of washed vaccinia elementary bodies and graded collodion particles coated with vaccinia antigen will migrate as a single component in a centrifugal or electrical field. In spite of these shortcomings and in lieu of an independent method of testing for biological purity (i.e., each physical particle equivalent to one infectious unit) physical uniformity has generally served as the criterion of purity.

The diameters of spherical or nearly spherical virus particles are usually estimated by ultrafiltration, electron microscopy, or ultracentrifugal sedimentation analyses. Results obtained by ultrafiltration yield a rough approximation of particle diameter at best. The surface tension forces of evaporating solvent flatten the virus particles in specimens prepared for electron microscopy. This effect can be minimized if the particles are spheres of uniform size and align themselves in two-dimensional crystalline arrays on the collodion membrane. Thus, an average diameter can be computed from a linear array of about six or more particles. Preparation of specimens by freeze-drying (Williams, 1953) completely eliminates flattening of individual particles, since the ice is sublimed away in this technique, thereby avoiding the distortive effects of surface tension forces. The critical point method of Anderson (1951) also avoids structural artifacts of drying although specimens prepared in this way usually cannot be shadowed.

Sedimentation constants can be determined with a high degree of precision but an estimation of particle diameter from such data requires equally precise measurement of particle density which is more difficult to achieve. The partial specific volume (i.e., the reciprocal of the dry weight density) of purified virus particles can be determined by the pycnometric method. Because the amount of available purified virus is usually quite small, the pycnometer is being displaced by the ultracentrifuge as the tool of choice for virus density measurements (Sharp *et al.*, 1945, 1950; Schwerdt, 1957). Sedimentation constants of aliquots of the purified virus are estimated in media of successively increasing density, corrected for viscosity, and then plotted as a function of the density of the suspending medium. An extrapolation of the best-fitting curve to the density value corresponding to zero

sedimentation rate yields an estimate of the virus particle density. Sucrose, bovine serum albumin, and  $D_2O$  have been used to increase the density of the suspending medium. The results obtained with  $D_2O$  are the easiest to interpret since mixtures of  $D_2O$  and  $H_2O$  act as a one-component solvent and the particle density measured in such a system is the dry weight density essential for the computation of the virus particle mass and numbers per unit volume of suspension as described above.

All of the above methods of measuring particle diameter and density have their inherent sources of error. Assumptions made in the calibration of average pore diameter of gradacol membranes and adsorption of virus to pore walls give cause for uncertainty in ultrafiltration studies. The flattening of virus particles as well as possible shrinkage during dehydration in preparation for electron microscopy make this method of measuring virus particle diameter equally uncertain. The estimations of particle dry weight density and diameter by sedimentation analysis in mixtures of  $D_2O$  and  $H_2O$  may easily be in error because of the long and hazardous extrapolation necessary to estimate the solvent density at which zero sedimentation occurs (Schachman, 1957). The extrapolation is long because the density of  $D_2O$  is approximately 1.1 while the dry weight density of viruses may be as great as 1.35 (or greater), depending upon their nucleic acid content. Since the volume of a spherical particle varies with the cube of the radius, even small errors in the measurement of particle diameter greatly magnify errors in the estimation of particle mass. Sharp (1953) points out that particle diameters obtained from sedimentation velocity, ultrafiltration, and electron microscope data on a given virus which agree to better than  $\pm 5\%$  are probably accidental and that even this conservative estimate of the error represents a 30% difference in particle mass.

Recognition of these difficulties has resulted in the replacement of this indirect method of estimating particle concentration by the direct counting procedures made possible by electron microscopy. Before the latter techniques had been developed, however, vaccinia (Smadel *et al.*, 1939), Shope papilloma (Bryan and Beard, 1940a), and influenza (Friedewald and Pickels, 1944) virus particle counts had been calculated from estimates of particle mass.

## 2. Based on Estimate of Number of Hemagglutinating Particles

Attempts have been made to estimate the number of hemagglutinating particles of influenza and Newcastle disease viruses on the assumption that a single particle may cause the agglutination of two red blood cells. The concentration of red cell dimers which will sediment more rapidly than single cells can be estimated by simple colorimetric means. The procedure is carried out as follows: A small amount of virus is mixed with a suspension of red blood cells of known concentration. The concentration of virus must be such

as to cause the dimerization of only a fraction of the red cells present; in other words, the estimated number of hemagglutinating particles should be considerably less than half the concentration of red cells. Since the paired red cells will sediment more rapidly than the single cells, the optical density of the suspension measured at some point midway between the meniscus and the bottom of the tube will decrease more rapidly than that of the control suspension without virus as the dimer boundary passes the light path. Once the dimers have passed the beam of light, the slopes of the optical density versus time curves from both the virus plus red cell and the red cell control suspension should be equal. At this stage the difference in optical density between the parallel slopes then represents the concentration of red cells which have sedimented as dimers. One-half this number equals the number of hemagglutinating particles present if the original premise is correct, namely, that each particle present succeeds in agglutinating two red cells.

This method was developed independently by Levine and associates (1953) and by Horsfall (1954) and has been referred to as the "absolute assay" of virus hemagglutinins. The former workers compared their hemagglutinin counts with parallel particle counts by the electron microscopic spraying technique of Backus and Williams (1950) and found them to be approximately equal.

The validity of this technique has been questioned by Isaacs (1957) and by Tyrrell and Valentine (1957). Their principal objection is directed against the original assumption that the hemagglutinating particles are 100 % efficient in the formation of red cell dimers. They present a body of evidence from which they conclude that the estimates of hemagglutinin particles by the "absolute assay" method is erroneously low by a factor of approximately 10. Levine *et al.* (1953) and Horsfall (1954), on the basis of their hemagglutinin particle counts, estimated that, under optimum conditions, 1 or 2 particles represent 1 egg-infective unit. Donald and Isaacs (1954a), on the other hand, found 10 particles visible by electron microscopy for each egg-infective unit present in freshly harvested preparations produced under equally optimum circumstances. Tyrrell and Valentine (1957) made a comparative study of particle counts by the hemagglutination technique and by the two electron microscopic methods of spraying and adsorption on lysed red blood cells and again found 10 times more physical particles by the latter direct methods than by hemagglutination. They suggest that the correspondence Levine *et al.* (1953) found between their "absolute assay" and spray droplet counts was fortuitous and that the results by the electron microscopic method might have been falsely low because of technical difficulties. Furthermore, Donald and Isaacs (1954b) have shown filamentous influenza virus to be 7 to 8 times more efficient than spherical virus in hemagglutination, thus casting doubt on the presumed 100 % hemagglutinating efficiency of the

spherical particles predominating in the preparation used by the advocates of the "absolute assay."

The hemagglutination method of counting influenza and Newcastle disease viruses is not yet firmly established. Whether discrepancies between this and the direct methods of counting reflect strain differences, technical difficulties, or, in general, a lower efficiency of agglutination than assumed requires clarification by further study. Evidence, so far, suggests that these particles are not fully effective or efficient in dimer formation.

It would seem, then, that the direct procedures of enumerating morphologically identifiable virus particles by electron microscopy are the methods of choice, since they demand that fewer assumptions be made and are applicable to a wide variety of viruses without precluding viruses which do not possess the special biological property of hemagglutination.

#### IV. RELATIONSHIPS BETWEEN TOTAL PARTICLE COUNT AND BIOLOGICAL FUNCTION

##### *A. Initiation of Infection by a Single Virus Particle*

Before the possibility existed of determining actual virus particle count by direct observation, attempts were made by statistical means to test the hypothesis that infection of a host may be initiated by a single virus particle. It has been generally found in assays of the local lesion type that the concentration of the virus suspension (i.e., the number of physical particles per inoculum) is directly proportional to the number of lesions produced. Thus a linear curve is produced (ideally with a slope of unity), provided both variates are plotted on the same scale, either arithmetic or logarithmic. This relationship has been found over an appropriate dilution range for phage (Ellis and Delbrück, 1939) as well as for various animal viruses assayed by the pock count method on chick embryo chorioallantoic membranes, such as vaccinia (Keogh, 1936; Burnet and Faris, 1942; Overman and Tamm, 1956a; Westwood *et al.*, 1957), infectious laryngotracheitis (Burnet, 1936), canary pox (Burnet and Lush, 1936a), ectromelia (Burnet and Lush, 1936b), myxoma (Fenner and McIntyre, 1956), and Rous sarcoma (Rubin 1955; Prince, 1958). Plaque assays in tissue culture of western equine encephalomyelitis (Dulbecco, 1952), vaccinia (Noyes, 1953), poliomyelitis (Dulbecco and Vogt, 1954a), influenza (Ledinko, 1955), and foot-and-mouth disease (Sellers, 1955; Bachrach *et al.*, 1957) have exhibited a similar proportionality.

Luria (1940) and Dulbecco and Vogt (1954a) have concluded from statistical reasoning that the linear relationship between virus dilution and lesion count means that only one virus particle is necessary to initiate a lesion. If the production of a single lesion required the cooperative efforts of several

virus particles to infect a single susceptible cell, the lesion count might be expected to increase more rapidly than linearly with increasing virus concentration, in contrast to the usual experimental findings (Luria, 1953). Furthermore, if virus particles are randomly distributed in dilute suspension, the frequency distribution of such particles in samples taken from the suspension should follow Poisson's law of small numbers. This, in turn, should be reflected by a similar distribution of lesions upon inoculation of samples, provided the infective particles act independently. And, indeed, such a distribution of plaques has been found by Ellis and Delbrück (1939) upon plating aliquots of a dilute suspension of coliphage.

Although it appears that under appropriate conditions (i.e., at low multiplicity of infection) lesions are produced by single virus particles, this gives no information about the total number of virus particles present in the inoculum. The latter number can only be ascertained with assurance from direct particle counts by electron microscopy (Section III, A), from which one can then estimate the "efficiency of plating" or the probability that a virus particle will infect.

There has been some question recently of the assertion that the distribution of pock counts on the chorioallantoic membrane of chick embryos follows the Poisson series. If the pock counts truly reflect random distribution of virus particles in suspension prior to inoculation, the variance,  $V$ , of the mean count is expected on a Poisson distribution to equal the mean count,  $\bar{x}$ , from which the expected coefficient of variation ( $100\sqrt{V/\bar{x}}$ ) is readily calculated. Westwood *et al.* (1957), upon comparing the theoretical and observed coefficients of variation of vaccinia virus pock counts on chorioallantoic membranes, usually found the latter to exceed the former, indicating that the degree of scatter of experimental counts was in excess of that expected, although by careful control of experimental conditions the distribution of counts approached that predicted by the Poisson equation. Kaplan and Belyavin (1957), using the same virus assay system, found not only that the variances of mean pock counts exceeded expectation (on the assumption that the distribution was Poissonian) but also that they "wandered" excessively, making impossible valid estimates of coefficients of variation. In a detailed statistical study of the variability of pock count data obtained by others, Armitage (1957) also concluded that the variance was considerably greater than that expected if the count distributions were of the Poisson form. By way of a possible explanation of the excessive deviation of pock counts from theoretical, the hypothesis frequently offered is that the chorioallantoic membranes are heterogeneous with respect to virus susceptibility. Thus a secondary distribution of host susceptibility is superimposed upon the random sampling distribution of virus particles in suspension.

Another approach to testing the hypothesis that one virus particle may initiate infection is through a statistical analysis of the dose-response curve in which the percentage incidence of positive test animals (i.e., animals showing an all-or-none reaction, such as death or survival) is plotted against log virus dilution. Parker (1938) made such an analysis using vaccinia infection in rabbits as his model system. According to the Poisson law of small numbers, successive sampling of a suspension of particles independently distributed in a liquid medium will yield aliquots containing 0, or 1, or 2, etc., particles in proportions related in a definite way to the mean number of particles per aliquot. From this, in turn, the proportion of aliquots containing at least 1, or 2, or 3, etc., particles can be calculated. A plot of the per cent. of positive aliquots (i.e., aliquots containing at least some arbitrarily selected minimum number of particles such as 1, or 2, or 3, etc.) against the log of the mean number of particles per aliquot yields a family of S-shaped curves, the slopes of which increase in steepness with increasing values of the minimum number of particles required for a positive result. When Parker (1938) constructed such a set of theoretical curves and superimposed upon them experimental dose-response curves, he found the latter to fit best the curve calculated on the hypothesis that one particle per aliquot is sufficient to yield a positive response. This correspondence between experimental curves for vaccinia titration in rabbits and the so-called hypothetical "one-particle" curve was confirmed by Sprunt *et al.* (1940) and also found with Shope papilloma (Bryan and Beard, 1940b) and myxoma viruses (Parker, 1940).

Agreement on the above statistical inference that infection can be initiated by a single particle is by no means unanimous. Bryan and Beard (1940b) contend that the usual apparent correspondence between experimental and theoretical "one-particle" dose-response curves is not necessarily proof of the hypothesis that infection is due wholly to the chance presence or absence of an infective virus particle in the inoculum. They interpret the dose-response curves observed with Shope papilloma virus, as well as those observed by Parker (1938) with vaccinia virus in rabbits, as expressions only of the variable susceptibilities of the test animals employed. According to their postulate, the hosts exhibit a distribution of sensitivity to infection by virus at any one dilution similar to that found for response to drugs. Thus they have derived an expression which fits virus titrations as well as or better than the model proposed by Parker, particularly in those instances where the dose-response curve is somewhat flatter than that predicted by the "one-particle" hypothesis.

The decision as to which of these two hypotheses is better able to explain the dose-response curve is difficult to make with certainty at this time. It may be that both mechanisms play more or less of a role in determining the shape of the curve, depending upon the virus-host system under study. In

favor of the theory that one virus particle can initiate infection is the observation that each phage particle counted by electron microscopy can produce a plaque (Luria *et al.*, 1951; Section IV, B) although there is, at present, no such direct evidence from the quantal-response type of animal virus assay data. Lauffer and Price (1945), upon examination of a variety of virus infectivity data drawn from the literature, concluded that they were, for the most part, consistent with the "one-particle" theory and noted that the alternative theory requires the improbable assumption that the variability in the response of all virus-host systems is of the same order, since the slopes of their titration curves are almost always similar. Discrepancies that do occur (i.e., where the dose-response curve is flatter than the "one-particle" curve) may be attributed to variability of host response as a secondary factor. For example, Parker *et al.* (1941) found that titrations of vaccinia virus of moderate virulence yielded somewhat flattened curves when carried out in several rabbits, in contrast to the "one-particle" type dose-response curve obtained with virulent strains. However, when this same strain of moderate virulence was titered by the inoculation of all dilutions into *individual* rabbits, the titration curve obtained from each rabbit was adequately described by the Poisson equation, assuming that a single particle initiates infection, although the individual 50 % end points varied considerably.

Armitage and Spicer (1956) also have shown from statistical analysis of dilution counting experiments that departure of the quantal response curve from a "one-particle" curve may occur, particularly at higher virus concentrations, in the form of fewer positive responses than expected as a consequence of variation of host susceptibility. The over-all results is a general flattening of the curve.

### *B. Observed Ratios between Virus Particle Count and Infectious Units*

The tentative conclusion reached by many on the basis of statistical inference that a single virus particle can initiate infection has awaited confirmation by direct observation. It must be admitted that, except for bacterial viruses, confirmatory evidence for a 1 : 1 correspondence between countable characteristic physical particles and infectious units is scant. The difficulty may lie in finding the optimal conditions for measuring infectivity. If the assumption that one particle is sufficient to initiate infection is correct, the observed ratios of successful to total particles, which are usually less than one, can be considered estimates of the probability of infection by a virus particle under the conditions of the titration. Failure to observe 1 : 1 ratios may be due, in part, to the selection of host and/or route of inoculation or may be inherent in the particle itself. The methods employed for particle counting are incapable of distinguishing between fully infectious particles

and those noninfectious due to thermal inactivation or incomplete synthesis if their gross physical characteristics are similar.

### 1. *Bacterial Viruses*

There is little doubt today that plaque assays of bacterial viruses can be ideal under appropriate conditions, thus supporting the concept of infection by a single virus particle. At least this can be said of the T series of coliphages. Luria *et al.* (1951), in an investigation of the particle-to-plaque relationships of purified T-even phages, found ratios varying approximately between 1 and 2 when particle counts were made by the electron microscopic, spray droplet technique using polystyrene latex reference particles. This has been confirmed by Kellenberger and Arber (1957), who did electron microscopic counts of stock T<sub>2</sub>, T<sub>4</sub>, T<sub>5</sub>, and lambda phages by their agar filtration technique and found a range of particle-to-plaque ratios lying between 1 and 3 with an average value of 1.4. The bacterial virus-bacterium host system has proved most satisfactory in establishing the 1 : 1 relationship between statistical (infectious) and physical particles.

### 2. *Poxviruses*

Vaccinia was among the earliest of the animal viruses to be studied with respect to particle per infectious unit ratios, no doubt because of its large size and relative ease of purification. At first, elementary body concentrations were determined indirectly on the basis of estimates of particle mass from physicochemical data. Smadel *et al.* (1939) divided the dry weight of an aliquot of purified vaccinia virus suspension by the calculated dry particle mass and compared this figure with the number of rabbit ID<sub>50</sub>'s in an equal volume of the same preparation. They found that values for ratios of elementary bodies to ID<sub>50</sub> varied between 2.4 and 9.2 with an average of 4.2. Similarly, Sprunt *et al.* (1940) counted vaccinia elementary bodies, using nitrogen content, however, as a measure of total purified virus mass per aliquot. They observed an average value of 366 particles per ID<sub>50</sub>. This figure exceeds significantly the 4.2 value found by Smadel *et al.* (1939). It seems unlikely that this 90-fold discrepancy represents great differences in the degree of purity of the preparation employed by the two groups of workers and thus differences in enumeration of elementary bodies. Rather dissimilarities in virus virulence and host susceptibility (Sprunt, 1942; Sprunt and McDearman, 1940) as well as in conditions of titration, such as volume of inoculum (Sprunt, 1941), may have been responsible.

With the advent of direct particle counting by electron microscopy, the particle per infectious unit ratio for vaccinia virus has been reinvestigated. Overman and Tamm (1956b) used a modification of Sharp and Beard's (1952) sedimentation technique for counting particles and estimated the

somewhat surprising average ratio of less than one particle (0.67) per pock by titration on chick embryo chorioallantoic membranes. Also Dumbell *et al.* (1957) estimated elementary body per infectious unit ratios for vaccinia and variants of cowpox viruses. Particle counts in this instance were made by the spray droplet technique. They observed average ratios of approximately 100 and 20 for the cowpox variants assayed for infectivity on chorioallantoic membrane and in rabbits, respectively. Those for vaccinia virus were found to approximate 18 particles per pock or  $ID_{50}$ , indicating no detectable difference in susceptibility between the two hosts used for assay. Again it might be postulated that inherent differences in the virus-host systems employed account for the disparate results obtained in the two laboratories. However, Overman and Tamm's (1956b) finding that infectivity titer exceeds total count suggests that technical difficulties may also have been responsible in part. The latter workers made particle counts using impure preparations in which debris may have obscured elementary bodies in the electron micrograph; thus their estimate of physical particle numbers may have been low.

### 3. *Myxoviruses*

Although this group includes the mumps, Newcastle disease, fowl plague, and influenza viruses, only the results with influenza virus will be presented briefly, since it has been the most extensively studied. Influenza virus, in common with the other myxoviruses, possesses the property of hemagglutination as well as infectivity for developing chick embryos. Both properties are associated with the virus particle yet can be studied independently. Thus it has been possible to investigate simultaneously the quantitative relationship between actual particle count and two functional activities of the virus particles.

Infectivity titers are generally expressed as the number of  $ID_{50}$  per unit volume for mice or eggs, principally the latter. Hemagglutinin titers are usually determined by the pattern test and are expressed as the dilution of virus present at the partial agglutination end point. The conditions for carrying out this titration may vary from one laboratory to the next. Isaacs (1957) described the partial agglutination end point as that pattern of agglutinated cells intermediate between complete agglutination and absence of agglutination and defines an agglutinating dose as, on the average, the amount of virus present at the partial agglutination end point in a pattern test using 0.25 ml. of a 1 % suspension of chick red cells (approximately  $10^{7.15}$  cells). For greater precision a 50 % hemagglutination end point may be determined by a photoelectric densitometer as described by Miller and Stanley (1944), but the results are not readily compared with the pattern test end point.

Friedewald and Pickels (1944) estimated the number of influenza virus particles required to infect mice and eggs as well as agglutinate chick red cells. Virus particle count was determined indirectly by dividing the dry weight mass of an aliquot of purified virus by the dry mass of a single particle estimated from physicochemical data. Donald and Isaacs (1954a) and Isaacs and Donald (1955), in more extensive studies, counted particles of representative strains of all four viruses of the myxovirus group by electron microscopy, using both the spray droplet and red cell adsorption techniques. The results of both groups, in summary, indicated that at the 50 % agglutination or partial agglutination end points the number of influenza A virus particles equaled the number of red cells present. They also agreed on an estimate of approximately 10 particles corresponding to one  $ID_{50}$  from titrations in embryonated eggs conducted under optimal conditions. These results compare favorably with those of Werner and Schlesinger (1954), who found an average ratio of 1.2 virus particles per cell at the partial agglutination end point, and of Graham and McClelland (1950) and Miller and Schlesinger (1955), who estimated 16 and 10 particles, respectively, per  $ID_{50}$  in chick embryos.

Such findings receive additional support from the many observations, reviewed by Isaacs (1957), that the ratio of the number of infective doses per hemagglutinating dose (I/HA) averages  $10^{6.3}$  for virus preparations of high infectivity (i.e., virus harvested from eggs infected at low multiplicities to avoid the production of noninfective or "incomplete" virus (von Magnus, 1946)). Since, as inferred from above,  $10^{7.15}$  physical particles constitute one hemagglutinating dose, an I/HA ratio of  $10^{6.3}$  indicates that approximately 7 particles correspond to one  $ID_{50}$ .

When influenza virus particle counts are made by the so-called "absolute assay" hemagglutination method (Section III, B, 2) devised independently by Levine *et al.* (1953) and Horsfall (1954), lower ratios of particles per  $ID_{50}$  and per hemagglutinating dose by pattern test were found, in contrast to the results cited above. These workers estimated that 1 or 2 virus particles represent 1 egg-infective unit and that only 1 virus particle per 20 red cells is present at the partial agglutination end point by pattern test. Tyrrell and Valentine (1957) have made a comparison of the indirect "absolute assay" method of counting particles with direct electron microscope counts and found that the indirect method yielded, on the average 10-fold lower estimates of particle numbers. It would appear that the hemagglutination method of enumerating virus particles has approximately a 10 % rather than 100 % efficiency in forming red cell dimers.

#### 4. Other Viruses

With the new, direct techniques for enumerating virus particles by electron microscopy now available, studies on the quantitative relationship between

identifiable virus particles and infectivity of more and more viruses can be expected. Among the more recent investigations are those of Crocker (1954) on meningopneumonitis virus. Counts of this virus by the spray droplet technique revealed a minimum value of 43 elementary bodies per  $ID_{50}$  when infectivity titrations were carried out by yolk sac inoculations in chick embryos. Poliovirus particles, after purification from infected cotton rat brains and spinal cords, were identified by electron microscopy and also counted, again by the spray droplet technique (Bachrach and Schwerdt, 1954). Approximately 20,000 characteristic physical particles were found equivalent to one  $ID_{50}$  by infectivity assays in cotton rats. In more recent studies, particle counts of tissue culture poliovirus were compared with infectivity assays by the sensitive plaque technique using monolayer cultures of primary human amnion cells. Here an average of 36 particles per plaque was observed for the Mahoney strain of type 1 poliovirus (Schwerdt and Fogh, 1957).

In those few instances where tumor-producing viruses have been sufficiently purified to permit identification of the virus particle, the count per infective dose has been found to be very high. Bryan and Beard (1940a) estimated the mass of the papilloma virus particle from centrifugation and filtration data. On this basis they calculated the number of virus particles present in the amount of purified virus necessary to cause infection in 50 % of the rabbits inoculated and found it to be approximately  $8 \times 10^7$ . The virus of avian myeloblastosis exhibited a particle per  $ID_{50}$  ratio in chickens of the same order of magnitude, namely,  $2.6 \times 10^7$  (Eckert *et al.*, 1955). These high ratios reflect, in part, the enormous range of host susceptibility to tumor viruses. For example, Eckert *et al.* (1955), upon extrapolating the linear plot of log number of virus particles inoculated against per cent. positive responses on a probit scale, observed that at the 5 % level of incidence approximately  $6 \times 10^3$  particles infect this proportion of chickens, while  $1.45 \times 10^{11}$  particles are required to infect 95 % of the hosts.

Quantitative comparisons between direct particle and lesion counts for plant viruses reveal high ratios, as do the tumor viruses but for different reasons. They are concerned largely with the inefficiency inherent in the inoculation technique. Virus suspensions of known particle concentration are rubbed on susceptible leaves in as reproducible a manner as possible. The number of lesions produced may be an exceedingly low estimate of viable particles present, however, since the number of suitable entry points per leaf is limited. Steere (1955), taking great precautions to apply his inoculum as effectively as possible, demonstrated, at best, a particle per lesion ratio of 50,000 for tobacco mosaic virus.

## V. SUMMARY AND CONCLUSION

The various methods of determining virus concentration in terms of infectious units and absolute particle count have been presented. Infectivity

assays, whether based upon the all-or-none or upon the local lesion type of response, are useful in estimating relative virus concentrations but give no clue as to the total mass or numbers of virus particles involved. If a virus can be purified or if the physical particle with which infectivity is associated has a characteristic size and shape readily recognized by electron microscopy, absolute particle counts can be made and correlated with infectious units. Several excellent techniques for direct counting by electron microscopy have been devised and applied to a number of bacterial, animal, and plant viruses. Although there are a few indirect methods of enumerating virus particles, they would seem to be less desirable, since they require assumptions regarding purity of preparation, particle mass, or the quantitative aspect of some biological activity, such as hemagglutination, which are not always readily verified.

Almost all viruses studied so far have revealed a particle per infectious unit ratio greater than one, with the exception of bacterial viruses (T series of coliphages), for which the ideal 1 : 1 relationship has been observed. In spite of the failure of most viruses to exhibit the ideal behavior of one particle per infectious dose, it is the consensus of many that infectivity may be initiated at the host as well as the cellular level by a single viable virus particle. This opinion has been arrived at by inference from statistical analyses applied to titration data of both the quantal and local lesion type of response and is supported, of course, by direct observation in the case of bacterial viruses. There is a dissenting opinion, however, that the probability of infection does not depend upon the chance presence or absence of a single virus particle in an inoculum but rather upon the variable susceptibilities of the individual members of the host species inoculated, particularly in those systems where large numbers of virus particles are required to induce infection. Variations in host susceptibilities may well play a role in determining the nature of the dose-response curve for some virus-host systems yet not necessarily vitiate the "one-particle" concept of infection under optimum conditions.

The usual experimental observation of a virus particle per infectious dose ratio greater than one may have several explanations. It may be due to a general, more or less uniform, resistance of the host animals. It may also reflect the fact that only a portion of the entire population of particles is infective. The fraction which is noninfective may be so by virtue of thermal inactivation or faulty synthesis during replication. There is a further possibility, proposed by Isaacs (1957), that the virus particles present a spectrum of infectivity with some particles showing a high probability and others a very low probability of initiating infection. Whatever the reason, the reciprocal of the ratio of particles per infectious unit expresses the probability that a particle will infect under a given set of conditions, assuming that the "one-particle" theory of infection is correct.

The potential that particle counting offers to experimental studies concerned with the mechanism of animal virus replication has been realized mostly for influenza virus to date. For example, the agglutinating behavior of "incomplete" and standard influenza virus has been found to be similar in each case (Werner and Schlesinger, 1954; Donald and Isaacs, 1954a) although it has not yet been possible to determine by particle count methods whether "incomplete" virus preparations consist of a mixture of totally noninfective and infective particles or represent a population of virus particles of uniformly low ability to infect (Isaacs, 1957).

The filamentous forms of influenza virus have been compared with the spherical forms with the aid of particle counting techniques and have been found to possess the same infectivity but about seven or eight times the hemagglutinating efficiency of spheres on the basis of filament and particle counts (Donald and Isaacs, 1954b). Ada *et al.* (1957) have recently confirmed this finding of greater hemagglutinating efficiency of the filamentous forms but noted that they also exceed the spherical particles in specific infectivity. They calculated from their data that two or three filaments are equivalent to one 50 % infective dose.

The problem of the effect of multiplicity of infection (i.e., the number of physical particles adsorbed per cell) upon "incomplete" virus production is being currently investigated. The quantitative aspects of interference by inactive particles is also under study. One difficulty in interpretation of such experiments is the uncertainty with which the total number of available, susceptible entodermal cells lining the allantoic membrane is estimated. Isaacs (1957) has summarized the results of findings in these areas. It appears that multiplicities ranging from one to one hundred may produce "incomplete" virus, depending upon the strain of virus, while twenty to several hundred particles per cell are necessary to produce interference, again depending upon strain of virus used and conditions of inactivation.

One can hope, now that more and more viruses are being purified and identified by electron microscopy, that further clarification of their mechanisms of replication as well as of their chemical and physical nature will also be aided by virus particle-counting techniques.

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## Chapter V

### Inactivation of Viruses

S. GARD AND O. MAALØE,

*Karolinska Institutet Medical School, Stockholm, Sweden*  
*University Institute of Microbiology, Copenhagen, Denmark*

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## I. INTRODUCTION

There is only a small step from the subject of the preceding chapter to that of the present. Each new method by which the biological activities of a virus can be registered widens the area open to experimentation; usually, the first and simplest experiments to be carried out are concerned with the stability of the virus. We want to know under what environmental conditions the activities we register are preserved and how we can interfere with or destroy them in a controlled manner. Such questions are of obvious interest from theoretical as well as practical viewpoints.

A great variety of treatments and agents are known to inactivate viruses and, during inactivation, the individual biological activities of the virus tend to disappear one after the other. We shall restrict the treatment of this manifold phenomenon in two ways: in the first place, emphasis will be on the most important and, generally, the most sensitive property of the virus particle, its *infectivity*. We shall therefore leave out studies of noninfective, virus-like particles, and treat inactivation of such virus manifestations as interference or hemagglutination in less detail. Second, we propose to deal exclusively with free virus particles and not consider the effects of inactivating, or inhibiting agents on virus cell complexes.

Even with these restrictions our subject matter is large and heterogeneous. The other obvious subdivision, according to the physical, physicochemical, or chemical nature of the inactivating treatment, or agent, that has been adopted is not altogether satisfactory. In some cases it would have been more natural to treat inactivation for the sake of obtaining sterility separately from those experiments in which the process of inactivation is studied in order to gain information about the structure of the virus particles themselves. In other cases, the presentation might have been based on the mechanism by which inactivation is thought to occur. However, neither of these alternative classifications is practicable. The first, because many agents are important from practical as well as theoretical viewpoints. The second, because, very often, the structural changes involved in inactivation are poorly understood.

Two special types of inactivation: neutralization by antibodies, and block-

ing of virus particles by receptor substance, will be discussed in detail in other chapters and are therefore not treated here.

## II. PHYSICAL AGENTS

O. MAALØE

### A. Mechanical Treatments

#### 1. Sonic Irradiation

In virology, the use of sonic oscillators is primarily of theoretical interest. Even prolonged treatment with the high energy waves emitted by modern machines usually does not sterilize virus suspensions, and it has not yet been possible to produce vaccines using "sonic" inactivation alone.

Two types of generators, magnetostriction and piezoelectric oscillators, are being used. The physical principles involved have been reviewed by Gregg (1944) and by Pollard (1953). The forces which seem to be responsible for inactivation are those created by the rapid shifts between high and very low pressures that occur rhythmically as the sound wave travels through a liquid. If the energy applied is great enough, submicroscopic bubbles of dissolved gas develop as the pressure drops and rapidly collapse as the pressure again goes up; this phenomenon is known as cavitation, and very great shearing forces are generated in this way. Difficulties in interpreting correctly what happens in the minute regions within which these forces operate are due mainly to the inevitable changes in temperature accompanying the transmission of the sonic wave. When cavitation occurs, the local temperature may rise to over 200°C. for very short times (Harvey *et al.*, 1944). Nevertheless, heat inactivation seems not to play an important role; thus Oster (1947) has shown that the properties of sonically disrupted tobacco mosaic virus (TMV) are quite different from those of heat-denatured virus. Nor are the pressures generated by sound waves sufficient to cause inactivation (Pollard, 1953).

Elimination of the dissolved gases, as well as increase of pressure, is known to counteract inactivation (Stanley, 1934a), and lowering the surface tension is reported to have increased the effect on bacteria (Hamre, 1949). These observations indicate that inactivation by sonic treatment is due mainly to the process of cavitation. In this connection it may be mentioned that there seems to exist an upper limit for the biological efficiency of the applied acoustic energy: Horton and Horwood (1951), working with *Escherichia coli*, found that maximum efficiency was reached when the energy was about 18 acoustical watts cm.<sup>-2</sup>. The explanation offered is that, at high intensities of cavitation, the gas bubbles prevent the effective transmission of the sound wave.

It was early demonstrated that TMV can be inactivated by sonic treatment (Takahashi and Christensen, 1934; Stanley, 1934a), Kausche *et al.* (1941) demonstrated that the characteristic rods of TMV are actually broken by the treatment.

Oster (1947) has analyzed the effects of ultrasound on TMV by following the changes in birefringence, viscosity, size distribution, as well as the loss of infectivity. From the size distribution at various times he concluded that the 2800 Å virus rod is fractured near its center and that the smaller pieces are broken in the same way to give fragments about  $\frac{1}{4}$  and  $\frac{1}{8}$  of the original rod. The preferential breaking of the rods into halves is presumably due to maximum hydrodynamic stress occurring near the center, and need not imply the existence of weak points in the biological structure of these particles. It is an interesting consequence of this observation that the fraction of particles not yet broken should decrease exponentially with time of treatment, whereas the frequencies with which pieces one-half or less the length of the original rod occur should increase before beginning to drop. Since the infectivity of the preparations is observed to decrease exponentially, it seems justifiable to conclude that only the 2800 Å rods possess infectivity (the same conclusion has been reached independently on the basis of X-ray experiments; see p. 371).

Oster also observed that longitudinal aggregation of the TMV fragments did not result in reactivation; this also suggests that the virus rod constitutes an organized unit the fragments of which cannot be effectively reassembled by random aggregation.

Malkiel (1947), studying similar fragments serologically, has shown that their capacity for combining with antibody molecules is strikingly increased. No new antigens seem to be uncovered by the sonic treatment.

The work of Newton (1951) with high-intensity sound waves extends Oster's observation. Despite very complete fragmentation of the virus rods there appeared to be no denaturation of the antigens. Later, Newton and Kissel (1953) carefully analyzed the size distribution after various intensities of sonic treatment; they concluded that the virus rod may have a weak point at a distance of about 1000 Å from one end.

An extensive study by Rheins and Finlay (1954) on various preparations of influenza virus showed that highly purified virus is much more susceptible than are the particles in chorioallantoic fluid. Appreciable inactivation was observed if the temperature was allowed to rise to about 50°C., but not if the temperature was kept below 30°C. during treatment. The antigenic and hemagglutinating capacity of the virus remained unimpaired even when infectivity had been greatly reduced. The sonic treatment furthermore proved very effective in releasing virus from allantoic membranes, and the authors discuss the practicability of using large-scale sonic treatment as a means of improving the quality and the yield in vaccine production.

The tests carried out with other animal viruses are largely qualitative. Partial inactivation has been demonstrated for vaccinia (Yaoi and Nakahara, 1934; Rivers *et al.*, 1937) and polioviruses (Kasahara and Ogata, 1938;

reviewed by Elpiner, 1952). Added protein was found to protect against inactivation.

The seven coliphages of the T series were tested by Anderson *et al.* (1948). The T2, T4, T5, and T6 phages, which are large and complex structures, were found to be considerably more sensitive than the host cell, *E. coli*, strain B, which, in turn, is more sensitive than the small phages T1, T3, and T7. Except in the case of T3, infectivity was lost exponentially. The T2, T4, and T6 phages were changed into "ghosts"; i.e., the DNA was liberated into the medium, leaving empty heads with the tail still attached, as in the case of osmotic shock (see Section III, D, 2). Exponential killing has also been observed with staphylococcus phage (Kreuger *et al.*, 1941). No careful study seems to have been made of inactivation rates as function of temperature or ionic environment, or in suspensions to which protective compounds, such as proteins, were added.

## 2. Surface Inactivation

The surface tension at liquid/air or liquid/liquid interfaces is known to denature protein. Thus, certain enzymes and toxins lose their biological activity when shaken in dilute solutions; Langmuir and Waugh (1938) have shown that protein which is "spread" on a liquid surface is denatured and becomes insoluble.

Surface inactivation of viruses has not received much attention, despite the fact that rather drastic effects can be obtained. Campbell-Renton (1937) observed that phage particles gradually lost infectivity on shaking or when air was bubbled through the suspension. Grubb *et al.* (1947) observed a similar effect on influenza virus, and McLimans (1947), studying equine encephalitis virus, noted that the degree of inactivation resulting from agitation depended strongly on the pH of the suspension.

The only thorough study of surface inactivation of viruses was made by Adams (1948) with the coliphages of the T-series. The important points brought out by Adams are: (1) that all seven phages are inactivated exponentially (phage T4, which in most respects is very similar to the two other even-numbered phages, T2 and T6, is very resistant compared to the other two); (2) the rate constants increase with temperature (observations between 0 and 38°C.), and at pH values below about 5; (3) neutral proteins in small amounts protect the phages against inactivation. Gelatin was found to be particularly effective.

Careful control experiments proved that inactivation occurs at the liquid/gas interfaces, and that it is independent of the gas phase used (air, H<sub>2</sub>, or CO<sub>2</sub>) and of the surface properties of the container.

The gelatin employed as protecting agent could be shown to act by competing with the phage particles for the available surface. Thus, if a dilute

solution of gelatin was shaken before adding the phage, it afforded no protection. As little as one gamma of gelatin per milliliter protected effectively for 14 minutes; with bovine albumin, about 10 times as much; and with gum arabic and yeast nucleic acid, about 100 times as much had to be used. Adams points out that, in the last two cases, protection may have been due to contaminating protein.

It may be recalled that neutral proteins also were found to protect virus particles against inactivation by sonic treatment. It would seem likely that sonics act, at least in part, by creating a large liquid/gas interface on which inactivation can occur, as in the case of mechanical shaking or bubbling. The protective role of protein is probably the same in both cases.

### 3. *Inactivation at High Pressure*

Being of purely theoretical interest and requiring unusual equipment, few experiments of inactivation at high pressure have been made. Johnson *et al.* (1948) studied the heat denaturation of TMV at different pressures. As predicted by the theories of absolute reaction rates, denaturation was retarded by increasing the pressure, and consistent values for the volume increase of activation,  $\Delta V^\ddagger$ , were obtained.

Foster *et al.* (1949) extended this work by experiments with some of the coliphages of the T-series. Phage T7 was unique in the sense that inactivation was accelerated by increasing the pressure; there is no simple explanation for this unexpected observation. For phage T5, at 66°C. inactivation rates were obtained which corresponded to a  $\Delta V^\ddagger$  of 113 ml./mole.

Basset *et al.* (1956) observed slow inactivation of poliovirus at 37°C. when the pressure reached 6–8,000 kg./cm.<sup>2</sup>.

## B. *Ionizing and Nonionizing Radiations*

### 1. *General Aspects*

It is characteristic of the different types of radiation grouped together in this section that, under a variety of experimental conditions, inactivation proceeds exponentially. Before discussing individual cases in detail, we shall briefly consider what this implies:

As mentioned in Section A2, surface inactivation is exponential. The simplest way to account for this is to assume that a virus particle remains completely unaffected until it is caught at an inter-face, and that, once caught, it is irreversibly inactivated. If the conditions are such that the chance of being caught is the same throughout the experiment we shall find that, *per minute*, a constant fraction of the still active particles are inactivated. This describes a typical first-order reaction, which may be expressed by the equation

$$N/N_0 = e \exp(-kt),$$

where  $N/N_0$  is the fraction of virus particles surviving at time  $t$ , and  $k$  is a rate constant which usually depends on temperature, ionic environment, hydration, etc. In irradiation experiments the dose  $D$ , delivered at time  $t$ , is often substituted for  $t$  itself, and the above equation may then be written  $N/N_0 = e \exp(-D/D_0)$ , where  $D_0$ , or  $D_{0.37}$ , is the so-called "inactivating dose" which gives  $N/N_0 = e^{-1}$ , or 37 % survivors (Lea, 1947).

The striking discovery that inactivation by various types of radiation usually is first order led to development of the so-called "target theory."<sup>1</sup> In its general form the theory postulates that a biological unit (e.g., a virus particle) has within it a sensitive volume, the target, and that a *single hit* within that volume (e.g., an ionization or the absorption of a quantum of ultraviolet light) can, but need not, inactivate the unit.

In experiments with ionizing radiation it is generally assumed that the first ionization<sup>2</sup> to occur within the target volume causes inactivation. This simple and attractive model is, to a large extent, based on early experiments with certain viruses, which suggested that a single ionization, anywhere in a particle, sufficed to inactivate that particle (Lea, 1947). In other words, these viruses seemed to be all target, and primary target-ionizations seemed to be 100 % effective. Target volumes for some large viruses and cells have been calculated, assuming the same high ionization efficiency and, as a rule, it has been found that the sensitive volumes are small compared to the particles or cells of which they are part (Lea and Salaman, 1942; Lea, 1947; Pollard, 1953).

Considering that inactivation by ionizing radiation is frequently studied for the sake of estimating the size and shape of the target, it should be emphasized that the assumption that primary target-ionizations are 100 % effective (i.e., that the "ionization yield" is unity) may not be generally valid. It is true that this crucial assumption has not been disproved, but recent findings somewhat reduce its significance. Most important are probably the experiments which show that the result of applying a fixed X-ray dose to a dried virus depends on the method of drying, as well as on the temperature maintained during irradiation (Bachofer *et al.*, 1953; Bachofer, 1953; see Section B2).

It has always been thought that the genetic material constituted the radiation-sensitive target in a virus particle (Jordan, 1940; Lea, 1947); and, today, this idea is as plausible as ever. Present knowledge, which tends to identify the genetic material with the viral nucleic acids (Hershey and Chase,

<sup>1</sup>The theory was formulated by Timoféeff-Ressovsky *et al.* (1935). It has been described in detail by Jordan (1940) and by Lea (1947). A generalization of the theory, applicable to most biological systems, was presented by Atwood and Norman (1949).

<sup>2</sup>We refer to primary ionizations which have associated with them one or a few secondary and closely clustered ionizations.

1952; Jacob and Wollman, 1956), therefore points to these structures as the most likely target material. In an attempt to test this hypothesis, Epstein (1953), has compared estimates of target volume and nucleic acid volume for about 10 different viruses; he finds rather close agreement except for vaccinia virus, where the target volume is less than 10 % of the nucleic acid volume. Buzzell *et al.* (1956) have shown that, except for vaccinia virus, the agreement is improved by introducing more recent analytical data. Phage T1, which was not considered by Epstein or by Buzzell *et al.*, seems to be another exception: according to Pollard and Forro (1951), its target volume is about 3 % of the particle volume, as contrasted with 15–30 % for the DNA volume (Mennigmann and Schaechter, unpublished data).

The assumption that a virus particle is inactivated by the first primary ionization to occur within the nucleic acid volume would thus seem to be valid in many cases, but not in all. For larger biological units this assumption may be far from correct; in cells of *E. coli*, the target volume (calculated by Lea, 1947) is only about 1–2 % of the DNA volume (assuming between  $5 \times 10^{-15}$  and  $10^{-14}$  gm. DNA/cell). These considerations show that, in some cases, only one out of a rather large number of primary ionizations within the “genetic target” would seem to cause damage that results in irreversible inactivation.

In this connection, it is interesting to recall that phage particles carrying radiophosphorus ( $P^{32}$ ) in their DNA undergo exponential inactivation, *but that only one decay out of about ten constitutes an effective hit* (Hershey *et al.*, 1951; Stent, 1953). From what was said above, it would seem that, at least in certain DNA structures, a primary ionization is not more effective in causing inactivation than is the decay of a  $P^{32}$  atom.

A low efficiency of inactivation could mean that “weak spots” existed within the genetic structure and that ionizations (or  $P^{32}$  decays) were effective only if they occurred in these spots (Stent and Fuerst, 1955). If so, we should be dealing with “radiation targets” distributed within a chemically and genetically defined structure, and the target volume would mean something very different from what it is commonly thought to mean.

In the case of inactivation by ultraviolet light (UV), the situation is quite different. First, the efficiency with which the absorption of a quantum of UV causes inactivation is low; the “quantum-yield”,  $\phi$ , being of the order of  $10^{-3}$  –  $10^{-4}$ . Second, the UV energy is absorbed by characteristic chemical compounds; and, finally, UV causes inactivation by direct effects only (see Sections II, B, 2 and 3).

## 2. Ionizing Radiation

*a. X-Rays.* The introductory remarks about irradiation of viruses dealt with the *direct* effect of ionizations occurring in the target material. It has

long been known, however, that X-rays produce free radicals and other toxic products in the suspending medium that are responsible for the so-called *indirect* inactivation.

Direct and indirect effects can usually be separated quite well because neutral compounds, especially proteins and nucleic acids, protect against the indirect effect of X-rays by competing for the inactivating radicals. This phenomenon was studied in detail for various enzymes by Dale (1940); Friedewald and Anderson (1940) have shown that rabbit papilloma virus is about 20 times more resistant in crude, protein-rich preparations than in a semipurified state. This general rule has been amply confirmed (e.g., by Latarjet and Ephrati, 1948), and the analogy to protection of viruses against surface inactivation is obvious (see Section A2).

The addition of protein or peptone protects phage very satisfactorily against indirect effects (Luria and Exner, 1941a,b; Watson, 1950, 1952). In nutrient broth the rate of inactivation of phage T2 is the same as in media containing as much as 5 % gelatin, which shows that inactivation is caused chiefly by direct effects (Watson, 1950). This, however, is not necessarily true of virus activities other than infectivity. Latarjet and Frédéricq (1955) found that the ability of phage T6 to kill bacteria was only partially protected in a medium containing 9 % yeast extract and catalase; freezing further reduced the rate of destruction by a factor 5. In the frozen state the bacterial killing ability of phage T6 and of the closely related "colicin K" were destroyed exponentially at exactly the same rate, suggesting that the element on the phage particle, which is responsible for killing of the host cell upon adsorption onto it, is similar to, if not identical with, the colicin molecule. Both of these protein units are extremely susceptible to radiation-produced radicals, presumably because they are in direct contact with the aqueous phase. The same holds for free DNA units: DNA, in the form of transformation principle, is as susceptible to and as difficult to protect against inactivation by radicals as is colicin (Ephrussi-Taylor and Latarjet, 1955); whereas phage DNA, which is separated from the suspending medium by a protein coat, is easily protected.

Bachofer and Pottinger (1953, 1954a) have demonstrated that the effect of radicals on purified phage T1 varies with the salt concentration. The effect depends on the type of cation as well as anion and on pH; the "protecting" role of the salts is not well understood.

Freezing and drying are usually assumed to eliminate very efficiently the indirect effect (cf. Wood, 1954). However, Bachofer (1953) found that lyophilized and vacuum-dried T1 preparations differed in X-ray sensitivity; the former being about twice as sensitive as the latter. Bachofer *et al.* (1953), studying inactivation of phage T1 at temperatures ranging from  $-196$  to  $37^{\circ}\text{C}$ ., further observed a distinct decrease in sensitivity of the phage at low

temperatures; in fact, their data can be fitted to the common first order equation by introducing the absolute temperature,  $T$ , as variable; the equation then becomes:  $N/N_0 = e \exp. (-D(0.00027 T + 0.09))$ , where  $N/N_0$  is a fraction of phage surviving at the time when the dose  $D$  has been applied. The authors suggest that the temperature dependency may be due to residual indirect effects, and they proceed to show that estimates of target volume, based on measurements at room temperature, may be off by as much as a factor of 2. As discussed on page 365, these findings show that it is difficult to decide precisely where the indirect effect of X-rays ceases to play a role, and, consequently, that it remains somewhat uncertain what fraction of the total effect can be ascribed to ionizations in the target material.

Great efforts have been made to analyze the indirect effect chemically. A few aspects of these studies will be mentioned here: it is known from the work of Hollaender *et al.* (1951) that the presence of molecular oxygen during irradiation greatly enhances the bacterial effect of X-rays. Parallel observations have been made on tumor cells (Gray *et al.*, 1953). In contrast, several phage strains (the coliphages T1, T3, and S13) are more rapidly inactivated in the absence than in the presence of oxygen (Alper and Ebert, 1954; Ebert and Alper, 1954; Bachofer and Pottinger, 1954b). The conclusion drawn from this, and from irradiation experiments carried out in  $O_2$ ,  $N_2$ , or  $H_2$  atmospheres at various pH values (Alper and Ebert, 1954), is that *phage is inactivated by reducing rather than oxidizing radicals.*

Alper (1956) recently suggested that the interplay between direct and indirect action and protection might be interpreted in terms of target damage followed by more or less efficient *restoration*, rather than in terms of prevented or not prevented damage.

Various reaction sequences, starting from the primary reducing radicals H and OH, formed by ionizations in water, have been proposed to account for the inactivation of phage (Ebert and Alper, 1954), for the fragmentation of DNA in solution (Daniels *et al.*, 1953) and for the inactivation and denaturation of enzymes (Barron *et al.*, 1949). For some enzymes, two different reactions are postulated: a reversible inactivation of SH groups and, at higher doses, irreversible protein denaturation. No differentiation with respect to mechanism of inactivation has been made in the case of viruses, but it is known that, apart from the short-lived radicals just referred to, relatively stable, toxic components are produced that may interact with viruses introduced into preirradiated medium. In general, phage inactivated by direct effects, by short-lived radicals, and by stable, toxic agents, respectively, differ from each other in adsorption characteristics and in other respects as well (Watson, 1952).

Some interesting hints about the nature of the long-lived, toxic products come from studies of the radiomimetic effect of certain peroxides (Latarjet,

1956). Two compounds were used, cumene hydroperoxide and succinic peracid, which both inactivated the coliphages of the T series and phage  $\lambda$  (active on strain K12 of *E. coli*). Different resistance patterns were observed for peroxides and for X-rays, the small phages (T1, T3, and T7) being the most sensitive to peroxides but the most resistant to X-rays. The chemical complexity encountered in this field is borne out: (1) by the observation that, of the two peroxides tested, only succinic peracid inactivates free DNA (transforming principle); (2) by the occurrence of unpredictable interactions between the peroxide added and slight impurities in the phage preparations.

A striking feature of inactivation by reducing radicals was discovered by Alper (1952a,b) and termed "part-inactivation." After moderate X-ray irradiation, survivors of phage T3 or S13 show greatly increased sensitivity to the toxic components in preirradiated buffer: they have become *sensitized*, so to say. With S13, the ratio of sensitized to fully inactivated particles changed from 10 : 1 to 1 : 30 when increasing the dose from 1000 to 20,000 rad., and it could be shown that the fraction of particles not inactivated and *not sensitized* decreased exponentially (Alper, 1955). If this is taken to mean that a single event is sufficient to sensitize, inactivation, via sensitization, must be at least a two-step reaction. It has recently been found that X-rays sensitize phage T4 to inactivation by ascorbic acid; this radiation effect is mimicked by cumene hydroperoxide but *not* by succinic peracid (Maxwell, quoted by Latarjet, 1956).

The direct effect of X-rays is, by definition, the effect remaining when the indirect action has been effectively eliminated. Two main criteria have been used to evaluate how well this has been achieved: (1) that maximum protection has been reached; and (2) that inactivation is independent of dose rate (in Roentgens/min.).

The experiments of Luria and Exner (1941a, b) and Watson (1950) showed that, above a certain broth or peptone concentration, the rate of inactivation remained virtually constant, and it was concluded that all indirect effects had been eliminated. It is certainly true that most of the indirect effect can be eliminated in this way, but, as mentioned above, it is possible that a less significant class of indirect effects remains, which cannot be shielded against in solution (Latarjet and Frédéricq, 1955; Bachofer *et al.*, 1953).

The principle of *dose-rate independency* will be obeyed if nothing but direct, immediate, and irreversible inactivation occurs (Jordan, 1940; Lea, 1947). That it cannot be expected to apply to indirect effects is easy to see when considering that the *concentration* of radicals and toxic compounds attained during X-ray irradiation depends strongly on dose rate (Bonét-Maury and Lefort, 1950). Dose-rate independence was demonstrated in the phage experiments of Wollman *et al.* (1940) and in experiments with dry preparations of vaccinia virus and some phages carried out by Lea and co-workers (Lea, 1947).

When protected against indirect effects, viruses are, as a rule, inactivated exponentially by X-rays. This has been observed by Levin and Lominski (1936) for fowl plague virus, by Gowen and Lucas (1939) for vaccinia virus, by Friedewald and Anderson (1940) and by Syverton *et al.* (1941) for Shope's rabbit papilloma virus, by Wollman and Lacassagne (1940) for several phages, and Wollman *et al.* (1940) for phage C16.

Throughout this section, we deal with experiments carried out with "hard" X-rays (wavelengths  $<$  about 1 Å). The electrons produced by such radiation are fast, and the primary ionizations they give rise to may be considered to be *randomly distributed* in the specimen. This is the reason why the *volume* of a radiation-sensitive target determines the chance of its being hit and, therefore, the rate at which it is inactivated. In Section B, 2, *b*, we shall discuss experiments with other types of radiation that produce ionizations that are closely spaced along the track of the ionizing particles. The use of such radiation permits estimation of target *area*.

It was noted already by Wollman and Lacassagne (1940) that big phage particles are more sensitive than small ones, and the possibility of estimating virus size by means of X-ray inactivation has been thoroughly explored by Lea and co-workers. Lea (1947) states: "If the inactivating dose,  $D_0$ , were used to calculate the target volume (assuming spherical target) the diameter obtained would be within a factor two of the virus diameter for 20 out of 23 measurements (on 18 different viruses)." Some of these estimates of virus size and of  $D_0$  value are quite rough; Lea's analysis only goes to show that the ratio between the calculated target volume and the particle volume is usually between 1 : 1 and 1 : 10.

With the improved methods now available for measuring virus size (Williams, 1954), the rough estimates obtained by equating target and virus *volumes*, as suggested by Lea, are of little interest except, perhaps, in cases where purification and concentration of a small virus present great difficulties.<sup>1</sup>

An interesting series of studies has been reported by Lauffer and co-workers; these studies comprise inactivation experiments with phage T5, influenza viruses, and TMV:

Buzzell and Lauffer (1952) found that in "4 % broth" but *not* in "0.8 % broth," phage T5 was inactivated exponentially. In the first case, surviving particles and nonirradiated phage showed the same resistance to high temperature; whereas, in the second case, where indirect effects presumably played a role, the survivors were *more* sensitive to heat than normal phage. This apparently is another example of "sensitization" (cf. p. 369), and it constitutes an easily testable difference between phage particles inactivated by direct and by indirect X-ray effects, respectively.

<sup>1</sup> In such a case a better estimate would probably be obtained using  $\alpha$ -rays to estimate the target area (Lea, 1947; Bonét-Maury, 1948). See also Section II, B, 2, *b*.

For influenza viruses, Buzzell *et al.* (1955) also found exponential inactivation curves. Calculations indicated that infectivity may be associated with a single sensitive volume, about  $\frac{1}{8}$  that of the virus. (In the paper by Buzzell *et al.* (1956), Table II, this estimate was changed to  $\frac{1}{15}$ ; mainly because a different average value was assumed for the number of ionizations per cluster.) The authors also studied hemagglutination after irradiation of dried virus. They found an inactivation curve corresponding roughly to a "3-hit" phenomenon. The data did not permit a closer analysis of the mechanism of inactivation.

With TMV, irradiated in concentrated suspensions, autoprotection against radicals was observed by Lea *et al.* (1944). This was confirmed by Buzzell *et al.* (1956), who obtained indistinguishable inactivation curves in 0.8% and 25% broth, when the virus concentration was as high as 0.2% (by weight). As in the case of phage T5, survivors showed no change in heat resistance. On the usual assumption that one primary ionization in the target inactivates the particle, the  $D_0$  — dose of  $2.3 \times 10^5$  r gives a target volume which is almost equal to the RNA volume, as estimated by Hart (1955a). Moreover, according to radiation analysis by Pollard and Whitmore (1955), this target is asymmetric and about as long as the virus rod (see Section II, B, 2, *b*). This suggests a rod-shaped target about 40–50 Å in diameter and 3000 Å long, in very satisfactory agreement with present ideas about the genetic significance of the RNA (Gierer and Schramm, 1956) and its distribution along the axis of the virus rod (Hart, 1955b). The mean wavelength of the X-rays used was 0.2 Å; in a target of diameter 40–50 Å, most electrons produced none, and few more than *one* primary ionization while traversing the target. Altogether, it looks as if, in the case of TMV, primary ionizations that occur in the "genetic target" are 100% effective in causing inactivation. Furthermore, determinations of the viscosity of RNA prepared from irradiated TMV, suggested that *each target ionization produced one, randomly located, complete break of the RNA rod* (Lauffer *et al.*, 1956).

In this connection, multiplicity reactivation of phage inactivated by X-rays should be mentioned. Weigle and Bertani (1956) have shown that the absence of multiplicity reactivation under the usual conditions is due to X-ray-damaged phage not properly injecting its DNA into the cell after adsorption; presumably, because the inactivating ionization has caused rearrangements or breakage that interfere with the smooth passage of the DNA from phage to bacterium. If the inactivating X-ray treatment is applied immediately after injection into a bacterium of the DNA from 2 or more phage particles, powerful multiplicity reactivation is observed.

Of interest in connection with determinations of target volumes are the experiments of Adams and Pollard (1952), which show that, at temperatures above about 45°C., the estimates of the target volume of dried T1 increase

sharply. Pollard's interpretation of this temperature effect is that: ". . . transfer of energy through the virus to a sensitive part, or damage to a larger part of the virus, is facilitated when irradiating at high temperatures." As mentioned earlier in this section, close-range indirect effects may also be considered responsible. Pollard and co-workers have presented a number of other determinations of target volumes (Pollard, 1953, 1954); these will be discussed in Section II B, 2, b, together with attempts to estimate size and shape of the targets.

Epstein and Englander (1954) compared X-ray inactivation of the temperate phage  $\lambda$  and of a virulent mutant thereof, and concluded that the loss of ability to lysogenize associated with the mutation is accompanied by a small, but significant diminution of the target volume. As a supplement to the data previously compiled by Epstein (1953) they point out that the phages  $\lambda$  and T2 have the same DNA/target-volume ratio.

To conclude this section, we shall mention briefly that X- and  $\gamma$ -rays are being used in attempts to sterilize virus-containing material. General aspects of "electronic food sterilization" have been discussed by Nickerson *et al.* (1953). A series of animal viruses and phages have been subjected to the high intensity electron beam from a 3Mv "Capacitron"<sup>1</sup> it was found that the dose required to sterilize was inversely related to the size of the virus (Huber, 1952). Jordan and Kempe (1956) have used gamma rays from a cobalt-60 source to sterilize poliovirus-containing material (in these experiments, "sterilization" represented a reduction to roughly  $10^{-6}$  of the original as surviving particles). The main points of this investigation are that crude material requires more irradiation than semi-purified preparations, and that no antigenic change could be demonstrated after applying three times the "sterilizing dose."

*b.  $\alpha$ -Rays and Other Heavy-Particle Rays.* The potential usefulness of these densely ionizing radiations in virus research was discussed by Jordan (1940); Wollman *et al.* (1940) showed that, according to theory, the  $D_0$  (in Roentgens) for  $\alpha$ -rays was several times greater than for X-rays. Radon in solution was used to obtain uniform effect.

Lea and co-workers (1940-1944) proceeded to develop a practical procedure for drying viruses in thin films in order to permit the use of poorly penetrating radiations. In his classic monograph, Lea (1947) strongly emphasizes that the target theory requires the fulfillment of three different criteria: two of these have been discussed, i.e., exponential inactivation and dose-rate independence. The third demands that the efficiency per ionization (the ionic yield) decreases with increasing density of ionization along the track of the particle. If the average number of primary ionizations produced in the target by an

<sup>1</sup> Electronized Chemicals Corp., Brooklyn, N.Y.

ionizing particle is high, and if one is assumed to suffice to inactivate, only targets that are not hit by an ionizing particle will escape inactivation. Consequently, the smaller the *area* of the target, not the volume, the greater the chance of survival.

Formally, data obtained by irradiating dried viruses with  $\alpha$ -, X-, and  $\gamma$ -rays permit calculations to be made of area as well as volume. The interpretation of such calculations is simple if the data fit the assumption of a single spherical target (which usually they do not). In the case of vaccinia virus, the calculations suggested either a few hundred separate, spherical targets, all essential for reproduction, or a long, filamentous target (Lea and Salaman, 1942). To identify the many targets with as many genes, as was tentatively done, today appears highly speculative.

The case of TMV is a great deal more significant because agreement has been obtained between radiation analysis and independent chemical and morphological evidence (see p. 371). This success is probably due to the target being highly asymmetric and yet of simple cylindrical shape, and to the fact that, apparently, one primary ionization is sufficient to inactivate.

Structure analysis by means of ionizing radiation should be capable of yielding more than estimates of target volume and area. The ionization density along the track of a deuteron particle, for example, varies with its energy and, in principle, the thickness of the target, in the direction of the beam, can be estimated from the formal values for the target area obtained by varying the energy of the ionizing particles. For a detailed description of this experimental approach, see Pollard (1953).

A number of viruses have been analyzed by Pollard and co-workers, who have combined data from  $\alpha$ -particle or deuteron bombardment with X-ray data (Pollard and Forro, 1951; Pollard and Whitmore, 1955; Pollard and Kraft, 1955; Pollard and Setlow, 1956; Till and Pollard, 1956). It is difficult to evaluate the interpretations, some of which are illustrated by Pollard (1953) in the form of models depicting the possible distribution of different kinds of sensitive material within different virus particles. At the time of writing, it would appear that more pertinent information about viruses is being obtained from chemical and genetic studies, tracer experiments, and autoradiographic work. In the future, some of the models may derive new significance by being checked against independent evidence about the structure of these viruses; in this respect, the case of TMV, discussed above, is encouraging.

Very soft X-rays of limited penetration have been used in experiments which suggest that the sensitive material of phage T1 (presumably its DNA) is protected by a layer about 250 Å thick (Davis, 1954). This value seems very high considering that the diameter of T1 is not much over 500 Å. Guild (1952) attempted to use soft X-rays for analytical work and demonstrated that

energy absorption by P atoms was not significantly more effective in inactivating phage T1 than was absorption by any other atom.

### 3. Nonionizing Radiation

*a. Far UV Light (Wavelength below 3000 Å).* It has been mentioned that, in contrast to X-rays, UV inactivates by direct effects only. This was demonstrated by McKinley *et al.* (1926), who showed that phage, herpes, and Levaditi viruses, as well as bacteria, could be killed by direct irradiation but not by being introduced into preirradiated medium. Inactivation by UV is characteristically exponential. This was observed by Fisher and McKinley (1927) and Baker and Nanavutty (1929) for phages, and has later been confirmed for a variety of viruses by Bawden and Kleczkowski (1953) for small plant viruses, and by Fogh (1955), Dulbecco and Vogt (1955), and Stanley *et al.* (1956) for polioviruses. At low survival values the rate of inactivation is sometimes observed to decrease; this may be due to the presence in the virus population of a few relatively resistant particles.

The  $D_0$  dose (in ergs/mm.<sup>2</sup>) for inactivation by UV is usually found to be independent of dose rate. A departure from this rule was observed by Eckart (1954), who found that the  $D_0$  dose for inactivation of phage T1 by monochromatic UV increased slightly with increasing dose rate (i.e., low dose rates were most efficient). This effect has not been observed by others and its nature is obscure. Another abnormality connected with dose rate was noticed by Latarjet and Morene (1951): in experiments with phage T2 irradiated with UV of very low intensity, a deviation from exponential inactivation was observed which suggested a "3-hit" process. It is difficult to say what this means; published inactivation curves for T2 irradiated at higher intensities do not deviate nearly as much from exponentiality.

The rate of inactivation by UV is usually a stable and characteristic property of a virus, with the possible exception of host-cell-induced modifications. Salk *et al.* (1940) claimed that influenza virus harvested from the lungs of infected mice was more susceptible to UV than virus from tissue cultures, and the sensitivity of certain actinophages was found to depend on the strain on which the phage was propagated (Welseh and Minon, 1955b).

All speculations about the mechanism of UV inactivation are based on analysis of the relative efficiency of different wavelengths. The  $D_0$  dose determined by irradiation with monochromatic UV is a convenient expression of the efficiency at the wavelength ( $\lambda$ ) chosen. A plot of  $1/D_0$  against  $\lambda$  gives a curve somewhat resembling an absorption spectrum; i.e., the curve has maxima corresponding to the most effective  $\lambda$  values and vice versa. "Action spectra" of this type were determined for staphylococci and phages active on this organism, as well as for vaccinia virus (Rivers and Gates, 1928; Sturm *et al.*, 1932; Gates, 1930, 1934). Close parallelism could be demonstrated

between the different curves, which all showed a maximum near 2600 Å and more or less pronounced minima between 2300 and 2400 Å.

Very similar action spectra have later been established for influenza virus (Hollaender and Oliphant, 1944), for the coliphages T1 and T2 (Fluke and Pollard, 1949; Zelle and Hollaender, 1954), and for a megatherium phage (Franklin *et al.*, 1953).

There are, however, exceptions to this rule. Duggar and Hollaender (1934a,b) and Hollaender and Duggar (1936) showed that TMV was most efficiently killed by the shortest wavelengths used (2200 Å) and that the efficiency decreased sharply with increasing wavelength, except for a possible minor peak between 2500 and 2600 Å. Rous' sarcoma virus was found later to exhibit the same unusual pattern (Hollaender and Oliphant, 1944).

With these two exceptions, the known action spectra are characterized by a maximum which more or less coincides with the absorption maximum for nucleic acids or nucleoproteins, at about 2600 Å; a strong suggestion that, in the range between 2000 and 3000 Å, the principal photo-labile component of a virus particle is its nucleic acid. This important point has been tested by studying the quantum efficiency or quantum yield,  $\phi$ , of the inactivation process at different wavelengths. The quantity  $\phi$  may be defined as the number of virus particles inactivated per quantum absorbed in still viable particles.<sup>1</sup>

Oster and McLaren (1950) found that TMV is inactivated exponentially by UV and that the quantum yield, for  $\lambda = 2537$  Å, is about  $4 \times 10^{-5}$ . This means that, out of 23,000 absorbed quanta, only one is effective, and that this one alone is responsible for inactivation. The ionic yield for simple

<sup>1</sup> In a primary ionization the locally released energy is of the order of 100 ev and the killing efficiency of such an event is, as we have seen, high. The UV quanta responsible for inactivation have energies ranging from about 3.8 to 5.5 ev. *A priori*, nothing can be said about *their* efficiency. To estimate this, Zelle and Hollaender (1954) used the expression  $\phi = 1/(D_0 A_p)$ , where  $A_p$  is the absorption coefficient of the phage (in  $\text{cm}^2$  particle).  $D_0$  can be determined with as much precision as needed if monochromatic UV of sufficient intensity is available, whereas reliable estimates of  $A_p$  are difficult to obtain. In the first place, the total number of virus particles per milliliter, not only the number of viable particles, should be known; secondly, purification of the virus must be such that impurities do not contribute significantly to the absorption as read in the spectrophotometer; and, finally, correction for scattering must in some way be made.

Luria *et al.* (1951) found that the number of viable, or plaque-forming T2 particles, varied between 40 and 100 % of the total number, as determined by direct counts on electron micrographs (using the method of Backus and Williams, 1950). On the average, 50-60 % of the counted particles formed plaques. In the case of TMV and the coliphages, for which  $\phi$  has been calculated, adequate purification is possible. Correction for scattering is more problematic, since it rests on the validity of an extrapolation to the UV region from readings made in the range between 3200 and 4000 Å, in which virtually no absorption takes place (Luria *et al.*, 1951; Zelle and Hollaender, 1954).

reactions, such as rupture of peptide, disulfide, or pyrimidine bonds is of the order of  $10^{-2}$ , and it was suggested that, perhaps, the TMV particles contained about 50 critical bonds, the rupture of one of which by absorption of a quantum of UV causes inactivation. Since the action spectrum of TMV does *not* particularly point to the nucleic acid as the principal photosensitive element, it is difficult to guess what type of bond might be involved. Oster and McLaren summarized their discussion as follows: ". . . these approximate calculations simply serve to show that the quantum efficiency for destruction of nucleic acids as well as for protein linkages is much higher than for inactivation of viruses. The nucleic acid protein framework of the virus apparently serves to hold together the radicals formed by quanta and thus allows for a high probability of recombination without net chemical change and with an over-all low quantum efficiency."

In this connection it is perhaps significant that *infective* RNA prepared from TMV is about six times as sensitive to UV as the RNA protein complex of the intact virus particle (McLaren and Takahashi, 1957).

The coliphages T1 and T2 were thoroughly studied by Zelle and Hollaender (1954). For both phages, the action spectrum was determined and the quantum yield calculated for 10 different wavelengths in the interval between 2200 and 3000 Å. Over this whole range reasonably constant values of about 6 and  $3 \times 10^{-4}$  were obtained for T1 and T2, respectively. Fluke (1956) carried out similar experiments with dried T1. His estimate of the quantum yield was  $3 \times 10^{-4}$ , but his action spectrum (Fluke and Pollard, 1949) differs from that of Zelle and Hollaender for the shortest wavelengths and, in that region, the quantum yield therefore does not remain constant. Zelle and Hollaender (1954) suggest that nonspecific absorption by protein contained in the dry film perhaps accounts for this discrepancy.

The more or less constant quantum yield registered in the 2000 to 3000 Å region indicates that inactivation of T1 and T2 phages is due to UV absorption in a particular substance; and, as mentioned, the action spectrum already strongly suggested that this substance is the virus nucleic acid. The theory of the action spectrum and the assumptions involved in interpreting data of this kind have been detailed in a review by Loofbourow (1948).

Bawden and Kleczkowski (1955) have remarked that the rule of McLaren (1949), that the quantum yields for proteins are approximately inversely proportional to the molecular weight, does not apply to TMV, which is too *sensitive* according to this rule. They ascribe the relatively high sensitivity to the presence of nucleic acid in the virus. For phage T2 the deviation from McLaren's rule is much more striking: the quantum yield is 10 times that of TMV and, at the same time, T2 is bigger than TMV. In T2, it is reasonably certain that it is the nucleic acid that determines the sensitivity to UV.

The discoveries in 1947 and 1949 of multiplicity reactivation and photo-reactivation, two phenomena associated with UV inactivation, greatly influenced the direction of UV research. These processes will be treated in detail in another chapter, but should be discussed briefly here because they reveal something about the state of UV-inactivated particles:

*Multiplicity reactivation* (Luria, 1947; Luria and Dulbecco, 1949; Dulbecco, 1952) occurs when two or more UV-damaged phage particles infect the same cell. Under these conditions the chance of that cell producing normal phage is much higher than one would expect on the basis of the viability of the individual infecting particles. *Photoreactivation* of viruses (Dulbecco, 1949, 1950) is observed when a cell has been infected with a UV-damaged particle and subsequently is exposed to visible light. The action spectrum for the reactivation process has its maximum at about 3500 Å; wavelengths under 3100 or above 4500 Å are almost ineffective.

These reactivation phenomena show that the structural changes caused by moderate doses of UV are remarkably stable; thus, a UV-irradiated phage suspension not only retains its survival level unchanged almost indefinitely, but, what is more, the response to reactivating light remains unchanged. Quantitative experiments have shown that a well-defined fraction of the UV damage can be reversed by light, and that a single quantum suffices to return the damaged site to a functional condition (Bowen, 1953). Furthermore, photoreactivated phage-bacterium complexes were found to be sensitive to a second dose of UV to the same extent as nonreactivated complexes (at identical survival levels); this suggests that, once reactivated, a complex is as good as new (Lennox *et al.*, 1954).

Several other phages and a number of plant viruses have been tested for reactivation after UV inactivation. Fluke (1951) tested phage T1 inactivated by UV of different wavelengths, and found that a constant fraction (about 70 %) of the inactivated phage could be reactivated by light. This high degree of photo-reactivability seems to disappear if T1 is irradiated with UV in the dry state (Hill and Rossi, 1952). The dry-irradiated phage was found to adsorb normally onto bacteria and the absence of photo-reactivation has not been explained (Hill and Rossi, 1954). Other coliphages tested are less reactivable than T1; the closely related phages T2, T4, and T6 differ significantly in reactivability. Price (1950) and Kleczkowski and Kleczkowski (1953), working with staphylococcus and *Rhizobium* phages, respectively, observed photo-reactivation but failed to elicit multiplicity reactivation. The reason for this failure is not known; but, considering that two or more particles must reproduce simultaneously in the same cell for reactivation to occur, exclusion phenomena, preventing entry of a second particle, should be considered.

Bawden and Kleczkowski (1953, 1955) demonstrated photo-reactivation in bushy stunt, tobacco necrosis, and, particularly strongly, in potato X virus. Five strains of TMV gave consistently negative results.

Along with theoretical studies a considerable amount of work has been done to test the usefulness of UV as a bactericidal and virus inactivating agent, e.g., in barracks and laboratories. Thus, Wells and Brown (1936), demonstrated the effects of UV on air-borne influenza virus and extensive studies of a similar kind are reported by Edwards *et al.* (1944). Inactivation by UV has also been considered in connection with vaccine production; e.g., by Levinson *et al.* (1945) for rabies vaccine, and by Taylor *et al.* (1957) for polio vaccine. In the latter case, safe and highly antigenic preparations are said to have been obtained by UV treatment and subsequent storage for a week at 37–40°C. Another combined treatment, with UV and  $\beta$ -propio-lactate, was suggested by Smolenz and Stokes (1954) for sterilizing hepatitis-contaminated sera.

Like X-rays, UV has been used to test for inactivation of properties other than infectivity. Salk *et al.* (1940) found that the antigenicity of UV-inactivated influenza virus was considerably reduced. Stanley (1945), in studies of the UV inactivation of purified PR8 influenza virus, noted that the loss of virus infectivity greatly preceded that of red cell agglutinating activity and that a fully potent non-infectious vaccine could be produced by using an amount of irradiation sufficient to cause loss of infectivity, but insufficient to cause a marked decrease in red cell agglutinating activity. A more thorough study of influenza viruses was made by Henle and Henle (1947). The properties examined were affected in this order: (1) infectivity; (2) toxicity to mice; (3) interfering property and inhibition of the development of the chick embryo; (4) hemagglutinating capacity (including the adsorption-elution mechanism, the ability to block red cell agglutination, and the adsorption onto allantoic cells); and (5) complement-fixing capacity. The immunizing capacity could not be definitely placed according to sensitivity to UV; certain differences between A and B strains were noticed, but, generally, antigenicity was at least as stable as the hemagglutinating capacity.

The action spectrum for the destruction of the interfering property of influenza virus has been established by Powell and Setlow (1956) and compared with the spectrum for the loss of infectivity. The spectrum for destruction of interference was found to have a broad maximum, extending to about 2800 Å, as compared with the usual, well-defined maximum at 2600 Å in the action spectrum for inactivation. This was taken to mean that, probably, absorption of UV by protein plays a significant role in destroying the interfering property.

Interference between plant viruses after UV treatment was demonstrated by Bawden and Kleczkowski (1953) and heat inactivation of preirradiated

TMV was studied by Kleczkowski (1954). The rate constant for heat inactivation was found to increase with preirradiation, but the process was first order also for suboptimal doses of UV. As pointed out by Kleczkowski, this suggests that the "sensitization to heat inactivation" is a multi-hit phenomenon. Similar sensitization was observed with a *Rhizobium* phage (Kleczkowski and Kleczkowski, 1953) (compare discussion of the indirect effect of X-rays, p. 367-69).

The rate of adsorption and the bacteria-killing ability of phage T6 remain unchanged after extensive UV inactivation (Frédéricq, 1952); the enzymatic activity of certain phages on the Vi substance is also very resistant to UV (Kozinski and Opara, 1955). It is therefore reasonable to assume that these phages are inactivated through absorption of UV in their nucleic acid moiety before the surface properties are significantly damaged.

*b. Near UV and Visible Light* (Wavelength above 3000 Å). In a system composed of virus particles and a suitable dye, light which is absorbed by the dye may inactivate the virus. This photodynamic effect is quite different from inactivation by direct absorption by the virus of UV, and is in some respects similar to inactivation by radicals formed by ionizing radiation.

Schultz and Kreuger (1928), Clifton and Lawler (1930) found that methylene and toluidine blue—but none of a long series of other dyes—might be toxic to phage particles. Further studies by Clifton (1931) revealed that oxygen is essential for this type of inactivation and that cystine, in low concentration, protects the virus. Divalent cations, which have a general stabilizing effect on many phages, also increase their resistance to the photodynamic effect (Burnet and McKie, 1930). Clifton concluded that the phage was inactivated by oxidation caused by photo-sensitized dye in the presence of oxygen. This theory has been further elaborated but not changed by later observations.

Photodynamic inactivation of a variety of animal viruses, as mediated by methylene blue, was studied by Perdrau and Todd (1933). The viruses of vaccinia, herpes, fowl plague, louping-ill, Borna disease, Fujinami's tumor, and canine distemper were found to be very sensitive, those of foot-and-mouth disease and ectromelia much less. In suspensions containing living cells some viruses were protected, others not.

The photodynamic inactivation of TMV in the presence of acriflavine was studied quantitatively by Oster and McLaren (1950). At low salt concentration, about 160 dye molecules were found to absorb to each TMV particle and exponential inactivation was observed upon illumination. At higher salt concentration ( $> 0.1 M$  NaCl) the virus-dye complexes were partly dissociated, with the result that the rate of inactivation dropped, although the inactivating blue light was absorbed to the same extent. Oxygen was found to enhance the effect greatly but some inactivation occurred even in a  $N_2$  atmosphere.

A second detailed study was made by Welsch and Adams (1954) with the coliphages of the T series and methylene blue. Inactivation kinetics were first order (with a short lag) and the rate constants varied with pH and with the concentration of the dye, both factors probably affecting adsorption of the dye to the phage surface. A 20-fold difference was observed between the highest and the lowest inactivation rate, and, as observed earlier by Burnet (1933) for dysentery phages, the sensitivity to the photodynamic effect varied considerably more between than within serological groups. There was no obvious correlation between sensitivity and morphological and physiological characteristics, and all the phages tested were more or less equally sensitive to hydrogen peroxide. The authors concluded that, most likely, adsorption of the dye to the phage surface was the rate-limiting factor.

Testing the effect of eosin on Newcastle virus, Torlone (1955) found that, upon illumination in the presence of oxygen, infectivity disappeared before the hemagglutinating activity. Without oxygen no inactivation was observed.

The fact that normal daylight may inactivate viruses is of considerable practical interest. Skinner and Bradish (1954) investigated the susceptibility of several animal viruses to light as a function of the intensity and duration of illumination and the composition of the suspending medium. Unfiltered fresh suspensions of egg-grown strains of the viruses of vesicular stomatitis, influenza, Newcastle disease, and fowl plague were strongly inactivated by exposure to daylight for 4 hours; foot-and-mouth disease virus was much more stable. The authors did not analyze the inactivation process, except to show that UV probably was not responsible. Some viruses were protected by the addition of 10 % rabbit serum; in experiments with Newcastle virus, cysteine was tried but no protection was observed (cf. Clifton, 1931).

Finally, the phages C16 and S13 have been shown to be slowly inactivated by near UV and by blue light in the absence of added dye (Latarjet and Wahl, 1945; Wahl and Latarjet, 1947). Unlike the photodynamic effect, this inactivation process was almost independent of temperature (between 17 and 37°C.). The influence of oxygen was not tested. In the far UV region, the rate of inactivation of phage C16 was 3 times that of phage S13; in the near UV region and with visible light, C16 was twice as resistant as S13, indicating that the mechanism of inactivation is probably different in the different wavelength regions.

#### *4. Inactivation Due to Decay of Incorporated Radioactive Phosphorus*

These very special experiments are most naturally treated at this point because they bear some resemblance to both X-ray and UV experiments: first, because the decay process, by which the  $P^{32}$  atom is changed into a sulfur atom, involves the ejection of a fast electron and the *local* release of considerable energy from the recoiling atomic nucleus; second, because it has been

shown that inactivation through  $P^{32}$  decay, like UV inactivation, is a result of damage to the nucleic acid portion of the virus particles.

The ejected electron has a mean energy of 0.7 Mev and behaves like the electrons produced by hard X-rays or  $\gamma$ -rays; i.e., it produces primary ionizations which, to begin with, are several thousand Å apart. The mean energy of the recoiling nucleus, of about 20 ev, may cause disruption of the polynucleotide chain in which the  $P^{32}$  atom formed a link, but, in most cases, the recoil effect would seem to remain confined to one chain (Stent and Fuerst, 1955).

The first experiments of this type were carried out with the phage T2 by Hershey *et al.* (1951). The following important conclusions could be drawn: (1) the instability of labeled phage particles is due to the nuclear transmutation and/or the recoil effect; only a small percentage of the total effect is accounted for by ionizations, very few of which occur in the virus particle; (2) inactivation is strictly exponential, with a rate proportional to the specific activity; and (3) *only one out of about 10  $P^{32}$  disintegrations causes inactivation.*

These findings could mean that not more than about 10 % of the DNA of phage T2 is essential for reproduction and that a single decay process occurring in this part causes inactivation. This target type interpretation is ruled out, however, because it has been shown that inactivation depends on temperature. Stent (1953) and Stent and Fuerst (1955) determined the rates of inactivation of  $P^{32}$ -labeled phages at 4° and at -196°C., respectively. For the coliphages T1, T2, T3, T5, and T7, and for phage  $\lambda$  they consistently found a ratio of about 0.6 between the inactivation rates observed at -196° and 4°C., respectively. The constancy of the temperature effect suggests that the mechanism of inactivation is the same for all the phages studied. It is perhaps significant that a temperature effect of the same magnitude was found for inactivation of phage T1 by X-rays (Bachofer *et al.*, 1953; see also p. 367).

In agreement with these experiments, Castagnoli and Graziosi (1954) have shown that a megatherium phage labeled with  $P^{32}$  was inactivated more slowly at -79°C than at 4°C.

Garen and Zinder (1955) studied the phage P22, derived from *Salmonella typhimurium*. The transducing capacity of this phage was much more resistant to the inactivating effects of both UV and incorporated  $P^{32}$  than were the lytic and lysogenic activities. The fraction of the sensitive material (DNA) engaged in transduction would thus seem to be small compared to that which is necessary for reproduction or lysogenization. The relative sensitivity to X-rays and to UV was found to differ for several properties of the phages P22 and T2; in all instances inactivation due to incorporated  $P^{32}$  gave results similar to those obtained with X-ray inactivation. These experiments support the idea that the damage caused by decay of incorporated  $P^{32}$  is similar to that produced by X-rays, and that it resembles the effect of UV only insofar as both are confined to the nucleic acid structures of the virus.

Stent (1953) and Stahl (1956) have actually shown that inactivation of a phage particle by  $P^{32}$  decay can destroy any one of a large number of genetic loci without affecting others. These experiments and a number of interesting applications of the  $P^{32}$  technique to the study of phage reproduction will be discussed in another chapter.

### III. PHYSICOCHEMICAL FACTORS

O. MAALØE

The description of a virus is not very useful or complete unless its stability under different conditions is included. In this section we describe the most important physicochemical factors to be considered when handling a virus in the laboratory; i.e., the resistance of the virus to high and low temperature, to desiccation, and to changes in pH and, last but not least, the importance of the ionic composition of storage medium or of the medium in which the virus is made to react with other agents.

#### A. Heat Inactivation

Heat inactivation of viruses may be discussed from two main viewpoints: First, it is an effective method for sterilization and, when a proper balance can be struck between destruction of infectivity and preservation of useful qualities of the preparation, heat treatment is convenient and cheap. Second, studies of the temperature sensitivity is a natural step in the analysis of the physicochemical properties of the virus. As usual, any of a number of properties of the virus can be chosen as index of inactivation.

##### 1. Sterilization

Sterilization of virus-containing material is a problem of growing concern: in the dairy and fermentation industries it may be a problem to get rid of phages, especially such as infect lactic acid bacteria; and, in vaccine production and in the manufacture of dairy products and certain pharmaceutical products, it is essential to safeguard against contamination with pathogenic viruses. In all such cases heat inactivation may be tried, alone or combined with other treatments.

A large number of streptococcus phages have been studied with the idea of establishing practically useful "thermal death points" (Whitehead and Hunter, 1937). The results of such tests are often reported in such terms as: at pH 7, the activity of most phages was destroyed in 15 minutes at  $70^{\circ}\text{C}$ .; in one case, it was destroyed in 5 minutes at  $65^{\circ}\text{C}$ . (Nelson *et al.*, 1939). Like their host organisms, streptococcus phages differ widely in heat resistance, and some members of this group of phages will resist  $82^{\circ}\text{C}$ . for at least 5

minutes (Mazé, 1946). The rate of inactivation of streptococcus phages has not been studied directly, but Nichols and Wolf (1945) report that most of the 26 strains tested at a concentration of  $10^6$  viable particles/ml. were not completely killed in 15 minutes at  $70^\circ\text{C}$ .

Yakovlev (1939) draws attention to the fact that big volumes are harder to sterilize than small. (This results because, first, it takes time for a big volume to reach the desired temperature; and second, the more particles originally present (i.e., the bigger the volume), the more survivors will be found after a given time of heating. This is common sense, and is mentioned only to emphasize how inadequate are statements in terms of thermal deathpoints. In this connection it should also be recalled that, although inactivation usually proceeds exponentially until few infective particles are left, these few are frequently much more resistant than the majority (Kaplan, 1958).

According to Prouty (1948), the heat resistance of most streptococcus phages is such that normal commercial pasteurization is inadequate to prevent phages entering dairy manufacturing establishments with contaminated farmers' milk. A recent survey of the situation from this point of view was published by Wilkowski *et al.* (1954).

In certain media sterilization may be particularly difficult, as shown by Whitehead (1944), who found that, in cream with 50 % fat, heating to  $100^\circ\text{C}$ . with live steam was necessary to inactivate streptococcus phages. Such special conditions of protection may also be a medical concern; thus, Kaplan and Melnick (1954) have shown that, in ice cream, poliovirus is considerably more resistant than in aqueous suspension. In their experiments, all 3 polio strains retained some infectivity for cotton rats after being heated to  $80^\circ\text{C}$ . in cream or in ice cream.

The heat resistance of polioviruses in tissue culture filtrate has been studied by Lépine and Nantel (1951). Their suggestion of a simple relation between temperature and the time of heating necessary to obtain sterility is, however, based on very hazardous extrapolations. Also, dried preparations of polioviruses have been tested for their heat resistance. Kraft and Pollard (1954) have demonstrated that, at temperatures between  $40$  and  $60^\circ\text{C}$ . the rate of inactivation decreases considerably with time, which shows that, in the dry state, some particles are highly resistant. It is not known whether the resistant particles differ genetically from the more sensitive.

In vaccine production, sterility must be obtained without destroying antigenicity and, since effective heat inactivation usually involves a certain degree of protein denaturation combined treatments are commonly used. Thus, Salk (1946a,b) has shown that low concentrations of formalin considerably increase the heat resistance of the hemagglutinating activity of influenza virus. The usefulness of such "fixation phenomena" will be discussed in detail in Section IV (inactivation by chemical agents).

## 2. Theoretical Aspects

Heat inactivation of viruses may also be studied from theoretical viewpoints. The aim has frequently been to characterize the process in terms of the energy changes involved; e.g., the activation energy,  $\Delta H$ , as defined by Arrhenius, or the heat and entropy of activation,  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$ , derived from the theory of absolute reaction rates (Stearn, 1949).

It has been pointed out (Pollard, 1953; Woese, 1956) that the precise meaning of these parameters is not too clear when dealing with large and complex units like virus particles. However, the fact remains that data obtained by studying thermal inactivation of a virus at different temperatures frequently lend themselves to calculations of internally consistent values for  $\Delta H$  or for  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$ . This shows (1) that, at least to a first approximation, the virus particles are inactivated according to first-order kinetics; and (2) that the rate constant is frequently found to depend on temperature in a simple manner.<sup>1</sup>

We shall now review some of the experimental evidence upon which such calculations have been based and, in particular, we shall consider the dependence of the inactivation rate on factors such as ionic environment and hydration.

Nanavutty (1930) exposed coliphages to temperatures around 50°C. and observed that the loss of infectivity was strictly exponential except for a "tailing-off" effect at the level of  $10^{-4}$  survivors. The rate constant characterizing such an inactivating process naturally depends on environmental conditions. A striking example was furnished by Burnet and McKie (1930), who studied the effect of heating different dysentery phages to 60°C. for one hour in solutions containing NaCl and CaCl<sub>2</sub> in varying concentrations. The contour maps constructed from the data obtained in this way are very revealing and show that, for each phage, maximum survival was obtained when the sodium and calcium ion concentrations were balanced in a characteristic manner. Departure in either direction from the balance greatly reduced the survival (from a maximum of 40% to less than 0.1%). The protecting effect observed when broth was added to suspensions of phage in NaCl solutions was ascribed partly to the calcium and magnesium ions and partly to reducing components and colloidal material contained in the broth. The authors

<sup>1</sup> The rate constant at the absolute temperature  $T$ ,  $k(T)$ , is estimated from the familiar first-order equation,  $N/N_0 = e \exp(-k(T)t)$ , where  $N/N_0$  is the fraction of virus remaining active after exposure to the temperature  $T$  for  $t$  seconds. According to Arrhenius,  $\log k(T) = C_1 - \Delta H/RT$ , where  $C_1$  is a constant and  $R$  the gas constant;  $\log k(T)$  plotted against  $1/T$  should therefore give a straight line with slope  $-\Delta H/R$ . The theory of absolute reaction rates requires that  $\log k(T) \simeq C_2 + \Delta S^\ddagger/R - \Delta H^\ddagger/RT$ , where  $C_2$  is approximately constant in the temperature intervals usually employed; as before,  $\log k(T)$  plotted against  $1/T$  should therefore give a straight line from which estimates of  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$  can be obtained.

stressed that the resistance observed in an optimally balanced salt solution was at least as high as in media to which has been added broth or similar "protecting" substances.

A particularly clear case of salt effect was later studied by Adams (1949a), who found that the coliphage T5 is inactivated quite rapidly even at 37°C. in a medium containing 0.1 *M* NaCl and less than  $10^{-5}$  *M* CaCl<sub>2</sub>. Raising the calcium ion concentrations to  $10^{-3}$  *M* reduced the rate of thermal inactivation by a factor of a million! The same effect could be produced with an even sharper transition by raising the NaCl concentration from 0.1 *M* to molar. Adams concluded that the phage was stabilized by complexing with the metal ions (see Section III, D, 2). Adams and Lark (1950) have shown that T5 mutants exist which do not require this kind of stabilization; such mutant phage stocks are as resistant in the absence as in the presence of calcium ions. This is probably the only case in which genetic heterogeneity in a virus population with respect to temperature sensitivity has been unequivocally demonstrated.

From inactivation rates estimated in the temperature interval between 51 and 62°C., Krueger (1932) estimated the activation energy,  $\Delta H$ , for staphylococcus phages to be about 100,000 cal./mole, i.e., within the range commonly found for protein denaturation. Similar and higher values were published later, by Price (1940) for four plant viruses, by Bourdillon (1944) for poliovirus, by Pollard and Reaume (1951) for the coliphages T1, T2, T3, T4, and T5, and by Welsch and Minon (1955a) for actino phages.

In some cases it may be found that a single value for the heat of activation does not suffice to describe the experimental data. For TMV, Price (1940) found 55,000 cal./mole in the interval between 68 and 83°C. and 195,000 cal./mole between 84 and 95°C.; this situation, it has turned out, is not uncommon. It is usually interpreted to mean that different inactivation processes are rate-limiting in the different temperature intervals. Suggestive data of this type have been obtained by Cherry and Watson (1949) for a streptococcus phage, by Chang *et al.* (1950) for a coliphage, and by Bachrach *et al.* (1957) for foot-and-mouth disease virus. For tobacco ringspot virus, Price (1940) also found two values but, in this case  $\Delta H$  was significantly *smaller* in the high than in the low temperature range; a simple interpretation in terms of two independent inactivation processes does not suffice to explain this situation. Even more complex are the data obtained by Kaplan (1958) for vaccinia virus; in this case, the change in rate of inactivation in the interval between 50 and 60°C. suggested a gradual *decrease* in activation energy. Finally, it should be noted that single, well-defined, but remarkably low  $\Delta H$  values have been found for tobacco necrosis virus (37,000 cal./mole; Price, 1940), for influenza virus (34,000 cal./mole; Lauffer *et al.*, 1948), and for Theiler's virus strain FA (34,500 cal./mole; Leyon, 1951).

Such low activation energies are usually only found for dried viruses: for TMV, Price (1940) obtained a  $\Delta H$  value of about 24,000 cal./mole, as opposed to the high values obtained for wet virus (see above); and Pollard and Reaume (1951) found even lower values for the phages T1, T3, and T7. The  $\Delta S^\ddagger$  values, which in wet preparations run between 100 and 1000, had dropped to around zero. As pointed out by Pollard (1953), the entropy of activation, in the wet as well as in the dry state, is not correlated with the size of the virus; this suggests that inactivation involves changes in small regions rather than uniform denaturation of the virus protein.

For heat denaturation (insolubilization) of TMV protein, at a given virus concentration and pH, Lauffer and Price (1940) found a single, well-characterized  $\Delta H$  value. Boyd and Eberl (1948) attempted to interpret the data of Lauffer and Price in terms of the number of H bonds which may have to be broken simultaneously to initiate denaturation; their figure is 25-30 out of more than 10,000. Normally, protein and RNA become separated when TMV is heated but, at very low salt concentrations, the virus rod is transformed into a ball in which the RNA is trapped (Hart, 1956b). This transformation seems to require an activation energy similar to the one found for denaturation of TMV protein.

As in radiation experiments, the hemagglutinating activity is much more resistant than infectivity. The activation energy is of the order of 100,000 cal./mole, but the reaction rate was found to depend in a complicated manner on the initial virus concentration (Lauffer and Carnelly, 1945; Lauffer and Scott, 1946; Scott and Lauffer, 1946a). Similar high values have been reported by Woese (1956) for the heat of activation of a number of influenza A strains, for one B strain, and for Newcastle, mumps, and swine influenza viruses. The A strains were characterized by identical high  $\Delta H^\ddagger$  values (170,000 cal./mole); for the other viruses, the values ranged between 100,000 and 340,000 cal./mole.

Pollard and Setlow (1953) followed the loss of the ability of phage T1 to combine with antibody. In the wet as well as in the dry state, the  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$  values were considerably higher than had previously been measured for inactivation. The low  $\Delta S^\ddagger$  values, characteristic of dry preparations, suggest that inactivation may occur without profound denaturation and therefore without loss of serological affinity and antigenicity (Pollard and Reaume, 1951).

A few observations suggest that heat treatment of a virus may sensitize it in a manner analogous to what was found in X-ray and UV experiments. Thus, Smith and Kreuger (1952a,b) have reported that a vibrio phage, when heated for a short time, becomes permanently sensitive to cold shock; Hart (1955b) has shown that heating in the presence of small amounts of the detergent Duponol C sensitized TMV to the action of RNAase. Subsequent inactivation occurs without removal of measurable amounts of RNA.

To conclude this section we want to draw attention to studies of the thermal denaturation of the complexes formed between TMV or influenza virus and urea (Stanley and Lauffer, 1939; Scott and Lauffer, 1946b), and to the studies of the thermal denaturation of different viruses at high pressure (see p. 364). These theoretically interesting investigations cannot be discussed in detail here.

### *B. Resistance to Low Temperature and to Desiccation*

In practice, the stability of viruses at low temperature becomes a problem in connection with longtime storage in the frozen or freeze-dried form. We shall therefore treat freezing and drying in direct succession and do so mainly by referring the reader to manuals and to a few pertinent publications.

#### *1. Freezing and Thawing*

The conditions prevailing in a frozen suspension of biological material depend on the way in which cooling is carried out, on the temperature at which it is held, and on the nature and concentration of solutes in the medium at the time of freezing. The physical principles of ice formation at different temperatures and of thawing, with special reference to biological material, have been reviewed by Meryman (1956). Most of our knowledge about the biological effects of freezing and thawing concerns animal cells and bacteria; relatively few viruses have been directly tested for resistance to repeated freezing and thawing.

From the studies of heat inactivation we know that measurable rates of inactivation are usually not encountered until the temperature is raised to about 40–50°C. In a neutral medium most viruses should therefore be extremely stable at temperatures near zero. From studies on cells and bacteria it would seem that damage caused by freezing, storage in the cold, and thawing is a result either of the high salt concentration created when most of the water has crystallized out, or of enzymatic activities which may continue at significant rates if the storage temperature is not too low. The salt effect is difficult to study and to control, since inactivation may depend on transient conditions prevailing only during freezing or thawing; such effects are probably best counteracted by adding glycerol or other protective substances. Inactivation *during* storage due to enzymatic activity can be dealt with by lowering the storage temperature; temperatures below about –40°C. are usually safe.

Rivers (1927) studied the effect of repeated freezing (at –185°C.) and thawing on several viruses in different suspending media. The vaccinia-like virus III was most susceptible; 12 cycles of freezing and thawing would kill most of the particles, even in a highly protective medium. Vaccinia virus,

though more resistant, could be killed by repeated freezing and thawing when diluted in Locke's solution. Herpes virus was stable in brain emulsion, but sensitive if diluted in Locke's solution. Finally, a coliphage was found to be stable in broth irrespective of phage concentration, but susceptible in salt solutions.

Instructive data have been collected for freezing of influenza virus at higher temperatures. Penttinen (1950) found that freezing at  $-79^{\circ}\text{C}$ . did not affect the hemagglutinating activity of centrifugally purified vaccine, but that subsequent storage at  $-10$  or  $-20^{\circ}\text{C}$ . gradually destroyed the activity. Virus preparations purified by adsorption elution were stable at these temperatures. Greiff *et al.* (1954) made the following statements about the infectivity of allantoic fluids: ". . . Five freeze-thaw cycles resulted in a fall in titre from  $10^{8.6}$  to  $10^{-0.8}$ , cycles, 2, 3, and 4 causing much greater losses than cycles 1 and 5. Rapid cooling to  $-40^{\circ}\text{C}$ . or slow cooling to  $-80$  or  $-190^{\circ}\text{C}$ . did not cause significant titre loss, but rapid cooling to temperatures above  $-40^{\circ}\text{C}$ . or slow cooling to temperatures above  $-80^{\circ}\text{C}$ . caused definite titre loss. Loss of titre on storage occurred only at temperatures above  $-40^{\circ}\text{C}$ ."

The protective effect of glycerol and other compounds has been studied for various phages by Panijel *et al.* (1957). The practical aspects of freezing and storage of viruses under different conditions have been reviewed by Harris (1954).

## 2. Desiccation

A dried biological preparation resembles a frozen one in that both are effectively desiccated; the first by sublimation or evaporation, the second by isolation of the water in the form of ice crystals. By analogy with freezing and thawing, the transitions between the wet and the dry state may be harmful. If effective drying is tolerated, storage at room temperature may be adequate; if drying is less complete, the specimen may have to be kept at low temperature and, from point of view of storage, the advantage of drying is practically lost.

We have seen that drying of virus preparations may be an essential step in inactivation experiments (see Sections II, B, 1 and 2, and III, A). The work of Lea and co-workers (Lea, 1947) shows that many viruses tolerate drying in thin films if a protective agent like broth is added to the suspension. Pollard and Reaume (1951) observed that the coliphages T3 and T7 could be dried without serious loss of infectivity only if sublimation occurred at  $-20^{\circ}\text{C}$ . or less. Phage T1 requires no such precaution and it has therefore been used extensively for studies of inactivation in the dry state.

The general principles of freeze-drying will not be discussed here; the reader is referred to the recent review by Harris (1954). The results obtained

with different freeze-drying equipments can only be compared if the temperature maintained during sublimation is known. This factor was carefully studied by Greiff *et al.* (1954) who found that the infectivity of influenza virus was best preserved if the sublimation temperature was maintained near 0 or below  $-80^{\circ}\text{C}$ .

As regards the polioviruses, Faber *et al.* (1951) found that drying at room temperature from suspensions containing mucoid material or stools resulted in complete inactivation, and Pollard (1951) found that infectivity as well as antigenicity was reduced by a factor 100 or 1000 under various freeze-drying conditions. Much more promising results were obtained by Kraft and Pollard (1954), who reported about 10 % recovery after drying of poliovirus when peptone or sodium thioglycolate was added to the suspending medium before freezing or *to the reconstituting fluid*.

### C. Virus Stability and pH

It is common experience that pH, like ionic environment and temperature, greatly influences the stability of viruses, as well as their interaction with other agents. As a general rule viruses will remain stable for hours, at temperatures below about  $20^{\circ}\text{C}$ ., if the pH of the suspending medium is between 5 and 6 on the acid and 8-9 on the alkaline side. However, there are great variations among viruses in this respect; if storage for long periods is envisaged, the optimal pH should be determined by "accelerated degradation tests," i.e., by subjecting the virus, at the desired pH values, to a temperature that produces a convenient inactivation rate.

The general resistance pattern just described was found to apply to the coliphages T1 and T4 (Pollard and Reaume, 1951; Gönner and Bock, 1955) to influenza, herpes, and Theiler viruses (Gönner and Bock, 1955), and to four adenoviruses (Ginsberg, 1956). A detailed study of influenza viruses by Miller (1944) gave a more differentiated picture, showing that stability depended strongly on the buffer used and that, as usual, infectivity was lost before the hemagglutinating activity. Quantitative studies of the stability of the coliphages of the T-series as function of pH *and* ionic environments were carried out by Ruegamer (1954). Very striking differences between closely related strains were observed; thus, the phages T4 and T6 were stable, but phage T2 was rapidly inactivated at  $2^{\circ}\text{C}$ . in  $2 \times 10^{-5} M$  buffer at pH 3.

The remarkable instability of foot-and-mouth disease virus at slightly acid reactions (pH 6.5-6.0) should be mentioned separately. Randrup (1954) has shown that inactivation is due to the virus particles splitting up into non-infective but serologically unchanged fragments. It is not known whether other viruses undergo a similar degradation at low pH.

### *D. Salt Effects*

#### *1. Stability as a Function of Salt Concentration and Ionic Composition*

The modifying effect of the ionic environment on different inactivation processes has been stressed repeatedly. Outstanding examples are furnished by Adams (1949a), who showed that moderate changes in the ionic composition may reduce the heat inactivation rate one millionfold (see p. 385), and by Jerne and Skovsted (1953), who discovered that the rate of inactivation by antibodies is extremely sensitive to the ionic strength of the reaction mixture. These examples alone suffice to demonstrate that the ionic composition of the suspending medium is as important a factor to control as are temperature and pH.

Experiments by Burnet and McKie (1930), by Stanley (1935); by Gratia (1940); by Lark and Adams (1953); by Friedman (1953); by Ruegamer (1954); by Bachofer and Pottinger (1953, 1954a); and by Northrop (1954, 1955) concur in showing that salts play a very significant role in determining the stability and reactivity of viruses. In general, minimum stability is observed in the absence of salts, or at certain relatively low concentrations of monovalent cations ( $0.1 M$  or less); if this concentration is raised, or if small amounts of divalent cations ( $10^{-4}$  —  $10^{-3} M$ ) are added, the stability is greatly increased. Presumably, stabilization is due to the formation, on the virus surface, of complexes between protein and cations.

Inactivation due to unfavorable ionic environment can be reversible or irreversible. In the case of reversibility, a clear distinction should be made between cases in which *an inactivated state can be recognized*, in which the virus fails to initiate infection when mixed with susceptible cells under optimal conditions, and cases in which failure to initiate infection is due to deficiencies in the test system. If the latter can be shown to be true, we are probably dealing with a case of cofactor requirement, either for adsorption of the virus to the sensitive cells or for an early step in the reproduction process. As examples of this type of deficiency in the test system we may mention the tryptophan requirement of phage T4 for adsorption onto *E. coli* B cells (Anderson, 1945) and the Ca-ion requirement for the initiation of the reproduction cycle in cells infected with phage T5 (Adams, 1949b). Systems of this type are discussed in detail in Chapter II of Volume II.

True reversible inactivation at low salt concentration has been described by Puck (1949). Phage T1, taken from a suspension in distilled water or in dilute buffer, is irretrievably lost if mixed with susceptible cells under optimal conditions for adsorption and infection (in broth). The phage can be shown to adsorb but no productive infection results. If the "inactive phage" is incubated in broth, it recovers slowly but quantitatively; when sensitive cells are added, normal productive infection results. Similarly, phage T1,

inactivated in dilute buffer at pH 3.8, is gradually reactivated upon addition of  $\text{CaCl}_2$  (Ruegamer, 1954). The experiments of Northrop (1954, 1955) showed that several megatherium phages were reversibly inactivated at pH 5.8 in media of low salt concentration.

Irreversible inactivation at low or intermediate salt concentrations has been observed several times. Gratia (1940) found several phage strains to be rapidly inactivated at NaCl concentrations between 0.25 M and 0.03 M; very low concentrations of Ca or Mg salts prevented inactivation. Friedman (1953) made very similar observations with several megatherium phages. Lark and Adams (1953) showed that phage T5, inactivated at low salt concentration, has lost its ability to adsorb onto sensitive bacteria and that, in addition, its DNA has been released into the medium. This double effect suggests that inactivation may be due to damage to the tail tip by which the normal phage adsorbs and through which the DNA of the damaged phage may be assumed to leak out.

Most studies on virus stability at low salt concentrations have been carried out with phage. It should be mentioned, however, that a thorough investigation of the stability of centrifugally purified influenza virus in various buffers of low ionic strength was made by Knight (1944). Reactivation was observed upon addition of 0.1 M buffer to virus which had been partially inactivated in distilled water.

## 2. Osmotic Shock

We have twice referred to coliphages as "losing their DNA," thereby being transformed into "ghosts" consisting of empty phage heads with tails still attached. This splitting of phage particles into a protein membrane and tail plus free DNA can be achieved by intense UV or sonic irradiation of the T-even phages (Anderson, 1945; Anderson *et al.*, 1948; see p. 363), by heat treatment of phage T5 in the absence of Ca ions (Lark and Adams, 1953; see preceding section); under certain conditions by freezing and thawing (Panijel *et al.*, 1957); and in some cases by *osmotic shock*.

This phenomenon is observed if a suspension of one of the T-even phages in a concentrated NaCl solution is diluted into distilled water (Anderson, 1949). The transition from high to low osmotic pressure must be rapid, hence the term osmotic "shock." For the shock to be effective the osmotic pressure must be reduced by about 70 atmospheres, or more, and the temperature at which the phage is equilibrated in the concentrated solution must not be above about 30°C.; NaCl may be replaced by any of a number of ionic and nonionic solutes (Anderson *et al.*, 1953). Under favorable conditions the shock inactivates 98-99% of the phage particles.

The effect of a sudden drop in osmotic pressure may be explained by assuming that the outer membrane of the phage particle is much more

permeable to water than to the solute. When the pressure drops, the slow diffusion into the medium of solute molecules, and the simultaneous rapid diffusion into the phage of water molecules apparently create a diffusion pressure high enough to open pores in the phage membrane and allow the DNA to escape (Anderson, 1953).

It has been found that the phage particle can exist in a different state in which it is not susceptible to osmotic shock. If phage T6 is equilibrated with 2.5 *M* NaCl at 55° C., it is resistant to osmotic shock, but if the suspension is cooled before being diluted, the phage returns to the sensitive state at a rate which depends strongly on the temperature at which it is held. At 0°C. the half-time for reversion to sensitivity is about 24 hours! In the resistant state, the phage membrane is assumed to be sufficiently permeable to solute molecules to prevent inactivation upon dilution into water (Anderson, 1953).

Phage T6, in the sensitive state, is inactivated directly in concentrated sucrose solutions. Apparently the membrane is impermeable to the sugar and inactivation results from dehydration. In the resistant state, the phage is stable in sucrose solutions but can be shocked by rapid dilution into water (Anderson *et al.*, 1953).

The reversible transition between the sensitive and the resistant state has a very high temperature coefficient, to which corresponds an entropy change of about 250 cal./degree (Anderson *et al.*, 1953). It is interesting to compare these two states with the active and inactive states of phage T1 (see p. 390); at relatively low temperature, *the transition from the resistant to the sensitive, as well as from the inactive to the active state, is remarkably slow.* In fact, it is the sluggishness of these reactions which first allowed the recognition of two states.

Phage ghosts adsorb onto sensitive bacteria almost as well as do normal phages; Herriott (1951) has demonstrated that the adsorption of a ghost may be enough to kill a bacterium. This shows that killing must be associated with a very early step in infection, since it does not depend on phage DNA entering the cell.

#### IV. CHEMICAL AGENTS

S. GARD

##### *A. Chemical Alterations of the Virus Particle not associated with Loss of Infectivity*

The first systematic attempts to induce specific chemical alterations of the TMV protein were reported by Schramm and Müller in 1940. These authors treated the virus with ketene or phenyl isocyanate. They found that the resulting acetylated or phenylureido viruses retained their infectivity even

after coverage of a major part of the amino groups. Attempts to remove the amino nitrogen with nitrite led, however, to complete loss of activity, probably on account of uncontrolled oxidation.

Independently, Miller and Stanley (1941a,b, 1942) attacked the same problems. These authors found that about 70 % of the amino groups and 20 % of the tyrosine + tryptophan groups could be covered without loss of infectivity. If the reaction was carried beyond these limits a gradual inactivation occurred. Analyzed in the ultracentrifuge, the acetyl and phenylureido derivatives appeared homogeneous; electrophoretically they were likewise homogeneous, with a mobility quite distinct from that of the native virus. As further derivatives, carbobenzoxy, *p*-chlorobenzoyl, and benzenesulfonyl viruses were prepared. Again, about 70 % of the amino groups and 20 % of the phenol + indole groups could be covered without loss of infectivity. Finally, Anson and Stanley (1941) found that oxidation of sulfhydryl groups with iodine could be carried out without appreciable reduction of the infectivity. The progeny of all of these derivatives were invariably found to cause the normal disease and to have the chemical composition of normal virus. Thus, none of the *in vitro* reactions produced any "mutants."

Most of the substitutions now described undoubtedly involve only surface groups; they cannot be considered to represent major chemical alterations and they may be more or less readily reversed after introduction of the virus into the cell, although Miller and Stanley were unable to demonstrate reversion after treatment with homogenates of fresh tobacco leaves. The significance of these early observations is, therefore, limited.

Later, however, more drastic changes of the protein with no or little effect upon the infectivity have been described. Harris and Knight (1952) found that treatment with carboxypeptidase removed about 7 % of the total threonine from the TMV protein without apparently affecting the infectivity.

Schramm (1947a,b), in confirmation of several earlier reports, had observed that TMV in a moderately alkaline medium was gradually broken down into smaller fragments. A systematic study of this phenomenon (Schramm *et al.* 1955) revealed the appearance of several fractions distinguishable by their sedimentation and electrophoretic properties. Of particular interest in this connection are those fractions designated as I and II, which had a greater electrophoretic mobility and higher nucleic acid contents than the native virus. In electron micrographs this material appeared as mutilated virus rods, more or less deficient in protein, and with the nucleic acid showing as a central strand. After careful preparation these fractions retained practically full infectivity.

Similar results were reported by Hart (1955a,b), who treated the virus with sodium dodecyl sulfate (SDS) at pH 8 and a brief exposure to

temperatures about 85°C. He found, in addition, that virus in which the nucleic acid had thus been exposed had acquired sensitivity to ribonuclease.

Fraenkel-Conrat and Williams (1955) prepared pure nucleic acid and pure protein from TMV and claimed that, although each fraction was by itself inactive, a partial "reconstitution" of the virus with restoration of infectivity was obtained if the protein was allowed to polymerize in the presence of nucleic acid. Similar observations were reported by Hart (1956a) and by Commoner *et al.* (1956). Since it has now been convincingly shown that the pure nucleic acid alone may produce infection, the true significance of the reconstitution phenomenon is at present somewhat doubtful.

Gierer and Schramm (1956), after treatment of the virus with 50 % phenol in the cold, were able to isolate a protein-free nucleic acid fraction with a residual activity corresponding to about 2 % of the original, i.e., of the same order as that reported for the reconstituted virus. The free nucleic acid was rapidly inactivated by ribonuclease, which has no effect upon the intact virus. On the other hand, its infectivity was not affected by anti-TMV hyperimmune serum, which completely neutralized the virus. Fraenkel-Conrat (1956, 1957) independently found that the nucleic acid fractions obtained by treatment with alkaline buffer, SDS, or 67 % glacial acetic acid are infectious as well.

Phenol treatment, according to Gierer and Schramm, has also been applied to encephalomyocarditis (EMC), West Nile, and poliovirus (Colter *et al.*, 1957a,b) and to eastern equine encephalitis (EEE) virus (Wecker and Schäfer, 1957), in all cases with successful isolation of an infective nucleic acid fraction. However, in these cases the starting material consisted of unpurified virus, hence the resulting preparations were composed largely of tissue cell nucleic acid. Myxoviruses similarly treated have so far failed to yield any active fraction (Schäfer, personal communication). A liberation of the nucleic acid containing group antigen of the latter viruses by means of the much milder ether treatment (Hoyle, 1952) likewise results in complete inactivation (Andrewes and Horstmann, 1949).

From these observations it is obvious that the TMV, and perhaps the EMC and EEE virus nucleic acids contain the entire genetic information necessary for the synthesis of the respective viruses, as was previously shown for bacteriophage. It seems not unreasonable to generalize this conclusion and assume that the nucleic acid in all viruses represents the *genome*, that it alone carries the reproductive capacity, and determines the type and rate of synthesis induced in the host cell as well as the ultimate fate of the infected cell.

The naked nucleic acid seems to be very labile, however, easily inactivated by heat and by treatment with nuclease. One of the functions of the non-nucleic acid components of the virus seems to be that of stabilizing and protecting the nucleic acid. In addition such components may serve the purpose

of mediating or facilitating the entrance of the virus particle into the host cell. In those viruses (e.g., bacteriophage and myxoviruses) which possess a highly specific receptor mechanism, the intact function of the receptor mechanism seems to be an absolute condition for maintenance of infectivity. EEE virus, in which similar although probably less specific mechanisms are supposed to play a part in infection, may apparently retain some infectivity, even if these mechanisms are destroyed. In plant viruses, finally, capacity of attachment to the cell surface is completely immaterial; apparently the only mode by which infection can be achieved being a direct mechanical introduction into the cytoplasm. The "reconstitution" phenomenon may indicate, however, that the virus protein in TMV also serves some purpose beyond that of a stabilizer in the extracellular phase.<sup>1</sup>

We no longer conceive of viruses as giant nucleoprotein molecules, but recognize a structural organization of the particles, clearly on a supramolecular level. As a consequence, we also have to assume a functional differentiation within the particle. We are not yet in a position to map out all the details of the composite picture. It is obvious, however, that the infectivity of a virus particle is not determined exclusively by the functional state of its nucleic acid. These other properties (for the time being poorly understood) which, together with the *reproductive capacity*, determine the *infectivity* will be referred to as *avidity* of the virus.

Present evidence indicates that an irreversible change in the nucleic acid is sufficient to cause a complete loss of infectivity. Whether or not it is also a necessary condition for inactivation is not equally clear. A chemical alteration not involving the nucleic acid would, if the infectivity were affected at all, presumably lead only to a reduction in avidity which might, however, well approach complete inertia. The more the virus is dependent upon receptor mechanisms, the more easily might such a situation become established. In such cases the result of an infectivity test will depend largely upon the conditions of the test, the type of host cell used, the physiological condition of the cell, the pH, the composition of the medium, etc. The tryptophan-dependent bacteriophages might be mentioned as an illustration to the general principle. More relevant, perhaps, is the observation by Miller and Stanley (1942) that excessive substitution of amino and phenolic groupings of TMV led to partial inactivation, the rate of which appeared to be much higher when measured in *Phaseolus vulgaris* than when *Nicotiana glutinosa*

<sup>1</sup>Spizizen (1957) recently published some interesting experiments on T2 bacteriophage, in which osmotic shock-inactivated virus was found infective for naked protoplasts not only of the natural host strain but also of some under normal conditions resistant strains of bacteria. In this case infectivity was destroyed by proteolytic enzymes, whereas DNA ase seemed to have no specific effect upon the virus. Pure nucleic acid prepared with Schramm's or Hart's techniques was not infective.

was used as a test plant. Problems of this nature assume particular importance in production and testing of "killed" virus vaccines, and further examples will be mentioned in the following sections.

Apparently, therefore, a virus particle can be considered as truly inactivated only if its reproductive capacity is definitely and irreversibly destroyed through a chemical alteration of its nucleic acid. A chemical agent, in order to produce such effects, must first penetrate the non-nucleic acid protective cover, or destroy and remove it. Apart from enzymes, to which the protein is most probably impermeable, no chemical agents could be expected to be sufficiently specific to react only with the nucleic acid. It is, thus, to be expected that inactivation by chemical means will involve not only the nucleic acid but, to an even greater extent, the non-nucleic acid components of the virus. Obviously, the mechanisms of chemical inactivation cannot be fully understood unless the structure of the virus and the various functions of its integral parts are known. Needless to say, our present knowledge is too fragmentary to permit a really rational approach to this problem. It is important, however, to keep the various possibilities in mind. Experimental data on inactivation, when carefully analyzed and correctly interpreted, are likely to add to our knowledge of the nature of viruses and their mode of action.

In the present situation it may be helpful, as a first approximation, to compare the structure of the virus particle to that of a bacterial cell. In both cases the vital interior is enveloped in a fairly resistant membrane, functioning more or less as a mechanical and chemical sieve. The capacity of a chemical agent to cause inactivation will, thus, depend, not only upon its reactivity toward the nucleic acid, but also upon its molecular size, charge, and chemical affinity to the "membrane." The kinetics of inactivation will be shaped by the balance of these various factors. For instance, a first-order type of reaction (when the chemical agent is present in excess) should be expected under two conditions only: either (a) when mainly surface reactions, involving a specific receptor mechanism, are responsible for the loss of infectivity, or (b) when no interaction between the agent and the surface structures takes place. All reactions with the latter will—in one way or another—disturb the rate-determining diffusion through the membrane. Thus, it should be expected, at very low concentrations of the chemical, that the diffusion pressure would remain insignificant until the surface was "saturated." In other words, as in the case of disinfectants versus bacteria, the chemical agent would have to exceed a critical concentration level in order to produce a measurable inactivation. Furthermore, any reaction taking place in the membrane must be expected to affect its permeability, causing a continuous change in the rate of diffusion. A "fixation" (in the histological sense) of the protein cover would be associated with a gradual slowdown of

inactivation; hydrolysis or any type of breakdown of the protein might produce the opposite effect.

The inaccuracy of the biological methods of assay is a severe handicap in studies of the kinetics of inactivation. It can be overcome only by systematic repetition of experiments under rigidly standardized conditions and a careful statistical analysis of the results. Even so, observations often have to be extended into extreme ranges in order to reveal significant trends. In the past this has not been clearly realized. Most of the kinetic studies, limited in number in the first place, were designed on the more or less conscious assumption of the molecular nature of the virus particle and aimed at identification of "essential" sites in the molecule. Time is actually overdue for a revision of this basic philosophy and a reappraisal of the data already collected.

Presumably, each and every link in the complex chain molecules that carry the specific biological activity is equally essential. The activity rests, not upon a number of individual active groupings, but upon a specific pattern of forces. For the maintenance of the pattern, secondary bonds, particularly H bonds, no doubt are of great importance. Thus, as a hypothetical example, a reaction involving an amino group may lead to permanent inactivation even if it is readily reversible and the amino group can be reconstituted. What may not be reconstituted, however, is the original pattern. If by the primary reaction an H bond is broken, the delicate balance of intramolecular forces is disturbed and a new equilibrium of tensions will be established, i.e., a distortion of the configuration will ensue. A reconstitution of the amino group will restore the net charge and the elementary composition, but hardly the specific pattern. In this example the amino group may or may not be essential; if so, only as part of the pattern. For such reasons, attempts at identification of essential sites will presumably tell less about the mechanisms behind the specific activity of the virus than about the mode of action of the chemical agent used.

From a practical point of view a study of the relative importance of avidity and the "membrane" effect of the protein cover seems to be of definite importance. It was once thought that viruses could be "attenuated" by chemical means. Later this concept was revoked and the effect interpreted merely as a reduction in titer. However, thermal inactivation has been found, under certain conditions, to exert a selective pressure upon a genetically heterogeneous virus population—thus producing a shift in the average biological properties of the surviving fraction. A similar phenomenon has not yet been described in connection with chemical inactivation but has to be considered as a possibility. What seems to be definitely established, however, is the occurrence of avidity changes in the course of chemical treatment. These are demonstrable primarily as a reduction of the rate of adsorption of the virus onto the host cell, i.e., as a lower collision efficiency, and they may

therefore be interpreted as the result of changes in the net charge of the surface of the virus particle. No doubt the virus in this condition has to be regarded as attenuated, although the property is not hereditary.

The basic mechanisms regulating the penetration of a chemical agent into the interior of the virus particle seem to be accessible to direct chemical studies. At least, a very promising technique was recently described by Meriwether and Rosenblum (1957). These authors measured the incorporation of  $C^{14}$ -labeled formaldehyde in TMV. They concluded from the shape of the curves that a diffusion process was involved and applied diffusion mathematics to the analysis of the results. The brief preliminary communication cannot serve as a basis for a detailed discussion; it seems highly probable, however, that further research along these lines will prove rewarding.

In the following sections individual chemical agents and groups of agents will be reviewed. Until the nature of the reactions involved is better understood a truly rational classification is hardly feasible. The one here adopted is somewhat arbitrarily chosen and far from rational in the sense that overlapping frequently occurs. Actually, it is based mainly upon the amount of information available rather than upon attempts at analysis of the nature of the pertinent reactions.

### B. Formaldehyde

Formaldehyde reacts readily with proteins and in many different ways (for reviews of formaldehyde-protein reactions see French and Edsall, 1945; Walker 1953). Reactive sites are amino and imino groups; peptide linkages; amido, sulfhydryl, and hydroxy groups; and several ring structures. The reactions are often initially reversible, later become irreversible. Formaldehyde seems to react mainly in the hydrated state, as methylene glycol; the second step of the reaction usually includes formation of methylene bridges between two reactive sites, giving rise to new ring structures, or tying side chains or adjacent molecules firmly together (Fraenkel-Conrat and Olcott, 1948; Fraenkel-Conrat and Mecham, 1949). The over-all effect is that of a tanning agent: the structure becomes denser and less permeable, charge and solubility decrease, and the protein becomes chemically more inert.

The reactions between formaldehyde and nucleic acids are less extensively studied. Zamenhof *et al.* (1953) found no measurable change in biological activity or physicochemical properties after exposure of the DNA of the *Hemophilus* transforming principle to 0.33 *M* formaldehyde for 5 hours. In the presence of 4 *M* formaldehyde, however, a gradual decrease in viscosity and a rapid loss of activity was observed, presumably attributable to substitutions in the amino groups and breakage of H bonds. Fraenkel-Conrat (1954) found an increase of about 20 % in UV absorption and a shift of the

maximum of about 50 Å towards higher wavelengths in RNA (but not in DNA) after treatment with 0.33 to 0.67 *M* formaldehyde. The phenomenon was interpreted as evidence of formation of Schiff's bases, involving the amino groups of purines and pyrimidines. The reaction appeared to be reversible. In addition to amino groups, hydroxy groups of ribose and secondary acid groups of phosphoric acid would seem to provide theoretically possible reactive sites.

As a chemical widely used for production of "killed" virus vaccines, formaldehyde is of considerable practical importance; for that reason formol inactivation of a number of viruses has been extensively studied.

### 1. *Tobacco Mosaic Virus*

Stanley (1936) and Ross and Stanley (1938), studying the effect of formaldehyde on TMV, found that the infectivity decreased in a regular fashion in the course of treatment. They observed that inactivation during the first 12–18 hours proceeded approximately as a first-order reaction, but that more extensive inactivation required much longer periods of treatment than would have been expected on the basis of such an assumption. Lauffer and associates (Fischer and Lauffer, 1949a,b; Cartwright *et al.*, 1956), quoting Ross and Stanley, state the reaction to be of the first order. Actually this statement implies only that the data were better fitted by an equation of order 1 than by equations of orders 0 or 2; it does not exclude the possibility that an order of 1.1 would have fitted even better, which, indeed, is the case in Ross and Stanley's experiments.

However, on the assumption of a first-order specific reaction rate, constants were calculated and the effect of external factors on the inactivation rate determined. Formol-treated virus had a lower electrophoretic mobility, indicating a loss of charge, no doubt mainly by coverage of amino groups. The rate of change in mobility was much lower than that of inactivation, indicating either that the two phenomena were not referable to the same chemical reaction or else that infectivity was maintained only as long as all "essential" groups remained intact, i.e. an irreversible change in one of a number of identical groups would mean complete loss of activity but only a small change in the net charge. As it was later shown that the inactivation rate was largely independent of pH in the range of 4 to 8.5, the amino groups, as well as several other reactive sites, could probably be considered of little significance in inactivation; the most likely essential sites would have to be looked for among the beta hydroxy groups of threonine and serine, the hydroxy groups of ribose, and the nitrogen of the purine and pyrimidine bases of the nucleic acid. Preinactivation oxidation of sulfhydryl groups with iodine, or coverage of amino groups by acetylation or coupling with diazosulfanilic acid had no

effect upon the reaction rate, an additional indication that these groups probably are unimportant in the present connection.

On the assumption of a first-order reaction and from the observation that the regression of log reaction rate on log formaldehyde concentration was roughly unity, it was concluded that the rate-determining step in inactivation was a single event: the reaction of one molecule of formaldehyde with one essential site. In support of this conclusion the energy of activation was calculated at 19,500 cal./mole.

According to Ross and Stanley, a partial reactivation of the formaldehyde-treated virus could be achieved by prolonged dialysis at pH 3.0. Kassanis and Kleczkowski (1944) could not confirm this observation. Fischer and Lauffer (1949a), on the other hand, reported a mean ratio of post- to predialysis infectivity of 1.84, possibly significantly different from unity.

## 2. *Bacteriophage*

In spite of the fact that bacteriophages have to be considered as ideal model viruses in inactivation experiments and have been extensively used in studies of irradiation effects, the chemical inactivation of these agents has attracted little interest. Andrewes and Elford (1933), comparing formaldehyde-inactivation and neutralization of phage by immune serum, presented curves clearly deviating from the course of a first-order reaction. Recently, Heicken and Spicher (1956) reported more systematic studies on the coliphage T3. These authors found inactivation to follow approximately a first-order course down to a survival of about  $10^{-4}$ . In the later stages, however a deviation became apparent, particularly at lower formaldehyde concentrations. The phenomenon was supposed to indicate a heterogeneity of the phage population with respect to formaldehyde resistance. Gard (1957) described similar observations but offered a different explanation (see Section IV, B, 5).

Heicken and Spicher reported a considerable reactivation of formaldehyde-treated phage after addition of various amino acids, serum, or bisulfite. Reactivation continued for several days and amounted to as much as six powers of ten. This observation has been confirmed in the writer's laboratory.

## 3. *Poxvirus*

The kinetics of vaccinia virus inactivation was studied by Keogh (1937). The reaction was described as being of the first order. The validity of this conclusion appears somewhat doubtful, however, in view of the fact that 4 of the 5 experiments reported show a very marked deviation from a first-order course. The author seems to have attributed this phenomenon to a progressive fixation of formaldehyde by nonviral components of the medium, resulting in a successive reduction in the concentration of free formaldehyde. The loss of formaldehyde was estimated at not more than 25 % in 30 minutes.

This, however, could hardly account for the reduction in the inactivation rate of 80 % in 9 minutes that was recorded in one of the experiments presented. Therefore, it must be concluded that Keogh's data show a deviation from the course of a first-order reaction similar to that found in other viruses, the explanation of which is not immediately clear.

#### 4. *Myxovirus*

Lauffer and Wheatley (1949) studied inactivation of PR8 influenza A virus and concluded that the course probably was that of a first-order reaction. On this assumption the energy of activation was estimated at about 20,000 cal./mole. In the pH range of 5.6 to 8 increments in log inactivation rate were slightly below those in log formaldehyde concentration; in the same range the rate of inactivation was clearly pH-dependent, increasing with pH. Below pH 5.6, which is close to the isoelectric point, the virus was increasingly unstable and the presence of formaldehyde added little or nothing to the rate of normal heat inactivation at the temperatures tested (25–40°C.). The authors concluded that formaldehyde acted over the nonionized form of an ionizable basic group and that inactivation was the result of a single event.

Inactivation studies on influenza virus are complicated by the fact that noninfective virus may still retain the capacity of interference. In order to eliminate interference in infectivity assays, one has to work with comparatively high dilutions of the test material, thereby narrowing the range of activity accessible for observations. If work over a wider range is called for, and higher concentrations for that reason have to be included, it is important that primarily negative results in infectivity tests be checked in subpassages. In the case of interference, the virus content in primary cultures may stay well below the level detectable by means of haemagglutination and the presence of active virus may become manifest only after further passages. With this fact in mind, Uhler and Gard (Gard, 1957) studied inactivation of influenza A virus, using tissue cultures for assay of residual infectivity. Under these conditions a systematic deviation from the first-order course was observed.

#### 5. *Poliovirus*

Salk *et al.* (1954) described poliovirus inactivation as a first-order reaction. When, after initiation of commercial formol-vaccine production, it became evident that deviations from a first-order course occurred, an explanation was sought in the formation of aggregates and precipitates presumably shielding the virus from the action of the chemical. As a means of ensuring regularity of the process, filtration through Seitz pads was recommended (U.S. Technical Committee, 1955, 1957).

Timm *et al.* (1956) found an initial rapid drop in infectivity followed after varying periods of time by a phase of a reduced rate of inactivation, but largely of a first-order type. They also showed that prefiltration of the material had no effect upon the shape of the inactivation curve, although sometimes a considerable retention of virus was observed.

A Swedish group (Gard, 1955, 1957; Gard and Lycke, 1957; Lycke *et al.*, 1957; Wesslén *et al.*, 1957) has reported observations indicating that the reaction is of an order apparently higher than one, and that it is satisfactorily described by the equation

$$\log y_0/y = a \log (1 + bt) \quad (1)$$

( $y_0$  = original activity;  $y$  = activity at time  $t$ ;  $a$  and  $b$  are parameters), the corresponding reaction velocity equation being

$$\frac{dy}{dt} = - \frac{ab}{1 + bt} \cdot y \quad (2)$$

It was shown that the rate of inactivation was independent of the initial virus concentration (Lycke, 1957), indicating that the reaction is basically of the first order. It was further shown that the apparent deviation from the first order could not be explained by a gradual loss of active formaldehyde. Filtration or continuous mechanical homogenization during treatment had no effect upon the course of inactivation (Lycke, 1957). Thus, physical inhomogeneity seemed not to be responsible for the deviation from the first order, nor was any genetically conditioned heterogeneity demonstrable. As the apparently only remaining possibility, it was assumed that the virus particles gradually acquired resistance to formaldehyde in the course of treatment and as a direct result of the action of formaldehyde, the progressive fixation or tanning of the protein coat being responsible for the effect (Gard, 1957, 1958). Haas *et al.* (1957) have presented a large amount of material that is in excellent agreement with the Swedish observations (Gard and Lycke, 1957).

Böttiger *et al.* (1958) found that the time needed for appearance of cytopathogenic effects in tube cultures, as well as in the form of plaques, was increasingly prolonged as the time of formaldehyde treatment was extended.<sup>1</sup> This phenomenon was at least partly explained by gradually diminished adsorption rates, but in addition a prolongation of the first cycle of virus multiplication may have played a part. However, once the process had started, it seemed to proceed just as rapidly as after infection with untreated virus.

Within the limits of experimental errors the parameter  $a$  of equation (1) seemed to be independent of environmental conditions, the effect of which was instead reflected in the numerical value of  $b$ . The product  $ab$ , representing the initial reaction rate ( $t = 0$ ), would correspond to the rate constant of a

<sup>1</sup>This phenomenon was first described by Schultz *et al.* (1957).

regular first-order reaction. On this condition the results reported by Lycke (1958) indicated an energy of activation of about 20,000 cal./mole; the reaction rate showed a minimum at pH about 7, increasing at more acid as well as more alkaline reactions.

#### 6. Foot-and-Mouth Disease Virus

Kinetic studies were recently reported by Bachrach *et al.* (1957) and by Wesslén and Dinter (1957). The latter authors observed inactivation curves of the same type as those obtained with poliovirus.

#### 7. General Remarks

As previously pointed out, the relative importance of reactions in the nucleic acid and the non-nucleic acid components, respectively, might be expected to vary from one virus to the next, depending on the extent to which receptor mechanisms play essential parts in infection. To all appearances, however, the activity of receptor groups of bacteriophage and myxoviruses is largely unaffected by treatment with formaldehyde in concentrations and for periods of time sufficient to cause practically complete loss of infectivity. Therefore, destruction of the reproductive capacity seems to be the principal mechanism in formaldehyde inactivation of all viruses. The kinetics of inactivation should thus be determined mainly by the accessibility of the nucleic acid, the composition and structure of the protective cover, and the type of interaction between the latter and the chemical agent. Under such conditions lipid-containing and lipid-free viruses might be expected to show some distinctive features. This problem has not yet been systematically studied. *A priori*, the "membrane effect" seems likely to be the decisive factor, any possible differences being by degree rather than in principle. As a matter of fact, in this writer's laboratory the polioviruses, Theiler's virus, influenza A virus, and a staphylococcus phage were all found to follow the pattern formulated in equation (1), which also fits Ross and Stanley's data on TMV, as well as Wesslén and Dinter's on foot-and-mouth virus.

Theoretically, formaldehyde might be expected to penetrate the protein coat either by diffusion or stepwise in a series of reversible reactions. The observation by Cartwright *et al.* (1956) that preparatory substitution of SH or NH<sub>2</sub> groups does not demonstrably affect the initial rate of inactivation of TMV speaks decidedly in favor of diffusion as the more important factor. Whichever is the case, the progressive "fixation" or tanning of the protein in the course of formaldehyde treatment will lower its permeability. Thus, a membrane effect is to be expected, manifesting itself in a gradual decrease in the rate of inactivation. The problem can be treated mathematically after the following simplifying assumptions: (a) the fixation of the protein is

proceeding as a first-order reaction, as are the reactions in the nucleic acid responsible for the loss of reproductive capacity; (b) the "affinity" or accessibility of the nucleic acid to formaldehyde is at any time proportional to the remaining "free" fraction of the protein surface. The result is a probability function of a Poisson expression, of which the empirical equation (1) offers a very close approximation (Gard, 1957). The isotope studies of Meriwether and Rosenblum (cf. p. 398) seem to point in the same direction. These authors describe three stages in the incorporation of formaldehyde: (a) during the first 15 hours the surface reactions seemed to predominate; (b) next, followed 3-4 days of an approximately semilogarithmically decreasing rate of incorporation; (c) later, the process was further slowed down and had not come to a standstill even after 36 days, when the experiments were interrupted. Probably the subdivision in stages (b) and (c) is artificial. The most likely explanation is that, as in inactivation studies, the deviation from a semilogarithmic course becomes significant only when a sufficiently wide reaction range is covered.

It was already pointed out, as a corollary of the "membrane hypothesis," that the ratio of inactivation rate/formaldehyde-concentration increments should be expected to be less than unity in the range of low concentrations of the chemical agent. With TMV, Cartwright *et al.* (1956) observed values of roughly unity; the four points on which this assumption is based are too scattered, however, to permit any definite conclusions. Lauffer and Wheatley (1949), studying influenza A virus, recorded a value of about 0.8. As yet unpublished Swedish observations on poliovirus and bacteriophage indicate that inactivation in formaldehyde concentrations below 0.002 *M* is very slow, the increment ratio approaching unity only from concentrations of 0.004 *M* and upward.

As another corollary, pretreatment with formaldehyde should be expected to render the surviving virus more resistant to inactivation by most other chemical agents as well. In spite of the fact that combined chemical treatment—formaldehyde followed by  $\beta$ -propiolactone (Lépine, 1957)—is being applied in commercial production of poliovirus vaccine, no kinetic studies of this problem have been published.

The question of avidity changes in the course of treatment has only recently become a matter of interest. It has been a general experience in large scale production and testing of poliovirus vaccine that formaldehyde-treated virus may produce cytopathogenic changes in tissue cultures as late as 3 to 4 weeks after inoculation, which never happens with untreated virus. As already mentioned, this phenomenon seems to be at least partly explained by a reduction in the adsorption rate, most probably an effect of charge and solubility changes in the virus protein. Similar observations concerning other viruses have not yet been reported. The immediate practical consequences of

the phenomenon for poliovirus vaccine production and testing are obvious. There are some indirect consequences as well. In order to produce a safe vaccine to be used in mass vaccinations, one has to extend inactivation into a range where assay of survival ratios is no longer practicable. One has, therefore, to build the procedure on an extrapolation from the accessible part of the inactivation curve. Obviously, it is of utmost importance that the latter is determined with sufficient accuracy, and particularly that the potential activity—the reproductive capacity—is recorded. When tissue cultures are used for assay of remaining activity, it is of decisive importance that readings are extended over sufficient periods of time. If this is not observed, titers will come out too low and the rate of inactivation too high. This fact is the most probable explanation of the discrepancies in the results reported by different authors (Gard and Lycke, 1957; Gard, 1957).

Another question of great theoretical and practical interest is that of reactivation. *A priori*, a reversible change in the configuration of the nucleic acid does not appear to be particularly plausible. The possibility of reversion or perhaps counteraction of chemical alterations leading to a reduction in avidity seems much more likely. Nagler (1957) reported that adjustment of the inoculum to pH 9.3 reduced the incubation period of formaldehyde-treated poliovirus to normal values without affecting the end-point titer. If, in this case, activity were assayed, as is the custom in many laboratories, by taking final tissue culture readings 6 or 7 days after inoculation, the pH adjustment would be considered to have brought about a reactivation. This example illustrates both the need for a stricter definition of the term reactivation and the desirability in studies of this nature of the establishment of optimal conditions for activity assays. Nagler's method of restoring the avidity of the virus to normal values might be better described as "deattenuation" than as reactivation. A situation of this kind might be anticipated, particularly with viruses dependent upon specific receptor mechanisms. Further studies are obviously needed before any definite conclusions can be drawn.

Formaldehyde inactivation has been treated at some length, as this seemed to be the best place to discuss the theoretical and practical problems encountered in the study of chemical inactivation of viruses. Speculation may seem to have been carried farther than the comparatively meager facts permit. The justification for this lies in the obvious potentialities of research in this field to contribute to our knowledge of the nature of viruses.

### *C. Protein-Denaturing Agents*

Several amides, aromatic acids, and detergents enter into reversible combinations with proteins. Applied in low concentrations, they cause swelling

of the proteins, at higher concentrations, denaturation—stretching of the molecules, unmasking of sulfhydryl groups, solubility changes, and often a dissociation into molecular subunits. The effect is supposed to be mediated by attachment of these agents to the sites of hydrogen bonds which are thereby broken.

Urea is the most extensively studied member of this group of substances. It has been found to have little or no effect in concentrations below 3 *M*, but seems to inactivate most viruses if used in sufficiently high concentrations. A review of the earlier literature is found in an article by Bawden and Pirie (1940a). The most comprehensive and systematic studies concern plant-viruses. The rod-shaped viruses (TMV, potato X) are broken down into smaller fragments with liberation of nucleic acid (Bawden and Pirie, 1937, 1940a; Mehl, 1938; Frampton and Saum, 1939; Frampton, 1939; Martin, 1939; Stanley and Lauffer, 1939; Lauffer and Stanley, 1943; Lauffer, 1943). In spherical viruses (tomato bushy stunt, tobacco necrosis) neither fragmentation nor release of nucleic acid was observed (Bawden and Pirie, 1940a). Denaturation demonstrable by chemical or physicochemical methods is, however, to be regarded as the last step in a chain of reactions. Inactivation is not a result of denaturation but precedes it, and the nature of the reactions directly responsible for the loss of infectivity is as yet unknown. The kinetics of denaturation of TMV was studied by Lauffer (1943). In this connection it may suffice to mention that the reaction rate increased with pH above the isoelectric point and that it showed a minimum at a temperature of about 20°C., rapidly increasing with decreasing as well as increasing temperatures. Unfortunately, inactivation has been less extensively studied. It shows, however, the same dependence upon pH and temperature as denaturation (Bawden and Pirie, 1940a). Only one experiment has been reported which gives an idea of the shape of the inactivation-time curve (Lauffer and Stanley, 1943). In this case infectivity in the first 6 minutes disappeared at a rate corresponding to about 4 log units per hour. Subsequently, the inactivation rate decreased gradually, reaching about 0.016 logs/hour in the interval of 30 to 96 hours, with a survival at the latter time of about  $10^{-3.5}$ . The continuous decrease in inactivation rate suggests the occurrence of side reactions leading to enhanced resistance, i.e., a mechanism of principally the same type as that discussed in the section on formaldehyde.

The effect of 2 to 3 *M* urea on a cofactor-dependent T4 bacteriophage was studied by Sato (1956). He observed a rapid loss of infectivity, but at the same time an increasing fraction of the surviving phage became cofactor-independent. Isotope studies showed the adsorbability of the treated phage and its activity to run strictly parallel. Both "activation" and inactivation displayed the pattern of temperature and pH dependence characteristic of protein denaturation. Apparently, urea acts primarily on the receptor

mechanism, causing an initial activation of the reactive sites in a manner similar to that effected by L-tryptophan; further treatment is followed by inactivation of the same sites.

Bawden and Pirie (1940b) examined several other denaturing agents and found them to have effects apparently similar to those of urea. The remarkable temperature dependence of the reaction rate seems to be a unique characteristic of urea, however. Of the substances tested, including guanidine, urethane, pyridine, phenol, salicylic, benzoic, and hippuric acid, most seemed to split off RNA from rod-shaped, but not from spherical plant viruses; they were usually active at much lower concentration levels than urea. SDS was also tested and found to differ from the others by giving water-soluble products and also by releasing RNA from tomato bushy stunt virus. Reports concerning the effect of aniline are somewhat controversial; Lauffer (1938) could not demonstrate any inactivation of TMV after 10 minutes exposure to 50 % aniline in glycerol; Bawden and Pirie observed an immediate reduction in infectivity of 80 to 90 % which, however, did not show any further progress even after 4 hours. Subsequently, Lauffer and Robinson (1949) reported that different strains of virus behaved differently in this respect.

As already mentioned, cautious treatment with denaturing agents like phenol (Gierer and Schramm, 1956) or SDS (Fraenkel-Conrat and Williams, 1955) might give a protein-free virus nucleic acid retaining a considerable activity. Obviously, protein denaturation per se is not directly responsible for the inactivation observed. The fact that inactivation was found to precede demonstrable denaturation also seems to exclude the possibility of a purely indirect effect, i.e., by exposure of the labile nucleic acid deprived of its protecting coat. For the time being, the question of the mechanism of inactivation must be left open.

#### D. Oxidizing Agents

##### 1. General Aspects

Oxidation of organic material may, apart from the introduction of oxygen into the molecule, result in dehydrogenation, deamination, opening of ring structures, or formation of new rings (for a review of oxidation of proteins see Herriott, 1947). All these reactions may lead to breakage of hydrogen bonds. Therefore, oxidation, carried beyond certain limits, will almost invariably cause denaturation. Biologically active proteins, e.g., enzymes, may be inactivated by a cautious oxidation, but the reaction is often reversible, provided no denaturation has occurred. Reports of similar reversible inactivation of viruses (Perdrau, 1931), must, however, be viewed with some doubt. Zamenhof *et al.* (1953) found that the *Hemophilus* transforming principle

could be completely inactivated by nitrite without viscosity changes or any other signs of denaturation; they concluded that inactivation could be accounted for entirely by deamination. According to these authors, removal of one single amino group was sufficient to render the nucleic acid molecule completely inactive.

Many of the reactions dealt with in this section are strongly affected by catalyzers. Of particular importance are certain metal ions, primarily Fe and Cu. Free radicals presumably appear by oxidation of ferrous ions which would explain the pronounced catalytic effect of this ion. In studies of virus inactivation very little attention has been paid to these facts; consequently, the scattered observations on the effects of oxygen, hydrogen peroxide, ascorbic acid, etc., are difficult to evaluate.

According to Sizer (1945), oxidation reduction stability curves are equally specific and characteristic for individual enzymes, as are pH stability curves. Some enzymes are stable over an extended  $E_h$  range; at potentials beyond the stability range increasingly rapid inactivation takes place. Others show a stability optimum, the rate of inactivation gradually increasing with shifts in potentials in either direction. Similar systematic studies on viruses have not been carried out. It is generally assumed, however, that reducing substances like cysteine have a preservative effect (Zinsser and Tang, 1929; Zinsser and Seastone, 1930; Long and Olitsky, 1930; Perdrau, 1931; Amos, 1953).

## 2. *Oxygen*

Oxidation through aeration is supposed to be one of the contributing factors in "spontaneous" inactivation of viruses. The underlying evidence is mainly indirect. Viruses are known to be sensitive to other, more potent oxidants; the preservative effect of lyophilization or addition of reducing substances points in the same direction; a few direct experiments on the effect of increased oxygen pressure were also carried out (Perdrau, 1931). The relative importance of aeration in this respect was never fully established, however. As already pointed out, inactivation of viruses must always be considered a complex phenomenon, the resultant of several simultaneous processes. In the so-called spontaneous inactivation in aqueous media at least four different factors are to be taken into consideration:  $H^+$  and  $OH^-$  ions, heat, and oxidation reduction potential. Within the "stability" range, where pH changes have little effect upon the rate of inactivation, the direct action of  $H^+$  or  $OH^-$  must obviously be negligible, the rate-determining factor being either oxidation or heat denaturation. These two reactions should be expected to differ considerably with regard to the energy of activation. As already pointed out, Arrhenius plots of "thermo"-inactivation have yielded values varying from less than 30,000 to 195,000 cal./mole; not infrequently,

higher values were obtained in higher than in lower temperature ranges. Poliovirus seems to fall into the latter category (Lycke, 1958). Thus, temperatures from + 7 to 37°C. yielded approximately linear Arrhenius plots, with slopes corresponding to about 20,000 cal./mole, whereas the inactivation rate at 50°C. indicated a much greater energy of activation. This fact is probably best explained by the assumption that the rate-determining factor at temperatures below 50°C. is not heat denaturation but a reaction involving a single bond.

This assumption obtains further support from Youngner's (1957) studies on heat-resistant variants of polioviruses. This author found that a temperature of 36.5°C. did not exert any selective pressure, whereas temperature-resistant variants were regularly segregated by exposure to 50°C. Since no systematic studies of these and similar phenomena seem to have been carried out so far, it cannot yet be stated that the oxidation reduction potential is the rate determining factor at lower temperatures, although this assumption appears highly plausible.

*Ozone* is reportedly much more active than oxygen (Kessel *et al.*, 1943).

### 3. *Hydrogen Peroxide*

Hydrogen peroxide was found to inactivate several viruses (Gordon, 1925; Yaoi and Kasai, 1931; Stanley, 1936; Theiler and Gard, 1940), although at comparatively slow rates and only in relatively high concentrations. It is actually to be doubted whether  $H_2O_2$  per se is much more active than plain oxygen. It may participate in various catalyzed reactions, however, often acting indirectly by oxidizing nonviral substances in the medium which in turn act upon the virus. Thus, traces of ferrous ions usually increase its effect. Of considerable interest is the reaction mediated by the leucocyte enzyme myeloperoxidase (Agnier, 1950), as this might represent one of the processes by which viruses are eliminated from the living organism. In this case the mechanism is supposedly oxidation of NaCl to hypochlorite, which has a strong virucidal effect. Another type example is the oxidation of ascorbic acid, which was reported to have an inactivating effect (Holden and Resnick, 1936; Holden and Molloy, 1937; Kligler and Bernkopf, 1937; Jungeblut, 1939; Knight and Stanley, 1944; Klein, 1945). Systematic studies by Lojkin (1937) and by Ericsson and Lundbeck (1955a,b) indicate that ascorbic acid is by itself inactive. However, by rapid oxidation with  $H_2O_2$  in the presence of  $Cu^{++}$ , a labile intermediate of great inactivating capacity is formed.

Organic peroxides were reported by Latarjet (1956) to inactivate bacteriophages and *Pneumococcus* transforming agent and also to sensitize phage to the inactivating effect of ascorbic acid.

#### 4. Halogens

Earlier reports on the action of hypochlorite and chloramines were somewhat conflicting. One of the reasons for this was unawareness of the role played by nitrogen-containing impurities present in the test material. Trask *et al.* (1945) studied this question and found, not only that much larger doses of hypochlorite were required to inactivate virus in a medium rich in organic material, but also that higher residual chlorine concentrations were needed. Thus, impurities seemed to act partly by consuming chlorine and partly by affording some kind of extra protection against the chemical agent. This phenomenon was later explained by findings concerning the mechanism of chlorination. Chlorine ( $\text{Cl}_2$ ) can exist as such in aqueous solution only at a very acid pH. Otherwise it reacts with water, forming hypochlorous acid. This in turn, at alkaline pH, dissociates hypochlorite ions. In the presence of ammonia or amines a chain of reactions is incited, by which successively mono-, di-, and trichloramines are formed. The latter, finally, are oxidized by addition of further chlorine with formation of chlorides ("break point" chlorination). Residual chlorine may be either "free" ( $\text{Cl}_2$ , hypochlorous acid, hypochlorite ions) or "bound" (chloramines). According to Lensen *et al.* (1949), the inactivating capacity of organically bound chlorine is poor, whereas free chlorine is highly virucidal, particularly in the acid pH range. At an alkaline pH the negatively charged virus protein presumably offers a greater resistance against diffusion of the likewise negatively charged hypochlorite ions, thereby affording a better protection of the nucleic acid. In addition, the oxidation reduction potential of hypochlorite is a function of pH.

In a recent study of the kinetics of chlorination, Kelly and Sanderson (1957) observed deviations from the first-order type of reaction, similar to those described for formaldehyde and urea.

Iodine, cautiously applied at slightly acid pH and in the presence of an excess of potassium iodide, acts specifically on sulfhydryl groups without any inactivating effect (Anson and Stanley, 1941). Depending upon the configuration of the virus protein sulfenyl iodides or  $-\text{S}-\text{S}-$ , linkages are formed (Fraenkel-Conrat, 1955). At lower concentrations of potassium iodide and higher pH primarily di-iodotyrosine substitution is achieved, which affords the protein a new immunological specificity (Boltralik and Price, 1954) without necessarily inactivating the virus. Like chlorine, iodine is a powerful oxidant in the acid pH range and under these conditions has a strong virucidal effect.

Scattered observations on inactivation by several other oxidants have been published, e.g., potassium permanganate in concentrations of 0.001 to 0.005 % (Schultz and Robinson, 1942; Dunham and MacNeal, 1944); potassium dichromate (Schultz and Robinson, 1942); nitrites (Schramm and Müller, 1940; Zamenhof *et al.*, 1953).

### *E. Alkylating Agents*

In this group are included a number of biologically active compounds, of particular interest because they possess carcinogenic, mutagenic, and prophage-inducing capacity. These biologically active substances fall mainly into the following classes: sulfur and nitrogen mustards, epioxides, and ethyleneimines. They are characterized by their instability in aqueous solutions and their tendency to polymerize. They either contain heterocyclic rings or form on hydrolysis intermediates containing ring structures that are supposed to represent the active principle. They react primarily with ionized acid groups, carboxyl, hydroxy, phenolic, sulfhydryl, and phosphoric acid groups, or nonionized amino and thio-ether groups. They generally display a somewhat higher affinity to nucleic acids than to proteins. The reactions of alkylating agents with macromolecules was reviewed by Alexander (1954).

Herriott *et al.* (1946; Herriott, 1948), compared the sensitivity to mustards of various biologically active agents. Most easily inactivated was the *Pneumococcus* transforming principle and then, in order of decreasing sensitivity, DNA-containing, RNA-containing viruses, and enzymes. Since subsequently the question of the chemical nature of some of the viruses studied by Herriott has been revised—Newcastle disease virus (NDV), for instance, is now considered to contain only RNA and no DNA—the validity of the general rule suggested by Herriott appears questionable. A theoretical treatment of the problem of the kinetics of inactivation was also attempted, the conclusion being that the experimental data satisfied a reaction equation of the first order. The activity range studied was, however, too narrow to lend too much weight to this conclusion. Actually, “membrane effects” are to be expected; as mustards are capable of forming crosslinks as well as splitting peptide bonds, the type of effect can hardly be predicted, however. Kinetic studies meet in fact with great technical difficulties. The process is extremely rapid, the reaction proceeding to completion in a few minutes. No reliable method of prompt interruption of the reaction seems to exist. Fong and Nematollahi (1954) found that thiosulfate, recommended for such purposes, in reality seemed to have less competitive power than the virus. According to Fong (1955), mustards are considerably more active at slightly acid than at alkaline pH. The limited solubility of mustards and their rapid hydrolysis in aqueous media create great difficulties in the establishment of reproducible experimental conditions (Schwerdt *et al.*, 1951); in the last few years these substances have not attracted much interest as virus-inactivating agents.

Ethylene oxide has been used in both gaseous (Phillips and Kaye, 1949; Klarenbeek and van Tongeren, 1954) and liquid form (Wilson and Bruno, 1950; Ginsberg and Wilson, 1950) for sterilization of food, milk, serum, growth media, etc. It has the advantage over most other disinfectants of

being volatile and apparently leaving the media intact. Applied in concentrations of 1 to 2 % it inactivates  $10^3$ – $10^6$  ID<sub>50</sub> of influenza A and B, NDV, Theiler's, and EMC viruses in 60 minutes at + 4°C., followed by 24 hours at 37°C. (Ginsberg and Wilson, 1950; LoGrippo and Rupe, 1957). The kinetics of inactivation do not seem to have been studied. LoGrippo and Rupe (1957) also found that diepoxybutane and butylene oxide inactivate the EMC and EEE viruses, as did several ethyleneimines.

Betapropiolactone (BPL) is a versatile substance insofar as the lactone ring may open at either the alkyl or acyl oxygen bond; thus, both alkylation and acylation may be achieved. On hydrolysis, beta-substituted propionic acids and readily polymerizing hydracrylic acid are formed. For a discussion of the chemistry of BPL and for further references the reader is referred to Kelly *et al.* (1957). The half-life of BPL at 37°C. is about 30 minutes. Thus, hydrolysis proceeds at a sufficiently slow rate to permit kinetic studies and make reproducibility of experimental conditions feasible. BPL was found to inactivate all viruses so far tested. Data on the kinetics of inactivation are still scarce; in studies by LoGrippo and Rupe (1957) and Hartman and LoGrippo (1957) a "tailing effect" was described, i.e., the final level of survival was not a linear function of the concentration of the drug. This fact indicates that also in this case membrane effects interfere with the penetration of BPL into the interior of the virus particle.

#### F. Organic Solvents

The group of organic solvents is rather heterogeneous, not only chemically, but also with regard to the mechanisms of action. Almost all agents to be discussed in this section denature proteins, particularly at elevated temperatures. Some of them, under carefully controlled conditions, precipitate proteins in the native state. The solvent character is of interest mainly in the specifically lipid solvents.

The short-chain aliphatic alcohols precipitate viruses from aqueous solutions. Applied in the cold they leave infectivity virtually intact. For that reason methanol and ethanol have been used for purification purposes (Cox *et al.*, 1947; Pollard *et al.*, 1949; Moyer *et al.*, 1950; Schwerdt and Schaffer, 1956). At elevated temperatures denaturation and inactivation occur. Higher alcohols of low solubility can be used for deproteinization of crude virus suspensions without demonstrable inactivation. Bachrach and Schwerdt (1952) applied butanol for this purpose in purification of poliovirus.

Glycols are apparently somewhat less active as denaturing agents. They have been used in aerosol form for disinfection purposes and, in this connection, were also tested for their capacity to inactivate air-borne viruses

(Henle and Zellat, 1941). Liquid 90 % propylene glycol applied for 24 hours at 4°C. caused a considerable reduction in infectivity of EMC virus (Klarenbeek, 1954).

Glycerol not only has no inactivating effect but actually seems to preserve virus activity. This may possibly be explained by a replacement of hydration water by glycerol, leading to a reduction in the rates of the various "spontaneous" inactivation processes, which all depend upon the presence of water.

Ethyl ether is of particular interest on account of the relative specificity of its effects (Andrewes and Horstmann, 1949). It seems to be completely innocuous to lipid-free viruses. Of the lipid-containing viruses, some seem to be ether-resistant, e.g., vaccinia (4 % lipids) and, surprisingly enough, Western equine encephalitis (WEE) virus, reported to contain more than 50 % lipids (Beard, 1945). The group B arthropod-borne viruses are reported to be rapidly and completely inactivated by 20 % ether. As shown by Hoyle (1952), influenza A virus and, by Schäfer and Zillig (1954; Schäfer, 1957), fowl plague virus are disrupted by ether treatment, with release of two components with distinct biological and chemical properties: (a) nucleic acid-free hemagglutinin (type specific antigen) and (b) nucleoprotein group antigen. The latter is to all appearances identical with the "soluble antigen" that can be extracted from infected cells. There remains little doubt that the nucleoprotein represents the reproductive unit of the virus particle (Ada, 1957). So far, however, all attempts to demonstrate any infectivity of soluble antigen, the nucleoprotein liberated from virus particles, or a mixture of the disintegration products after ether treatment of the virus have failed. The reasons for this failure are at present unknown. *A priori*, it does not seem likely that exposure to ether would precipitate any significant structural changes within the nucleoprotein unit resulting in inactivation. As a more probable explanation it might be assumed that the nucleoprotein by itself is completely nonavid and that its introduction into the cell is entirely dependent upon the receptor mechanism. The lipid would then mainly serve the purpose of tying the two functionally different and equally essential moieties together. In the case of WEE virus one would have to assume either that the lipid is ether-insoluble or else that the virus hemagglutinin plays a minor role in the mechanism of infection.

Several nonpolar lipid solvents, in addition to the direct effect upon lipid-containing viruses, have the capacity of denaturing proteins at the solvent-water interface. As the virus protein seems to be very resistant to this type of denaturation such solvents can be used for deproteinization and purification of crude virus suspensions, with little or no damage to lipid-free viruses. Methods analogous to Bachrach and Schwerdt's butanol technique were applied by Polson and Selzer (1954), using chloroform-amyl alcohol (Sevagtreatment), and by Gessler *et al.* (1956), using fluorocarbons.

*G. Enzymes*

Lojkin and Vinson (1931) reported that TMV was inactivated by trypsin, presumably on account of the proteolytic action of the enzyme. Stanley (1934a), while confirming the inhibitory effect of trypsin, found no indication of a protein breakdown. Characteristically, the effect was established immediately upon addition of trypsin and did not proceed further on incubation. Full infectivity could also be restored by separation of the components in the mixture, e.g., by means of isoelectric precipitation.

A similar reversible reduction in infectivity by enzymes has later been repeatedly described, e.g., of TMV by papain (Bawden and Pirie, 1937) and by ribonuclease (Loring, 1942); of influenza virus by papain (Towarnizki, 1948); and of bacteriophage by ribonuclease (Jerne and Maaløe, 1957). Trypsin was supposed by Stanley (1934a) to reduce the infectivity of TMV, not by interaction with the virus, but by affecting the susceptibility of the host plant. In the other cases, virus and enzyme seem to combine to form an inert complex, displaying neither infectivity nor enzyme activity. The TMV-ribonuclease complex is insoluble in the absence of electrolytes, under these conditions forming a fibrous precipitate; the complex dissociates on dilution, releasing fully active virus (Loring, 1942). Reduction in infectivity of bacteriophage T4 is observed primarily at low ionic strengths and infectivity is restored after digestion of the complex with trypsin (Jerne and Maaløe, 1957). The mechanism is not yet fully understood. As enzyme treatment of the host cell alone can have an adverse effect upon its viability and synthetic capacity (Jerne and Maaløe, 1957), the apparent inactivation might at least partly be explained by a damage to the host cell and impairment of the substrate of the virus. That this is probably not the whole explanation is evident, however, from the fact that enzymatically inactive proteins with high isoelectric points, such as clupeine or globin (Bawden and Pirie, 1937; Towarnizki and Karlina, 1950), likewise reduce the infectivity, indicating that a complex formation of oppositely charged substances may be the decisive factor. Studies of virus/cell adsorption rates might serve to throw more light upon this question.

Under such conditions reports on inactivation by enzymes, presumably caused by enzymatic breakdown of the virus, must be cautiously judged. In addition, most of the early studies were carried out with very crude enzyme preparations and equally impure virus material; it is not always evident that effects observed were attributable to the action of enzymes. Since crystallized enzymes have become available, it has been shown repeatedly that neither RNAase nor DNAase attack the native viruses, a fact that has been utilized for purification purposes (cf. Schwerdt and Schaffer, 1956; Hershey *et al.*, 1951). The observations that free virus RNA is broken down by RNAase (Hart, 1955b; Gierer and Schramm, 1956) and that the *Hemophilus*

transforming principle is depolymerized and inactivated by DNAase (Zamenhof *et al.*, 1953) indicate that the resistance of the native viruses is attributable to the protected situation rather than to any intrinsic resistance of its nucleic acid. It is hardly likely that the nucleic acid is more accessible to other enzymes with less specificity; for that reason, the statements that phosphatases inactivate TMV (Pfankuch and Kausche, 1939) and herpes virus (Amos, 1953) seem to need confirmation; in neither case was "re-activation" attempted.

The action of proteolytic enzyme on viruses is a different matter. As already mentioned, Harris and Knight (1952) found that carboxypeptidase attacked TMV, splitting off about 7 % of the total threonine but no other amino acid. The infectivity of the virus remained unchanged. Apparently, threonine is the only C-terminal amino acid of the TMV protein. Knight (1955) showed, furthermore, that a number of TMV variants behaved similarly, yielding only threonine, as distinct from several other plant viruses, each of which seemed to possess a characteristic pattern of C-terminal residues. Fraenkel-Conrat and Singer (1954) attempted by various means to split off N-terminal residues from native virus protein, but without success. They concluded, therefore, that the protein consists of a closed polypeptide ring with "fringes" of C-terminal amino acids. As neither the individual amino acids nor the general composition of virus proteins seem to possess any distinctive features, the possibility of such a closed structure and perhaps other configuration peculiarities offers for the time being the only clue to the riddle of the remarkable resistance of many viruses to proteolytic enzymes. After denaturation the virus protein seems to lose this resistance (Kleczkowski, 1944).

Not all virus proteins are equally resistant to proteolytic enzymes. The study of this question, however, requires a number of safety measures to yield conclusive results. Since A. Pirie (1935) found virus-inhibiting impurities in crude enzyme preparations, it is obvious that only enzymes of satisfactory purity should be employed. The possibility of effects upon the host rather than the virus and of reversible inhibition of the nature previously described should be ruled out. Identification of split products and analysis of the residue (the procedure applied by Knight) is highly desirable. Finally, denaturation must be avoided and evidence presented that the enzyme attacks the native virus protein. So far, few studies come up to this standard.

Stanley (1934a) found no demonstrable change in the diffusion rate of trypsin in a mixture with TMV and concluded that virus and enzyme did not combine *in vitro*. Inhibition of infectivity, which appears immediately upon mixing, was consequently assumed to be the result of enzyme effects upon the host cells. Later Hills and Vinson (1938), using lower trypsin concentrations, did observe a retardation of diffusion of both the virus and the enzyme,

and Kleczkowski (1944) presented conclusive evidence of an *in vitro* combination of the two. Neither the enzyme nor the virus was inactivated and the combination was readily reversible on dilution. Thus, as in the case of phage ribonuclease, two factors—a direct reduction of avidity and damage to the host cell—might cooperate in reducing infectivity. Bawden and Pirie (1936) had shown that potato virus X was readily hydrolyzed and inactivated by trypsin. Kleczkowski (1944), confirming this observation, found that this virus, in spite of its sensitivity, did not bind more enzyme than the resistant TMV. Most animal viruses seem to be trypsin-resistant; for that reason, trypsin digestion has been applied as a means of purification. However, Merrill (1936) presented conclusive evidence to show that pseudorabies virus was readily inactivated by trypsin; the infectivity of vaccinia virus was slowly reduced by trypsin alone, more rapidly by trypsin + chymotrypsin; EEE and swine influenza virus were resistant. The effect of chymotrypsin upon the same four viruses followed a different pattern: EEE and pseudorabies virus were inactivated, vaccinia and swine influenza virus were resistant.

The effect of pepsin is less easily studied, as comparatively few viruses are stable at the pH optimal for the activity of this enzyme. Stanley (1934b) observed a slow inactivation of TMV, presumably the result of enzyme action on virus protein gradually denatured by low pH. Bawden and Pirie (1937) and Kleczkowski (1944), on the other hand, found this virus to be resistant. Kleczkowski showed that pepsin, which unlike trypsin combines specifically only with proteins serving as substrates, did not combine with native but did so readily with denatured virus, which also was readily digested. In contrast, potato virus X combined with the enzyme and was inactivated. Of animal viruses, poliovirus (Barski *et al.*, 1954) and presumably the other members of the group of intestinal viruses are pepsin-resistant.

Bawden and Pirie (1937) observed that commercial papain formed precipitates with plant virus suspensions as with various nonviral nucleoproteins and nucleic acids. The precipitate included virus as well as enzyme. With certain proportions of the reactants, precipitation was complete. TMV was thus removed from the solution by papain but not inactivated. The infectivity of potato virus X, on the other hand, was destroyed by papain in the presence of KCN but not in its absence, an indication that the proteolytic action of the enzyme was the factor responsible for inactivation. According to Lépine (1948), rabies virus is inactivated by papain. Polioviruses are resistant to papain and ficin (Gard and Östlund, 1951).

Other enzymes have not attracted much interest. Thus, nothing is known about the effect of pure lipolytic enzymes on lipid-containing viruses.

It is not yet possible to tell to what extent specific enzyme resistance-sensitivity patterns of virus proteins exist. Merrill's observations might be an indication that such is the case. As to the kinetics and the mechanism

of inactivation, very little can at present be said. Most probably the effect is mainly indirect, a "spontaneous" inactivation of the labile nucleic acid after destruction of the protective coat.

### H. Miscellaneous Agents

Some heavy metals were reported to inactivate viruses. Thus, inorganic mercury is virucidal, although higher concentrations than those needed for bactericidal effects are usually required. Kassanis and Kleczkowski (1944), studying the kinetics of inactivation of TMV by mercuric chloride, observed little or no effect at pH below 6 in the presence of molar KCl or NaCl, or of  $\text{HgCl}_2$  concentrations of 0.01 % or less. Under suitable experimental conditions, infectivity at first disappeared rapidly, the process soon slowing down and reaching near equilibrium in about 30 minutes. The virus could be removed from the reaction mixture by low-speed centrifugation, a fact suggesting that reduction of solubility and precipitation play a significant part in the process.

Several authors reported at least partial reactivation after treatment with  $\text{H}_2\text{S}$ , thioglycollate, or other —SH containing substances (Kreuger and Baldwin, 1934; Stanley, 1935; Pérez *et al.*, 1949; Sinkovics and Markos, 1956). Sinkovics (1956) presented data on influenza virus, exposed to  $\text{HgCl}_2$  for different periods of time and titrated for infectivity before and after treatment with  $\text{H}_2\text{S}$ . His results are not inconsistent with the assumption of a reversible surface reaction as the primary effect, followed by a more slowly progressing, apparently irreversible process. Since the interior of the host cell ought to provide good conditions for a reversion of the first stage, the loss of infectivity in this stage has to be explained either by failure of the cell to incorporate the virus, i.e., loss of avidity, or else by a toxic effect upon the cell of the mercury-laden virus. The nature of the second irreversible reaction is unknown. Of organic mercuric compounds, Merthiolate is of interest as a widely used bacteriostatic and bactericidal agent. It has generally been found to possess little or no virucidal activity. The stability of this substance does not seem to be absolute. Whether the slow inactivation observed with certain viruses is due to the organic compound or to traces of inorganic mercury has to be checked.

Silver appears to act in much the same way as mercury, i.e., by precipitation of the virus. Stanley (1935) obtained reactivation of silver-inactivated TMV simply by dialysis. The "oligodynamic" effect of silver ions, forming the basic principle in certain methods for purification of drinking water, does not seem to work on water-borne viruses. At least in the writer's laboratory, no inactivation of poliovirus was observed even at silver ion concentrations of 4000  $\mu\text{g}$  per liter.

Some protein-precipitating organic substances may also cause a reversible reduction in infectivity. Thus, Vinson (1932) reported recovery of active TMV from a noninfective safranine precipitate by treatment with amyl alcohol, picric acid, or acetone; Krueger and Baldwin (1935) reactivated safranine-treated bacteriophage by pH adjustment. Several tannic acids were reported to inactivate bacteriophage (Fischer *et al.*, 1954; Fischer, 1954); Thornberry (1935), however, observed reactivation of tannic acid-treated TMV after addition of gelatin or after filtration.

During an earlier period the action of bile and bile salts upon viruses attracted a certain interest. Recently Theiler (1957) emphasized the significance of sensitivity to sodium deoxycholate (SDC) as a criterion in virus classification. Systematic tests showed that SDC in a concentration of 1 : 1000, applied for 1 hour at 37°C., caused inactivation of 3-5 log ID<sub>50</sub> of all the arthropod-borne (arbor) viruses of both subgroups A and B, of influenza A and lymphocytic choriomeningitis (LCM) viruses, whereas polioviruses, Theiler's GD VII and FA strains, Coxsackie, and EMC viruses remained completely intact. It would thus seem that sensitivity to SDC and to ether run largely parallel. This fact does not yet justify any conclusions as to the mechanisms of inactivation. Generally speaking animal viruses can be divided into two classes: stable and labile viruses. Those described as labile are easily inactivated by heat, by acid pH, by lipid solvents, by enzymes, etc. Several viruses of this category are known to contain lipids. There are some notable exceptions from the general rule, however. The hardy poliovirus is abnormally sensitive to desiccation; the reportedly lipid-containing (54 %) WEE virus is ether-resistant as well as remarkably thermostable (Beard, 1945); influenza virus is not attacked by proteolytic enzymes. These irregularities at present appear somewhat puzzling; careful rechecking of the apparent inconsistencies and studies of the SDC effect seem desirable.

Some screening experiments, comprising large numbers of different chemicals, have been reported (Stanley, 1935; Schultz and Robinson, 1942; LoGrippe and Rupe, 1957). In general, relatively little specific information concerning the structure of viruses or the mechanisms of inactivation can be extracted from such reports. They will, therefore, not be reviewed in this connection.

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## Chapter VI

### The Chemical Basis of the Infectivity of Tobacco Mosaic Virus and Other Plant Viruses

H. FRAENKEL-CONRAT

*Virus Laboratory, University of California, Berkeley, California*

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A survey of the properties of those viruses which have been chemically characterized shows that the plant viruses consist only of nucleic acid and protein, while animal viruses range from this simplest composition to considerably more complex compositions. In regard to the nature of the nucleic acid, all known plant viruses contain only ribonucleic acid (RNA), while either deoxyribonucleic acid (DNA) or RNA may occur in animal and bacterial viruses. It appears possible that RNA viruses have a separate evolutionary history, possibly older than that of the DNA-containing viruses. On the other hand, the great resemblance in composition, both qualitative and quantitative, between many plant and animal viruses of both types suggests a close functional resemblance for the different groups of viruses. The occurrence of a seemingly constant amount of RNA in all viruses containing this nucleic acid, notwithstanding the great variations in their sizes,

is an intriguing observation (Frisch-Niggemeyer, 1956). The main architectural relationship of the viral components also appears to be constant. For in all cases that have been studied, the protein furnishes an outer shell for the more internally located nucleic acid. This appears to be true for the spherical plant or animal viruses, for the rod-shaped plant viruses, and for the tadpole-shaped bacteriophages.

### I. PURIFICATION OF PLANT VIRUSES

Since the isolation of pure tobacco mosaic virus (TMV) by Stanley in 1935, a number of plant viruses have been prepared in pure or near-pure state; several of these were obtained in crystalline or paracrystalline form. The chemical methods of isolation that enabled Stanley and others to achieve the first instances of success (Stanley, 1935a; Bawden and Pirie, 1937a) have in many instances been later replaced by the gentler physical methods—in particular, by differential centrifugation and density gradient centrifugation. As a typical example, the routine procedure for isolation of TMV in this laboratory will be described. Other plant viruses have been purified by variations of the same procedures as dictated by specific peculiarities of the particular viruses or the host plant material. The references listed in Table I will direct the reader to these procedures.

#### *A. Procedure for the Preparation of TMV*

Turkish tobacco leaves are harvested three weeks after inoculation with TMV. They are frozen in a deep-freeze and may be stored at this stage. They are then ground in a meat grinder, mixed with a concentrated solution of  $K_2HPO_4$  (3 gm. per 100 gm. leaf material), and allowed to melt. After centrifugation in a basket centrifuge, the juice is subjected to ultracentrifugation in the Model G Spinco ultracentrifuge (30-rotor, 22,000 r.p.m. for 1 hour). The pellet is dispersed in 0.1 *M* (pH 7) phosphate, clarified by centrifugation for 10–15 minutes at 4000–8000 r.p.m. and again ultracentrifuged. After about two more cycles of such alternate high- and low-speed centrifugation in 0.1 *M* phosphate, and two with water as solvent, the final pellet is dispersed in water and sterilized by filtration. The use of chloroform-saturated solvents throughout appears to be advantageous.

A convenient technique for near quantitative isolation of TMV from a few leaves or leaf discs was described by Schlegel and Rawlins (1953). It yields within a few hours a spectrophotometrically clean virus solution.

#### *B. Isolation and Properties of Other Plant Viruses*

A few of the better characterized plant viruses are listed in Table I, together with some selected data concerning procedures of isolation, the appearance and composition of the purified viruses, and a few key references.

TABLE I  
SOME PROPERTIES OF PURIFIED PLANT VIRUSES

Virus	Purification procedure	Crystallinity	Particles (shape and dimensions, $m\mu$ )	RNA (%)	References
Cucumber (CV3, CV4)	Chemical or centrifuge	(Tactoids)	Straight rods $17 \times 300$	5-6	Bawden and Pirie, 1937a Knight and Stanley, 1941
Potato X	Centrifuge	Liquid crystalline <i>no</i> tactoids	Flexible rods $16 \times 600$	6	Knight and Oster, 1947 Bawden and Pirie, 1938 Takahashi and Rawlins, 1946
Potato yellow dwarf	Centrifuge	—	Short rods $50 \times 200$		Black <i>et al.</i> , 1948 Black, 1955
Southern bean mosaic	Chemical or centrifuge	+	Spheres	21	Price, 1946
Tobacco mosaic (TMV)	Chemical or centrifuge	(Tactoids)	Straight rods $17 \times 300$	5-6	Miller and Price, 1946 Stanley, 1935a Stanley, 1936
Tobacco necrosis	Chemical or centrifuge	+	Spheres	17-22	Bawden and Pirie, 1943 Williams and Steere, 1951
Tobacco ringspot	Chemical and centrifuge	+	Polyhedra	24-26	Price and Wyckoff, 1939 Bawden and Pirie, 1945
Tomato bushy stunt	Chemical or centrifuge	+	Spheres	30	Steere, 1956
Turnip yellow mosaic (TYMV)	Chemical (centrifuge)	+	Spheres	26	Bawden and Pirie, 1943 Stanley, 1940
Wound tumor	Centrifuge	—	Spheres	80	deFremery and Knight, 1955 Markham <i>et al.</i> , 1948 Cosentino <i>et al.</i> , 1956 Brakke <i>et al.</i> , 1957

For a more detailed discussion of this subject the reader is referred to a review by Steere (1958), to whom this author is indebted for help in the preparation of Table I.

### C. *The Nature of Virus-Specific Components in Infected Plants*

Definite evidence has been obtained in recent years for the occurrence of noninfectious virus-specific products in the case of at least two plant virus diseases. The studies of Markham *et al.* (1948), as extended by Cosentino *et al.* (1956), have clearly demonstrated that purified turnip yellow mosaic virus (TYMV) isolates can be separated by ultracentrifugation into two components. The bottom component contained the infectious virus nucleoprotein, while the top component was rich in particles of the same dimensions, yet free from nucleic acid and devoid of infectivity. These protein particles showed the same electrophoretic behavior as the complete virus, and also were serologically almost indistinguishable.

Similar observations were made for noninfectious proteinaceous components isolated from TMV-infected Turkish tobacco plants by Takahashi and Ishii (1952, 1953), Jeener *et al.* (1954), and Commoner *et al.* (1953; Commoner and Yamada, 1955). A considerable amount of work performed by these groups has shown that this so-called X-protein resembles the viral protein in its tendency to aggregate below pH 6 to rod-shaped particles of varying lengths, but of the same diameter as TMV. This aggregation, which is also ionic strength-dependent, appears to be accompanied by a considerable increase in anodic electrophoretic mobility, which then approaches that of the complete virus. Exactly the same behavior has been observed upon reaggregation of the protein obtained by degradation of the virus, the so-called A-protein (see later). It is thus evident that in the case of both TMV and TYMV the electrophoretic mobility of the particle is a function of the protein alone and apparently only of its surface, as it is formed through the aggregation of smaller subunits. This is strong evidence for the interior location of the nucleic acid in those viruses (Kramer and Wittmann 1958).

It appears probable that the two or three components observed in some preparations of the X-protein represent comparatively stable intermediates in the aggregation process. Each of these, as well as the virus, is serologically closely related. Amino acid and end group analysis have also demonstrated great similarity between the X-protein and the protein isolated from the virus (Newmark and Fraser, 1956).

Concerning the metabolic significance of the formation of these proteins and their role in the replication process, no agreement has been reached among the various groups of workers, and this discussion may be regarded as not within the scope of this chapter. For the understanding of viral function

the crucial aspect of the observations is the noninfectivity of these mock-viruses. If one relates the mode of infection by bacteriophages, as elucidated by Hershey and Chase (1952) and Hershey (1957), with the occurrence of these inert proteins, then the hypothesis suggests itself that in all viruses it is the nucleic acid that carries the infectivity. This concept was not generally favored for plant viruses, because the analyses of different strains of TMV had revealed definite differences in amino acid composition, but not in nucleotide composition (Knight, 1947, 1952; Black and Knight, 1953; Markham and Smith, 1950; Cooper and Loring, 1954). However, since 1955 our knowledge about the nature of the infectivity of TMV has advanced beyond the stage of reasoning by analogy or inference. For we now have experimental proof that it is the nucleic acid that carries the infectivity of TMV. And the remaining inference that the nucleic acid is the infective component of all viruses is being experimentally supported at ever more frequent instances. The subsequent sections of this chapter will deal with those experimental results, largely concerned with TMV, which represent both the basis and the consequences of this new concept of the nature of viral infectivity.

## II. DEGRADATION OF TMV

Tobacco mosaic virus and its strains have probably been more intensively studied than all other purified plant viruses together. Separate chapters will be devoted to the chemical structure of TMV protein and nucleic acid, and particularly to the structural differences between strains. Much of the rest of this chapter will be concerned with the degradation of TMV into its two components, with their functional and some of their macromolecular chemical properties, and with the mechanism of reconstitution of complete virus from the two components. While none of these steps has been clearly duplicated with other plant viruses, there are sufficient hints and indications to establish confidence that the primary functional properties and relationships are the same for all viruses. This will surely become evident in the next few years, when techniques will be developed for the separation of other viruses into their native components.

### *A. Preparation of Nucleic Acid*

A great variety of agents, or conditions, almost all protein denaturants, cause the breakdown of the architecture of the viruses. There is probably no chemical agent which has not been so employed in early studies on TMV by Stanley (1935b) and by Bawden and Pirie (1937b). However, only a small number of these agents have been studied in detail, and have yielded practical

methods for the degradation of particles toward the end of isolating either or both components in pure and preferably native form. The last objective, that of separating both native protein and nucleic acid in good yield from the same batch of degraded virus, has actually not yet been realized.

A method for the separation of native (i.e., active) RNA which has proven very useful is by means of phenol. This method originally proposed by Morgan and Partridge (1941) was adopted by Gierer and Schramm (1956) for the purpose of the splitting of TMV and isolation of its nucleic acid. It has since proven applicable also to other viruses. The method resembles the older Sevag (Sevag *et al.*, 1938) procedure in using an interface separation of insoluble, denatured protein from water-soluble sodium nucleate, but it substitutes for chloroform the more water-soluble and more efficient denaturant, phenol. After several cycles of shaking and centrifugation of the pH 7 phosphate buffer-phenol mixture at 0°C., the aqueous phase is freed from dissolved phenol by ether extraction, and from ether by a stream of N<sub>2</sub>.

The alternate procedure developed in our laboratory makes use of the detergent, sodium dodecyl sulfate (SDS) (1 %) for the splitting of the virus (Sreenivasnya and Pirie, 1938) and of ammonium sulfate for the separation of nucleic acid and denatured protein (Fraenkel-Conrat and Singer, 1954, 1957). The most consistent results have been obtained when 0.002 *M* EDTA (ethylenediamine tetraacetic acid or Versene) was used as a buffer at an initial pH of 7.8, and the solution was heated to 50°C. until the viral opalescence had disappeared (1–10 minutes for different strains of TMV). Splitting occurs also in a few minutes at pH 3.5 and room temperature, and yields comparable nucleic acid preparations (Fraenkel-Conrat, 1957a). For the isolation of the nucleic acid, the protein is first precipitated by the addition of one-half volume of saturated ammonium sulfate. The nucleic acid precipitates from the supernatant upon cooling, and is reprecipitated twice from water by the addition of 2 volumes of ethanol, containing 1–2 drops of 3 *M* acetate sodium. The preparation is finally subjected to ultracentrifugation, in 2-ml. tubes, discarding the more viscous bottom drop (less than 10 %).

A third procedure which has yielded active nucleic acid is based on the original preparative method of Cohen and Stanley (1942). It consists in heat-denaturing and precipitating the protein in 0.1 *M* sodium chloride. No detailed description of this method as adapted for the purpose of isolating active nucleic acid has as yet appeared. It seems that the first two methods give preparations which are similarly infective and that the last, and other methods which have not been surveyed, give preparations which are at least one order of magnitude less infectious.

In the particular case of the TYMV, treatment with 30 % ethanol in presence of salts at room temperature was found sufficient to degrade the virus and yield pure nucleic acid (Markham *et al.*, 1948; Knight, 1957a).

### *B. Preparation of Protein*

The method most generally employed for the isolation of native TMV protein makes use of alkali for the degradation of the virus. Careful treatment near 0°C. with various buffers of pH 10–10.5 has given extensive splitting of the virus with little denaturation of the protein (Schramm *et al.*, 1955). Alkanolamine buffers appear advantageous for this purpose (Newmark and Myers, 1957). Ammonium sulphate can again be used to separate the protein from the nucleic acid. Electrophoresis has also been employed in preparing monodisperse protein fractions (A-protein), with a sedimentation constant of 4S, and approximate molecular weight of 100,000 (Schramm and Zillig, 1955).

An alternate degradation method, which in our laboratory has displaced the alkaline degradation, is by means of 67 % acetic acid (Fraenkel-Conrat, 1957b). This medium, an excellent solvent for most native and denatured proteins, dissociates the bonds between the two viral components in the cold and causes the precipitation of the free nucleic acid. The supernatant contains only protein, which can be isolated by dialysis. It aggregates as the acetic acid is removed, and can be sedimented in the ultracentrifuge. The protein can then be redissolved in dilute sodium hydroxide to a final pH of 7.5 to 8.0. This procedure is remarkably simple and convenient and yields native protein in excellent yield that corresponds in all properties to the alkali-prepared protein. The nucleic acid fractions, which can be isolated as by-products of these procedures, are not easily freed from contaminating protein, and are, as stated above, of low infectivity.

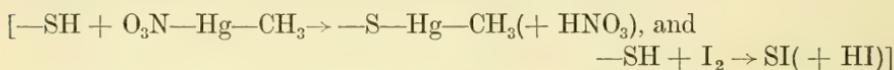
### *C. Structure and Function of Viral Proteins*

As previously stated, the proteins of TMV and TYMV have been found to occur naturally in the form of particles almost indistinguishable in shape from the complete virus. In the case of chemically degraded TMV, the small molecular protein (4 S) has also been shown to retain the ability to reaggregate to the general shape of the original virus under appropriate conditions of pH and ionic strength (Schramm and Zillig, 1955; Franklin, 1955). Thus, the shape of the viruses appears to be primarily a function of protein structure (Klug and Franklin, 1957). TMV protein consists of peptide chains of an approximate molecular weight of 18,000, held together only by hydrogen bonds and other coordinate or ionic linkages; it appears probable that spherical viruses are also constructed from separate subunits (Klug *et al.*, 1957). The tendency of many proteins to form specific and stable molecular aggregates is a well-known phenomenon. However, in only a few instances are proteins known to form particles approaching in size and in clearly defined architecture the virus protein aggregates, such as the TMV

protein, which aggregates below pH 5 to rods of uniform diameter but of greatly varying lengths, often exceeding that of the virus and thus consisting of more than the approximately 2300 peptide chains which build up one virus particle. This aggregation of the protein is associated with a marked increase in its electrophoretic mobility, which then approaches that of the complete virus.

While the aggregation of the protein is readily reversible by changes in pH or ionic strength, it becomes stabilized when viral nucleic acid (5 % of the protein) is also present, and becomes incorporated. In regard to heat denaturation, proteolytic enzymes, bacterial attack, and other agents, the resultant virus resembles the undegraded virus in being appreciably more resistant than the isolated protein. Thus, the result of aggregation is not only a protective shell for the nucleic acid but a self-stabilizing protein structure—a most fascinating case of biological adaptation, and a challenge to the protein chemist.

Some studies of the nature of the linkages which hold the subunits together have been initiated. It appears that the —SH groups (one per peptide chain) are masked in the sense that they react with some but not with other thiol reagents. The finding (Fraenkel-Conrat, 1955, 1957a) that these groups undergo substitution when they do react,



and that these reactions proceed without loss in the stability of the native protein structure, indicates that their H atom is present and unlinked, and that the bonding which prevents them from reacting like typical thiols must connect the sulfur with some other protein group. This finding is of general importance for the problem of the nature of masked —SH groups. However, there is no definite evidence that bonds involving the sulfur atom actually participate in inter-subunit linking. If they do participate in the interaction of native 4S subunits, then they must form new bonds within those units once the virus is degraded to this state, for they retain their masked character, although somewhat weakened, in the 4S protein, unless it be irreversibly denatured by detergents, heat, or other agents.

A similar conclusion can be reached concerning certain phenolic groups. For there is chemical and/or spectrophotometric evidence that these are masked in both the intact virus and the isolated protein; in the latter case, the masking is again less pronounced, and is a reversible, pH-dependent phenomenon. Thus, it is either not due to the same bonds as in the original virus, or it does not contribute to the inter-subunit bonds stabilizing the architecture of the virus.

As has long been known, all methods of degrading the virus architecture

cause a marked drop in the pH of the solution. Quantitative analyses of this reaction have indicated the dissociation of about two protons per subunit (Fraenkel-Conrat, 1957a; Koshland *et al.*, 1958). This finding is the most definite indication of the nature of some inter-subunit bonds, and has been interpreted (Fraenkel-Conrat, 1957a) as evidence that two carboxyl groups are involved in such bonding and thereby prevented from dissociating over the entire stability range of the virus, i.e., up to pH 9 for short time periods at low temperature. Further evidence that carboxyl groups are involved is the finding that divalent metals, particularly lead, are able to replace two protons at pH 6. Presumably the same carboxyl groups are involved; however, the metal in these cases does not disrupt the structure but actually seems to stabilize it when it replaces the not normally dissociatable protons (Fraenkel-Conrat and Narita, 1958). Much further work is required before this intriguing structural relationship will have been clarified.

The native protein gives clear solutions in water except over the range of pH 3.5 to 6.5, where it aggregates. Its characteristic ultraviolet absorption curve, with a sharp maximum and minimum at 280 and 250  $m\mu$  [ratio of  $A_{\max}/A_{\min} = 2.4$  for TMV;  $A_{\max}$  for 0.1 % solution (pH 8) is 1.3] can be used both as a measure of its concentration, and as an indication of its freedom from nucleic acid. Repeated freezing and thawing of the protein solutions, which are usually stored frozen to prevent spoilage, causes gradual denaturation, as indicated by decreases in the masked  $-SH$  titer and in solubility (Fraenkel-Conrat and Singer, 1957).

Considerable work has been done in several laboratories concerning the effect of chemical modification of the protein on the infectivity of the virus. These studies have been repeatedly reviewed, most recently by Knight (1954) and by Stanley and associates (1958). No additional work has been done in this field, and therefore it will not be discussed here. In the light of present knowledge, it is not surprising that extensive protein modifications often had little or no effect on the viral infectivity.

### III. ASSAY OF INFECTIVITY OF TMV AND TMV-RNA

Several varieties of tobacco give local lesions upon inoculation with TMV and can thus be used for quantitative evaluation. The preferred mutants in this laboratory at present are *Nicotiana glutinosa* and the Xanthi variety of *N. tabaccum*. The former host has the advantage of permitting the differentiation of one particular strain (HR) by the size and appearance of the lesions it produces (Fraenkel-Conrat and Singer, 1957). The latter host is the most sensitive variety of those compared by us.

In all tests for the activity of an unknown virus preparation, a standard solution is included for reference. This is obtained by diluting an aliquot of a

typical sterile virus preparation to a concentration of 0.1 mg./ml. with chloroform-saturated water, and storing this in a refrigerator for use over a two-week period. With each set of unknowns, a fresh secondary dilution of this stock solution in 0.1 *M* phosphate (pH 6.8) is tested. The dilution is 1–4  $\mu\lambda$  to 2 ml. for *N. glutinosa* (depending on the season), and an additional 10-fold dilution for the Xanthi variety. The most generally used level of 0.01  $\mu\text{g.}$  virus/ml. (in Xanthi) contains about  $10^8$  virus particles per milliliter and produces in average about 30 lesions per half leaf. Over the range of 10–50 lesions the dose-response curve appears to be nearly linear.

The standard, the unknown solutions (5–15) at appropriate concentrations in the same buffer, and the buffer alone are then applied to equivalent half leaves of 6–18 plants (3–6 leaves per plant, depending on size). Generally, 6 half leaves are used for each solution, and such assays are repeated at least once at levels selected to approximate the lesion number of the standard. For final, more exact evaluation, 12 half leaves are used, and, at times, 12 opposite half leaves are inoculated with unknown and standard.

Prior to inoculation, the plants are prepared for assay by cutting off all but the leaves which are to be used, and powdering these with carborundum. The solutions (0.02–0.05 ml. per leaf) are applied to the leaf and spread evenly over the entire half-leaf surface by means of a glass spatula with a rough-ground rubbing surface. The plants are then rinsed with water. All glassware, including the glass rod, is sterile. Nevertheless, there are occasional sets of assays in which contamination is present, as indicated by lesions appearing on the phosphate blank-inoculated leaves; if contamination is appreciable (more than 2 lesions), such sets are discarded.

The assay of nucleic acid preparations is performed by the same procedure with one important exception. Since the nucleic acid is more sensitive than the intact virus, particularly to salts, it is diluted in an ice bath with water of 0°C. to 90 % of the final volume. To each of these solutions is added at 0°C. one-tenth volume of *M* phosphate (pH 6.8) in the greenhouse just prior to application to the plants. Neither lower concentrations of phosphate nor other buffers investigated were found to give as many lesions as were obtained under these conditions.

Lesions on each half leaf are counted twice on the third to fifth day, and the highest number used in evaluation. Obviously, the accuracy of such assays is primarily dependent on the number of half leaves used in each group. Comparison of a series of solutions tested in one set of plants is most reliable. The significance of the differences of the group averages can be evaluated statistically if a sufficient number of leaves is used and some preliminary mathematical transformation is performed (Kleczkowski, 1949). The suggestion of Hart and Perez-Mendez (1957) of excluding the extreme individual cases and basing evaluation on the median of the remaining group may bear

some advantages. In general laboratory practice, groups of 6 and 12 half leaves appear to have errors of about 50 and 25 %, but accuracy can be improved when necessary by averaging many repeat assays (Loring, 1937). The inclusion of a standard virus solution in all tests permits calculation of the results in absolute terms, i.e., as percentage of the activity of the standard TMV. Quantitative comparison of the results of several assays is thereby greatly facilitated.

As previously stated, 0.01  $\mu\text{g./ml.}$  of TMV usually produces a convenient number of lesions. The nucleic acid comprises 5 % of the weight of the virus, and if it represents the infectious component, then one might expect it to be similarly infective at 0.0005  $\mu\text{g./ml.}$  Actually, 200 to 2000 times as much (0.1–1  $\mu\text{g.}$ ) of various nucleic acid preparations is required to produce the same number of lesions as is given by the 0.01  $\mu\text{g./ml.}$  standard virus. This discrepancy can be partly obscured by comparing the infectivity of nucleic acid and standard virus on the weight basis, when values of 10 % can be arithmetically reached. The same discrepancy can also be used to conclude that the infectivity of the nucleic acid is actually so low that it is obviously due to contaminating, undegraded virus, and thus not worthy of serious consideration. The following sections will show that neither of these trails of aberrant logic is favored by the author.

#### IV. RECONSTITUTION OF TMV

New impetus was given to the interest in the mode of viral infectivity when it was shown, in 1955, that native virus protein, together with suitably prepared nucleic acid, could form a co-aggregate consisting of rod-shaped particles many of which were indistinguishable from the undegraded virus in almost all respects (Fraenkel-Conrat and Williams, 1955). The discovery of this reconstitution reaction preceded the realization that the nucleic acid was infectious. The yields of reconstituted active virus were at first quite low; any residual activity detected in either of the two components in separate assays, usually at least two orders of magnitude lower, was regarded as a measure of its contamination with virus. It is thus not surprising that these results were first interpreted as indicating that viral infectivity was a property only of the complete 300  $m\mu$  particle, be it native or reconstituted *in vitro*.

As further work showed that the low infectivity of the nucleic acid moiety was actually an intrinsic property of the material (Fraenkel-Conrat, 1956; Fraenkel-Conrat *et al.*, 1957a; Gierer and Schramm, 1956) (see Section V) the significance of the reconstitution seemed at first to be eclipsed by that later finding. However, the quantitative difference between the infectivity of the isolated nucleic acid and the reconstituted virus has remained the same, as in the earliest experiments. Thus, combination of nucleic acid of relatively high infectivity (i.e., 0.5 % of that contained in the standard TMV)

with virus protein under the favorable conditions to be discussed below gives virus-like rods of full virus infectivity in 30–80 % yield (Fraenkel-Conrat, 1957c; Fraenkel-Conrat and Singer, 1958b). Thus the doubts mentioned at the end of the previous section are deprived of their basis, and the nucleic acid as isolated by either of two procedures is shown to be potentially almost fully infective. Reconstitution is thus the best method of functionally testing nucleic acid preparations, and is routinely used for this purpose in our laboratory. Other important uses of this reaction will be discussed in two subsequent sections.

The high efficiency of reconstitution now often attained has been the result of various changes of the original procedure. At present, the nucleic acid is added at room temperature to a 0.1 % solution of the protein in 0.1 *M* pyrophosphate (tetrasodium pyrophosphate adjusted with HCl to pH 7.0). For preparative purposes an approximately equivalent amount of nucleic acid is used, i.e., one-fifteenth to one-twentieth of the protein. Slightly higher relative yields in reconstituted infectivity are often obtained if relatively less nucleic acid (e.g., 1/100 or 1/200 of the protein) is used. For most nucleic acid preparations the use of pyrophosphate is crucial, while with an occasional sample a phosphate medium will give a similar extent of reconstitution (see later for discussion of the role of pyrophosphate). Lower salt concentrations and variations in pH below 6.5 or above 8.0 give distinctly lower yields.

Reaction mixtures containing reconstituted virus generally are directly tested for infectivity after suitable dilution, and the yield is calculated on the basis of the nucleic acid in the reaction mixture compared with the nucleic acid content of the standard virus, which is assumed to represent 5 % of its weight. Naturally, the total weight of the two viral components can be used and compared to the weight of standard virus when these are used in equivalent proportions (protein/RNA=20). The reaction can be terminated at any stage by dilution, preferably by simultaneous addition of ribonuclease (1 % of virus potentially present). This enzyme does not attack or inhibit TMV or reconstituted TMV when used at such concentrations, but it quickly degrades any free nucleic acid. The time required for the appearance of maximal infectivity appears to be about 6 hours in pH 7 pyrophosphate at 30° C., but 18 hours at 25° C. are more generally used.

Denatured protein precipitates under the influence of 0.1 *M* pyrophosphate. Thus, any precipitates appearing in reconstitution mixtures are removed by low-speed centrifugation. The amounts of protein in these precipitates are usually quite low, and with fresh, good protein preparations, negligible. The reconstituted virus can be separated from the reaction mixture by ultracentrifugation. The amount of material which is pelleted can be determined by weight, but is more usually derived from the absorbance of the pellet redispersed in water, using 2.7 as the absorbance at maximum (260–

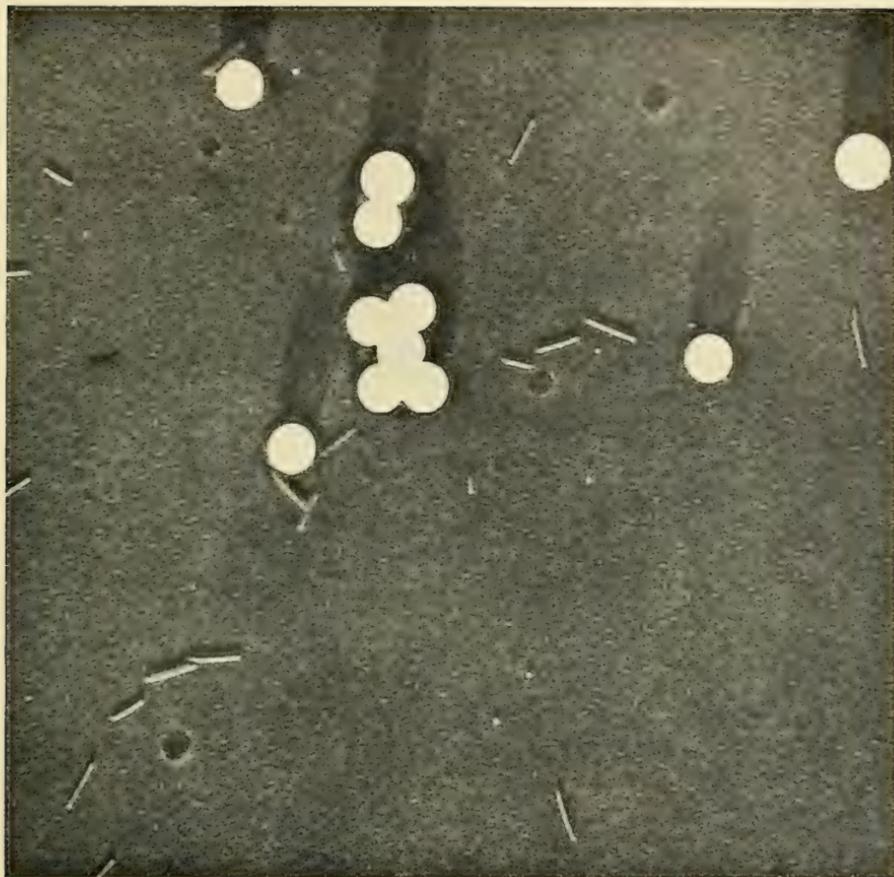


FIG. 1. Reconstituted TMV, twice sedimented in ultracentrifuge. The prevalence of particles of uniform length (about  $300\text{ m}\mu$  long) is evident. The polystyrene latex markers (white spheres) have a diameter of  $280\text{ m}\mu$  (Magnification  $\times 20,000$ ).



265  $m\mu$ ) of an 0.1 % solution of TMV. The amount of pelletable material found in a reaction mixture is generally quite similar to the yield of reconstituted virus, as calculated from the infectivity of that reaction mixture.

When poorly reconstituting viral nucleic acid (see later) or other nucleic acids, e.g., yeast nucleic acid, are used, very little material is sedimented by ultracentrifugation, and the character of its spectrum approaches that of the small amount of aggregated protein which is separated by the same technique from nucleic acid-free protein-buffer solutions. A second cycle of sedimentation from pH 7.0, 0.001 *M* tris-buffer produces essentially no sediment from TMV protein alone or from mixtures of protein with inactive (e.g., yeast) nucleic acid, while virus reconstituted by the use of highly active TMV-RNA, like undegraded virus, is recovered in at least 70 % yield. These findings show that there is limited significance to the electron microscopic observation of Hart and Smith (1956) that reconstitution of virus-like rods occurs with any RNA and even with polyribonucleotides. Apparently, these rods, the relative abundance of which was not determined, are of considerably lesser stability than is TMV or virus reconstituted from active TMV nucleic acid and protein. The latter material, after one or two sedimentations, shows an infectivity ranging from 50 to 100 % of that of the same amount of standard virus and proves, upon electron microscopic survey, to be composed, to at least 50 %, of its mass of rods of close to 300  $m\mu$  length (Fig. 1, electron micrograph). It is thus by all criteria almost indistinguishable from intact TMV.

The fact that the infectivity of TMV-RNA can be greatly enhanced by the reconstitution reaction has been confirmed in several laboratories (Siegel *et al.*, 1957; Commoner *et al.*, 1956; Commoner, 1957; Bawden, 1957; Bawden and Pirie, 1957). The use of pyrophosphate has given good reconstitution even with the phenol type of RNA (Fraenkel-Conrat, 1957a), which previously had been found not to reconstitute, in both Tübingen and Berkeley.

## V. INFECTIVITY OF VIRAL RNA

### A. TMV

The primary step toward identification of a viral component as the infectious moiety consists in its chemical separation from other components. Methods for the separation of nucleic acid and protein in comparatively pure forms from TMV and TYMV have been described. Differences in the efficacy of those methods for different strains of TMV have been recorded (Fraenkel-Conrat and Singer, 1957; Siegel *et al.*, 1956, 1957), and it is to be expected that for other viruses these procedures for splitting will have to be modified and additional ones developed.

The noninfectivity of the resulting protein fractions can generally be demonstrated without difficulty. Since proteins may inhibit viral activity, it is

usually advisable to demonstrate the detectability, by assay, of small amounts of added virus. Thus, in the case of TMV, protein at 1 mg./ml. is generally found to give no lesions, and added intact TMV (0.01  $\mu$ g./ml.) gives about 30 % as many lesions in the presence, as in the absence, of this amount of protein. Thus, it can be concluded that the protein is less than  $10^{-6}$  times as infectious as TMV, or, in other words, remarkably free from virus contamination (Fraenkel-Conrat and Singer, 1957).

Tests of the nucleic acid fraction, as previously mentioned, indicate that a 10- to 100-fold concentration by weight (0.1 to 1  $\mu$ g./ml.) is required to obtain the same lesion numbers as given by the standard virus. It thus has to be demonstrated that contamination with 1-10 % of intact virus is not the cause for this infectivity. This has been done by a variety of techniques, which can be segregated into two groups. They attempt either to exclude the presence of contaminating virus, or to demonstrate in a positive manner the nucleic acid nature of the infecting agent.

The identification of a biological effect with a chemical agent would be a simple matter if the possibility of contaminants could ever be completely ruled out. In the case of TMV-RNA, it can be shown analytically that after removal of salts by dialysis, the dry residue consists almost exclusively of ribonucleic acid, sodium, calcium, magnesium, and traces of aluminum, copper, manganese, and nickel; the metal content (total about 1 % after dialysis) can be further reduced by treatment with EDTA. Loring and Waritz (1957) found calcium, copper, iron, and magnesium in all preparations, and found traces of the latter two to be retained by the nucleic acid after treatment with chelating agents. However, the significance of this is not clear, and we have not found any iron in some preparations. The P/N ratio and the absorption per mole of phosphorus ( $A_p = 9200-10,000$ ) are all within the range of typical ribonucleic acid preparations.

Notwithstanding the fact that the viral nucleic acid has long been identified as RNA, the search for traces of DNA has continued. The presence of any such material might be of functional importance, since DNA has often been regarded as the only genetic substance. There are recurring statements in the literature, the latest by Holden and Pirie (1955), that DNA (up to 2.5 %) occurs in the viral nucleic acid, and the microbiological detection by Hoff-Jørgensen (1952) of a little thymidine in TMV appeared to support this suspicion. However, in searching for traces of DNA, the possibility of its being of bacterial origin must be considered, in addition to that of chemical contamination, which renders the interpretation of all trace components so difficult. In analyses of purified preparations, as contrasted with the cruder virus preparations previously used (Holden and Pirie, 1955), Pirie (1956, 1957) found no DNA; analyses of the infectious RNA performed by us recently (unpublished) by the diphenylamine reaction have also shown no

detectable traces of DNA, while 0.1 % of added DNA could be quantitatively recovered. Also microbiologically no DNA could be detected in sterile TMV (0.001 %, Hoff-Jørgensen, personal communication).

Of particular importance naturally is the extent of contamination with any virus protein, and much work has been expended on this question (Fraenkel-Conrat and Williams, 1955; Fraenkel-Conrat *et al.*, 1957a; Gierer and Schramm, 1956; Gierer, 1957). Colorimetric tests, using a sensitive biuret test, have indicated the presence of less than 0.4–0.5 % of protein. Application of a microadaptation of the Folin-Lowry test has shown a definite color, indicative of the presence of 0.1 to 0.5 % of protein in different preparations (Ramachandran and Fraenkel-Conrat, 1958); since no type of nucleic acid was available which gave less than this amount of color, and since guanylic acid also gave a certain amount of color, which in TMV-RNA would contribute about 0.1 % of spurious protein, the significance of the observed, slight chromogenicity is doubtful. Were it undegraded TMV, it would be 1–2 orders of magnitude below that required to account for the infectivity of the nucleic acid, and 4–5 orders below that of the reconstituted infectivity obtainable from it. Also, the variations in chromogenicity are in no way correlated with the infectiousness of different preparations.

Of other chemical tests for protein, amino acid analyses have been most intensively used (Gierer and Schramm, 1956; Fraenkel-Conrat *et al.*, 1957a). The fact that nucleic acid yields considerable amounts of glycine, and possibly of other amino acid-like products under the conditions of protein hydrolysis (16 hours, 108°C., sealed evacuated tubes, 6 *N* HCl) complicates this approach, or rather its interpretation. A technique which circumvents this difficulty is to degrade the nucleic acid by alkali (*N* KOH, 23°C., 18 hours), precipitating any undegraded protein by TCA, and analyzing this precipitate after hydrolysis by the FDNB method. Both this and the direct method have been repeatedly applied. At times only traces of glutamic and aspartic acid were detected, while other preparations have yielded more complex patterns of amino acids, not usually in the proportions characteristic of the virus protein, and ranging in total amount from 0.02 to 0.04 % of the amount of nucleic acid used (Fraenkel-Conrat, unpublished). Again, such findings cannot be interpreted in terms of viral contamination. If the results were more consistent, then one might conclude that some peptide-like material was associated with the active RNA and might possibly play a functional role in the genetic process.

Serological tests have also been used to search for the presence of native virus protein in TMV-RNA, and these have given a figure of less than 0.02 % (Gierer and Schramm, 1956). The use of S<sup>35</sup> to detect the presence of virus protein was advocated by Knight (1957b):

Electron microscopy has been used as a specific means of searching for TMV particles in nucleic acid preparations. Control experiments showed that added traces of TMV (1%) can be sedimented almost quantitatively from nucleic acid in the microtubes (2 ml.) available for use with the 40-rotor of the Spino preparative ultracentrifuge (Model L). Yet no, or very few, particles were detected in many infectious detergent preparations of nucleic acid (Fraenkel-Conrat *et al.*, 1957a). With a somewhat different technique, McLaren and Takahashi (1957) failed to find TMV particles in the phenol type of preparations.

The positive aspect of this search for active contaminants is the actual identification of the infectivity with the main component of the preparation, i.e., with the RNA. The foremost tool for such identification has been the enzyme, ribonuclease (RNAase). Thus, extremely low concentrations of pancreatic RNAase were found to inactivate, while other pancreatic enzymes, including deoxyribonuclease (DNAase), depressed the infectivity only at much higher concentrations (Fraenkel-Conrat *et al.*, 1957a). The infectivity of the intact virus was not affected by the same or by several higher orders of magnitude RNAase concentrations. Evidence was also adduced that the physicochemical parameters of the RNA were affected by enzyme action that caused incipient loss of infectivity (Gierer, 1957). The sensitivity to ultraviolet light (2537 Å) shown by the infectivity is also a strong indication that it is a property of nucleic acid, and in some strains differentiates the nucleic acids from the corresponding virus (Siegel *et al.*, 1956; McLaren and Takahashi, 1957). The finding that in various types of fractionation experiments the infectivity parallels the RNA concentration is additional evidence. Thus, the sedimentation experiments mentioned earlier not only failed to reveal the presence of virus particles but showed that infectivity and RNA concentration were proportional at various levels of the tube. Similar results were obtained in sucrose gradient centrifugation (unpublished experiments).

Anti-TMV sera were used, as previously mentioned, to search for contaminating virus protein. But they were also employed to differentiate viral from nucleic acid infectivity. When the latter was found not to be inhibited by such sera or the corresponding  $\gamma$ -globulin fraction at levels that inhibited the intact virus, the nonprotein nature of this infectivity was further substantiated (Gierer and Schramm, 1956; Fraenkel-Conrat *et al.*, 1957a).

Additional criteria for the differentiation of the two types of infectivity are based on the greater lability of the nucleic acid infectivity. Thus, exposure at room or incubator temperatures to 0.02 to 0.1 *M* salts generally caused rapid loss of nucleic acid infectivity, but not that of TMV (Gierer and Schramm, 1956; Fraenkel-Conrat *et al.*, 1957b). The mechanism of this unexpected sensitivity of the RNA to salts will be discussed below. The shift

to about pH 8 of the pH of optimum stability also differentiates the isolated nucleic acid from intact TMV.

Finally, an important functional difference was revealed by the studies of Siegel *et al.* (1957). These authors found that the development of lesions after inoculation with TMV could be largely forestalled by ultraviolet irradiation of the inoculated leaves within the first 2-5 hours, this time span varying for various strains. In contrast, no such protracted sensitive period existed for nucleic acid-infected leaves. This time interval was therefore tentatively interpreted as the time required by the plant for the separation of the nucleic acid from its protein shell. Similar conclusions were drawn from recent experiments in which the rate of lesion appearance was compared after inoculation with TMV or its nucleic acid, respectively (Fraenkel-Conrat *et al.*, 1958). The concept that the free nucleic acid would rapidly initiate the replication process in a manner not allowing for its continued presence is in accord with other data on the mode of infection by bacteriophage and various viruses.

### B. Other Viruses

The splitting and reconstitution of TMV and the recognition and acceptance of its RNA as the infectious moiety have led to an intensive search for similar evidence with other viruses. At present, only a few animal and bacterial viruses have yielded some success; since these will be discussed in later chapters, the pertinent experiments will be only briefly listed here. Colter *et al.* (B. B. Colter *et al.*, 1957; J. S. Colter *et al.*, 1957) have applied the phenol method to several virus-infected tissues and have isolated RNA fractions containing viral infectivity. Several of the criteria listed above were applied to differentiate the isolated infectivity from that of the corresponding intact virus. In some instances, sedimentability (i.e. precipitability) in MNaCl was an additional criterion differentiating the RNA from the virus. Wecker and Schäfer (1957) have performed similar experiments with Eastern equine encephalitis virus-infected tissue. Naturally, studies in which neither the starting material nor the final nucleic acid fraction is purely or even largely viral in nature must be regarded as no more than preliminary. But they suggest the possibility that techniques can be developed to separate all kinds of viruses into native protein and infectious nucleic acid, and possibly to reconstitute virus from such components. It remains to be elucidated whether the protein fractions can be used as noninfectious antigens, and whether the nucleic acids can in some manner be attenuated or genetically modified to render them advantageous for use in vaccines.

Other studies searching for infective DNA have centered on bacteriophages. In this instance, the protein supplies the seemingly crucial apparatus for infection of bacteria, but the use of protoplasts as hosts may serve as a means

to circumvent this difficulty (Fraser *et al.* 1957; Spizizen, 1957). Another instance in which a nucleic acid fraction is held responsible for a viroid effect (mouse leukemia) has been announced (Hays *et al.*, 1957).

### *C. Natural Occurrence of Infectious Nucleic Acid*

When infectious nucleic acid is isolated from tissue extracts or culture fluids, even by methods which disrupt many viruses, the possibility must be envisaged that some or all of this nucleic acid could have been present in the free state in that tissue. This would be a logical corollary to the known occurrence of viral protein components in plant tissues. Evidence that this can actually be the case was recently obtained by Cochran and Chichester (1957), when they fractionated crude homogenates of TMV-infected Turkish tobacco plants on ion exchange columns. For they obtained two widely separated infectious fractions, one of which resembled RNA in its behavior and comprised a variable proportion of the main infectious fraction (TMV), depending on the age of the infection.

## VI. CHEMICAL AND PHYSICOCHEMICAL PROPERTIES OF INFECTIOUS TMV-RNA

### *A. Molecular Weight*

A detailed discussion of the chemical composition of viral nucleic acids will be the subject of a later chapter. Here, only a few general properties of the macromolecular infectious material will be discussed. Such studies would appear to have little significance, since the low infectivity of all preparations suggests that over 99 % of the material is noninfectious. However, the recovery upon reconstitution of about 50 % of the original infectivity seems to exclude that possibility, and to justify the assumption that the main component of isolated TMV-RNA preparations is actually infectious.

One of the important physicochemical properties of a bioactive material is its molecular weight. Pertinent data have been obtained by Schuster *et al.* (1956) and by Gierer (1957) by means of sedimentation, diffusion, viscosity, and other determinations on phenol-prepared nucleic acid. These, as well as earlier light-scattering data obtained by Hopkins and Sinsheimer (1955) with heat-salt-prepared nucleic acid of unknown infectivity, have given molecular weight values between 0.9 and  $4 \times 10^6$ . The X-ray sensitive volume of TMV-RNA prepared by the SDS method, as determined by Ginoza and Norman (1957), falls within the same range. Since the calculated weight of the total complement of nucleic acid in a virus particle is about  $2.4 \times 10^6$ , the various authors favor the idea that the infectious nucleic acid represents

such undegraded nucleic acid particle-complements, possibly in the form of a single polynucleotide chain composed of about 7000 mononucleotides.

In contrast to this concept, some sedimentation analyses performed at Berkeley with the SDS-type of infectious nucleic acid preparation had given much lower *S* values, comparable to those obtained by Cohen and Stanley in 1942, and interpreted in terms of a molecular weight of  $0.2-0.3 \times 10^6$ . Further unpublished studies performed by Sue Hanlon, Pearl Appel, and H. K. Schachman showed that the sedimentation of TMV-RNA was to a remarkable extent dependent upon the salt concentration of the medium. Thus, low sedimentation constants ( $S_{20} = 5-10$ ) were obtained only at very low ionic strengths and were then very concentration-dependent, extrapolating at zero concentration to values similar to those obtained in the more concentrated ionic media ( $S_{20} = 20-30$ ). There appeared to be no basis for the earlier belief that there was a difference in the two types of nucleic acid preparations, since the sedimentability of both was found to depend in a similarly crucial manner upon the salt concentration.

One interpretation of the observed effects was that it represented an indication of the formation of higher molecular aggregates under the influence of the salt. It thus appeared possible that the various techniques listed above had measured the aggregate weight of a number of subunit polynucleotide chains, possibly of about  $0.25 \times 10^6$  molecular weight, and existing in free state only at low concentrations in very low ionic strength media. However, an alternate interpretation of the observed sedimentation behavior is that sedimentation at very low ionic strength is abnormal, owing to the primary charge effect (Svedberg and Pedersen, 1940), and that it therefore cannot be regarded as evidence for the formation of aggregates at the higher ionic strength. A similar though somewhat less marked effect of ionic strength was noted also for the sedimentation behaviour of DNA.

Thus, while the weight of evidence may favor the concept that the infectious component of nucleic acid preparations consists of intact nucleic acid units of  $2-3 \times 10^6$  molecular weight, several independent experimental observations are definitely not in accord with this concept, but seem to point to the existence of aggregating subunits. One of these is the hypochromic effect observed with TMV-RNA in the presence of salts. When salt ( $0.1 M$ ) is added to a mixture of the synthetic polynucleotides, polyadenylic and polyuridylic acid, the absorbance (*A*) decreases by as much as 30 %, an effect which is interpreted as an expression of the base-pairing aggregation of two or more polymer strands (Warner, 1957; Felsenfeld and Rich, 1957). Quite a similar effect is noted with infectious TMV-RNA (17-20 % depression at  $0.01 M$  or higher salt concentrations, be it NaCl, KCl, phosphate, etc., or at  $10^{-4} M$   $MgCl_2$ ), but to a smaller extent with degraded TMV-RNA, and yet less with yeast RNA. When it is taken into consideration that these same

preparations show the same decreasing order in their sedimentability in the presence of the same salts, the conclusion is strongly suggested that the salt causes a specific base-paired aggregation in nucleic acids of appropriate structure, and that this leads to an increase in molecular or particle weight in the solution, and possibly to the production of infectious nucleic acid.

Another series of observations in support of this concept is described in detail in a subsequent section. It consists of a study of the nature of virus reconstituted from a mixture of two nucleic acids. Only if subunits exist in solution would one expect a definite effect of one type of nucleic acid on another, whereas complete viral nucleic acid complements should not be greatly affected by the presence of another nucleic acid. The finding of evidence for definite interactions between two types, if mixed prior to addition to protein and buffer, seems again to require the existence of active subunits.

One approach to the problem of the molecular weight of an unbranched chain polymer, such as RNA is now regarded, would appear to be an analysis of the number of end groups. This is complicated in nucleic acids by the potentially varied nature of the terminal residues, which can be 5' phosphates, 3' phosphates, 2'-3' cyclic phosphates, or unphosphorylated terminal residues from either end of the chain. Earlier claims that there were many 5' phosphate end groups in TMV have been corrected (Reddi and Knight, 1957) and withdrawn (Matthews and Smith, 1957). At present there is little positive evidence for the occurrence of measurable amounts of any end groups of the various types listed. However, if one considers that the expected chain lengths are within the range of 1000 to 10,000 units long, the technical difficulties in detecting the 0.1 % or fewer nucleotides which might be terminal loom large.

### *B. Lability*

The fact that TMV-RNA degrades upon storage has been known since its first description by Cohen and Stanley (1942). Hopkins and Sinsheimer (1955) made similar observations with higher molecular weight preparations. Since the discovery of the infective activity of RNA, this activity was found a useful criterion in the study of the instability of RNA. According to Gierer and Schramm, only freshly prepared nucleic acid is fully infectious and of high molecular weight. With the SDS-type of preparations, the infectivity was generally found to be stable during many months of storage at  $-60^{\circ}\text{C}.$ ; also at  $0^{\circ}\text{C}.$ , many preparations decreased only slowly in infectivity. It then became evident that the difference in stability in the two types of preparations was due to the presence of 0.02 *M* phosphate in the phenol type of preparation, and the virtual absence of salts from the SDS-type of preparation. Most preparations of either type were found to degrade rapidly in the presence

of 0.02 to 0.1 *M* salts at room temperature or upon incubation, but to be very much more stable in 0.001 *M* or lower concentrations of salts. This degradation was evident from infectivity tests, from the decrease in sedimentability as determined in 0.02 *M* salts at 0° C., and finally from the appearance of material that was dialyzable and not precipitated by 67 % ethanol (Fraenkel-Conrat and Singer, 1958a; Reddi and Knight, 1957).

The variability in the rate of this degradation has been very great with different preparations, and even occasionally with the same preparation upon repeated tests, as well as in the comparative rates of inactivation at pH 5 and 7. Actually some preparations have been found which showed no definite loss in infectivity after several hours of incubation in 0.1 *M* salts, others which became completely inactive in pH 5 phosphate (0.1 *M*) but remained unchanged at pH 7, while the majority of the samples, lost over 90 % of the infectivity in one hour at 36°C. at any pH and in any 0.1 *M* salt, except pyrophosphate. Whenever tested, sedimentation tests showed a parallel behaviour, i.e., in experiments in which no loss of activity was observed there was also no decrease in sedimentability, while another nucleic acid preparation under the same conditions showed both.

The singular position of pyrophosphate was remarkable in that degradation, as measured by all criteria, was greatly retarded by this anion, as compared with others. Thus, little loss of infectivity was observed upon incubation for one hour in 0.1 *M* pyrophosphate of pH 7, even with preparations that were completely inactivated in phosphate under the same conditions (Fraenkel-Conrat and Singer, 1958a). The mechanism of this effect is not clear. It seems, however, quite probable that this is the reason why pyrophosphate was found, as previously stated, a favorable medium for the reconstitution reaction, yielding more reliably active virus preparations. It would seem that the high ionic strength required for extensive reconstitution makes the nucleic acid more susceptible to attack by trace enzymes prior to its incorporation into virus rods, and that this competing degradative reaction is minimized by the pyrophosphate medium.

The mechanism of this degradative reaction is naturally of great importance, particularly because its understanding may lead to its prevention. In general, sensitivity to salts is not a property of nucleic acids. DNA is actually sensitive to the absence of salts. The variability in the rate of degradation suggests that we are dealing with a variable trace contaminant. In view of the known sensitivity of the infectious RNA to enzymes, it would appear most likely that traces of ribonucleases of plant and/or possibly bacterial origin could account for the observed phenomena. It is known that the enzymatic activity of ribonucleases is dependent on the ionic strength of the medium. Particularly, when the enzyme concentration is limiting, a dependence of its efficiency on the salt concentration is to be expected. That this is in fact

the case was demonstrated by the addition of trace amounts of tobacco ribonuclease to stable TMV-RNA preparations. It was then found that an enzyme concentration sufficient to inactivate at 0.1 *M* salt concentration did not cause inactivation in 0.001 *M* salt (Fraenkel-Conrat and Singer, 1958a). Thus the probability that the lability of TMV-RNA is due to traces of enzymes is greatly strengthened. Unfortunately, however, no solution of the problem as to how regularly to prepare stable nucleic acid from the virus is as yet at hand.

### C. Miscellaneous Reactions

With any bioactive material the question arises as to how far the entire molecule is required for its functioning and how this is affected by specific chemical modifications. Little is known about chemical modifications of RNA, but recent studies have indicated that certain "protein reagents" definitely react with nucleic acids. The high toxicity of the mustard gases may well be due to their attack on the nucleic acid (Carpenter *et al.*, 1948). The inactivation of viruses by formaldehyde also is probably due to its affinity for the nucleic acid (Fraenkel-Conrat, 1954). Recent studies with  $C^{14}$ -labeled formaldehyde (Stachelin, 1958) and iodoacetate (unpublished) have shown that the amino groups of the nucleotides combine with the reagents, and that substitution of as little as one such group per 100,000 molecular weight unit (20 per particle nucleic acid complement) causes inactivation.

Inactivation of viruses, or of isolated nucleic acid, by ultraviolet light must also be listed here, although the extent of chemical alteration is as yet not too clearly defined. The mode of inactivation of TMV-RNA by traces of metals (particularly  $Cu^{++}$  and  $Fe^{+++}$ ) is also uncertain. The slow rate of this effect, and its irreversibility by later addition of chelating agents indicates that changes more extensive than metal binding are involved (Fraenkel-Conrat, 1958).

## VII. RECONSTITUTION OF VIRUSES FROM DIFFERENT STRAINS

TMV, like most viruses, represents a family of related viruses, usually referred to as strains or mutants. Only in rare instances are the genetic relationships of plant virus strains clearly established. These aspects will be discussed elsewhere. In the case of TMV strains, chemical criteria have become established in recent years which clearly define "membership in that club." Thus, all TMV strains seem to be composed of protein subunits of a molecular weight of about 18,000, containing one cysteine SH group and C-terminal prolyl-alanyl-threonine (Niu and Fraenkel-Conrat, 1955); all that were

tested have also proved to contain the same *N*-terminal acetyl-seryl-tyrosine group (Narita, 1958a,b); all appear to contain nucleic acid of the same overall composition (Knight, 1952; Black and Knight, 1953). That these criteria are not seriously restrictive to the mutation drive of viral genetic material is attested to by the existence of hundreds of mutants of TMV, and to the existence of at least one strain, the Holmes ribgrass (HR), characterized by remarkably wide areas of differences. There are hardly any points of resemblance between the HR protein and that of common TMV beyond those listed above as required for membership. It is thus not surprising that this strain can be serologically differentiated from common TMV, quite in contrast to most others. HR is also biologically the most segregated strain, detectable even in local lesion appearance (as stated previously). For all these reasons HR has been used more extensively than other strains in the strain reconstitution studies which will now be discussed.

#### A. Mixed Viruses

Before the infectivity of nucleic acid had been recognized, reconstitution of virus from nucleic acid and protein of two different strains appeared of singular importance and interest. This was possible for quite a number of different pairs; in each case the nature of the disease was the same as that of the parent strain supplying the nucleic acid. This appears now not surprising (Fraenkel-Conrat *et al.*, 1957b; Fraenkel-Conrat and Singer, 1957).

As expected, results were most clear-cut and definitive in the case of the HR and common TMV mixed viruses. These experiments unequivocally proved the reconstitution of active virus rods at a time when the yields were still relatively low and contamination with undegraded virus had to be seriously considered. The evidence was as follows: When HR-NA was reconstituted with TMV-protein, an HR-like disease was produced, as indicated already by the assay of the test solutions on *Nicotiana glutinosa*, and further proven by single lesion propagation on *N. tabaccum* and *N. sylvestris*, both of which react very differently to the two parent strains. Yet, addition of anti-TMV serum inhibited this HR-like virus, while anti-HR serum had little effect on it. Thus, its dual nature, i.e., HR-nucleic acid core and TMV-protein coat was clearly demonstrated (Fraenkel-Conrat and Singer, 1957). The expected opposite behavior was noted when HR-protein was combined with TMV-nucleic acid. Similar mixed virus reconstitutions were performed by Bawden (1957) and Commoner (1957), and similar conclusions were reached.

When the progeny of such mixed virus was isolated and subjected to detailed tests, all its properties naturally corresponded closely to those of the original virus supplying the nucleic acid. This was the case for both

qualitative and quantitative aspects of its infectivity and for its resistance to degradation. The most important question is, however, the nature of the protein coat of virus originating from an infective nucleic acid applied in a "foreign" protein coat. Does the nucleic acid carry the complete genetic information, even including every structural detail of its less important partner, and is the plant cell not confused by the presence of the other protein? Serological and preliminary amino acid analyses attest to the complete and infallible domination by the nucleic acid. But detailed and exact amino acid analyses are required (and are as yet in progress) to establish more firmly the identity of the original with the mixed-virus progeny protein.

### *B. Mixed Nucleic Acid Viruses*

In the light of present knowledge, no genetic effects were to be expected from the above mixtures of protein and nucleic acid from two strains. However, it seemed that this could possibly be achieved if nucleic acid from two strains could be incorporated artificially into one virus particle through the reconstitution reaction. This was conceivable only if the nucleic acid occurred in the form of active subunits in solution, several of which entered into each particle. Such was the author's concept, but it was in conflict with that of Gierer and Schramm (1956; Gierer, 1957), who postulated that only intact complete virus-nucleic acid complements or cores, of about  $2 \times 10^6$  molecular weight were infectious. Of such units, naturally, no more than one could ever occur in one 300-m $\mu$  particle, containing 5-6 % nucleic acid. While the question of the actual minimal molecular weight of the active unit cannot be regarded as settled, it seemed advisable to attack the above problem experimentally. The resulting data should in any case contribute information concerning the nature of the active unit.

Thus, a considerable number of experiments were performed in which TMV-RNA was mixed with various other nucleic acids, and subsequently reconstituted with TMV-protein at a constant excess (a 20-fold amount of the total nucleic acid) (Fraenkel-Conrat and Singer, 1958b). The various nucleic acids used for mixing (HR-RNA, inactivated TMV-RNA, yeast RNA) had very little, if any, affinity for this protein, and alone gave very few or no lesions upon reconstitution. The results of all these experiments can be briefly summarized as follows: When the two nucleic acids were in contact at low ionic strength, prior to addition to protein and buffer, and when the ballast nucleic acid comprised 70 to 90 % of the mixture, then the reconstituted activity of the TMV-RNA was greatly depressed (usually to about 40 % of the activity given by it upon undiluted reconstitution). Control tests showed that there was no inactivating action of any of these RNA preparations on the direct

infectivity of TMV-RNA. It would thus seem that these experiments are in accord with the existence of active subunits, which can interact with added RNA upon addition of salt, instead of aggregating with like molecules, and thereby lose much of their efficiency in reconstituting active rods. From that finding one must further conclude that packing with largely, if not exclusively, TMV-RNA is required for the formation of active virus rods.

When HR-RNA was used instead of yeast nucleic acids, it acted in a similar manner. This nucleic acid alone reconstitutes poorly with TMV-protein and, like yeast nucleic acid, etc., it acted as a depressant in mixtures with active TMV-RNA. The use of  $P^{32}$ -labeled virus nucleic acid preparations has supplied an additional tool to ascertain whether an infective nucleic acid actually favored the joint incorporation of another inactive RNA into a virus particle. However, no definite evidence for this was found. The single strand hypothesis thus seems indirectly supported.

### C. Search for *in Vitro*-Produced Mutants

Since it has become possible to demonstrate infectivity in degraded and reconstituted virus preparations, the aim has been to produce at will a new genetic (i.e., replicating) species of molecules. Reconstitution experiments with protein and nucleic acid of two different TMV strains were initiated prior to our realization that the nucleic acid alone was infectious and carried all the genetic information (Fraenkel-Conrat and Singer, 1957). These experiments were thus actually done in the hope of producing particles of an intermediate character, and obviously had to fail in this regard. Although an occasional mutant was observed, this also occurred in similarly rare instances with the progeny of the nucleic acid alone. For it seems that the chemical handling of the nucleic acid *per se* may be slightly mutagenic.

Contrary to these conclusions, Commoner (1957) has reported evidence that virus reconstituted from TMV-protein and HR-nucleic acid produced lesions of a size intermediate between those characteristic for the two strains. This effect is described as "temporary, long-term infections resulting in the predominance of virus which appears to follow the character of the nucleic acid donor." Neither Bawden (1957) nor this author have observed such fleeting intermediate effects with the same and other mixed virus systems.

It might be advisable to interject here a brief description of the procedure used by us in the search for *in vitro*-produced mutants, since this differs from the customary way of looking for "normal" or spontaneous plant virus mutants. The virus preparation suspected of containing genetically mixed particles is first applied to a local lesion host (*N. glutinosa*), and the nature of these lesions is observed. Single lesions (6-24) are then excised, ground up, and aliquots of the homogenate are transferred to several different varieties

of tobacco known to respond differently to the two parent strains of virus that may have become genetically mixed in the given experiment. If evidence is thus obtained that a given lesion contains genetic material not corresponding to one or the other parent strain in any one respect, then this material is further cycled through the same hosts, thus passing again through a single local lesion, to ascertain whether any difference from the parent strains has become genetically fixed. Only if this aim is achieved is the infectious agent regarded as a mutant, and only if the same type of change is observed in the progeny of an appreciable fraction of the first crop of local lesions (i.e., 30%), does it appear justifiable to assume that the observed mutation is the result of the *in vitro* mixing of two parental components.

As stated, the mixing of protein and nucleic acid from different strains has not resulted in such mutants. The seemingly more promising approach was then taken of mixing two nucleic acids derived from different strains, and then reconstituting virus particles by the addition of one or the other protein and buffer. Evidence that a mixing of two types of nucleic acid may actually occur at low ionic strength has been presented in the preceding section. Most of these experiments were performed with common TMV and the HR strain, because the marked biological and chemical differences between those two would have facilitated recognition of an intermediate character. In this case, the first local lesion test already supplied quantitative evidence for the relative amounts of the two viral types in a given solution. For, as previously stated, HR, in contrast to all other TMV strains, evokes smaller lesions on *N. glutinosa* which do not spread appreciably with time and are thus clearly distinguishable about one week after inoculation. It appeared from such experiments that HR-type lesions could be obtained only if the mixture of nucleic acids used for reconstitution together with TMV protein contained appreciably more HR-RNA than TMV-RNA (preferably 9:1). It furthermore appeared that every nonspreading (i.e., HR-type) lesion contained virus resembling HR in all respects upon further transfers, while the other lesions contained TMV-like material. In the rare instances when mixed symptoms were observed upon transfer, these generally disappeared upon further propagation. The conclusion from a considerable amount of work of this type was that no predictable mutant could be produced by mixing the nucleic acids of HR and common TMV. This failure to obtain genetic evidence for mixing might be interpreted as evidence that each infecting particle was purely of one or the other strain type, and that the postulated mixed virus rods were actually noninfective. However, since the observed symptoms are the outcome of countless replication cycles, the interpretation appears equally probable that, depending on the proportion of the two components in a given infectious particle, one or the other type of progeny becomes dominant and leads to exclusion of the other.

This failure to mix genetically TMV and HR-nucleic acid might be attributable to the marked genetic and structural differences between these two strains. Thus, it seemed advisable to repeat this type of experiment with a pair that was more closely related and yet biologically well distinguishable.

Yellow aucuba and common TMV were selected for this purpose. Virus giving symptoms characteristic of both parental forms was repeatedly obtained, and proved genetically stable upon repeated passage through single lesion hosts. However, the incidence of this occurrence was far too low to be regarded as evidence that several subunits of nucleic acid entered into each virus particle.

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## Chapter VII

### The Comparative Chemistry of Infective Virus Particles and Their Functional Activity: T2 and other Bacterial Viruses

E. A. EVANS, Jr.

*Department of Biochemistry, University of Chicago, Chicago, Illinois*

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#### I. INTRODUCTION

Although many bacterial viruses are known to the microbiologist, practically all of our quantitative chemical information concerning this group of infectious agents is derived from the T series of coliphages. Among these, attention has been concentrated on one or two strains and much remains to be done before our chemical information, even with respect to this limited group of viral agents, is adequate. The chemical study of the sperm-shaped coliphage particles, formed of headpieces ranging in diameter from about 45 to 80  $\mu$  and tails of varying lengths ( $15 \times 10 \mu$ – $15 \times 170 \mu$ ) is complicated by the vexatious problems of purification and homogeneity, so that it is difficult to evaluate the significance of differences in chemical composition reported with various preparations. Since a discussion of methods of purification and criteria for purity is not called for here [see Putnam (1953) for discussion of these topics], it suffices to say that the data selected represent, as far as possible, analyses of homogeneous and purified strains.

In general, studies of the N distribution of the T series of coliphages indicate that about half of the N is present as nucleic acid and half as protein, with small amounts of N (7 %) present as acid-soluble material. In purified preparations P is present as nucleic acid, although small amounts (up to 1 %) of acid-soluble P are observed (Taylor, 1946; Kozloff and Putnam, 1949; Herriott and Barlow, 1952). The nucleic acid seems exclusively of the DNA

type; recent work (Volkin and Astrachan, 1956) would seem to exclude rigorously the presence of RNA previously reported. The T7 and, probably, T3 phages appear to be exceptions to the above; only 71 % of the P is present as DNA (Lunan and Sinsheimer, 1956). Most virus preparations contain measurable quantities of lipid material (about 2 %), but it is uncertain whether this is a contaminant or an integral part of the viral structure.

## II. GENERAL STRUCTURE OF COLIPHAGE PARTICLES

Figure 1 represents a summary of current information with respect to the functional anatomy of coliphage T2. Many of these structural features

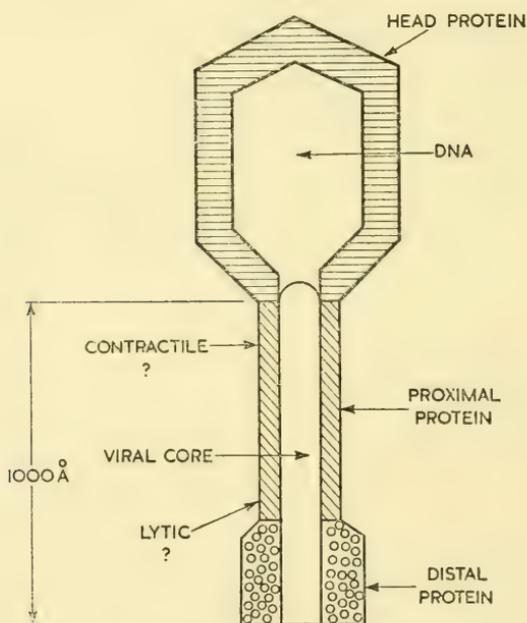


FIG. 1. Diagrammatic representation of coliphage T2.

probably occur in other members of the T series (most of them have been confirmed for T4) but the other T phages have not yet been studied in this respect. It is generally believed that the nucleic acid of the virus particle is concentrated within the head and is sheathed by a protective coat of protein. This view is based on the facts: (1) that the intact virus particle is resistant to the action of DNAase; (2) that on rapid dilution of concentrated saline solutions of T2 the viral nucleic acid is liberated into the medium and the remaining protein "ghosts" are seen (under the electron microscope) to retain the spermlike shape of the intact particle, although the head is

empty<sup>1</sup>; (3) that in infected host cells prematurely lysed or in infected host cells treated with proflavin one finds so-called "donuts" (resembling viral head-pieces), as well as particles resembling viral tails. These are noninfectious, protein in nature, and possess the serological characteristics of the intact infectious particle.

### III. THE PROTEIN COMPONENTS OF COLIPHAGE PARTICLES

#### *A. Amino Acid Content of Coliphage Proteins*

Table I represents a summary of the available analytical data for the amino acid composition of the proteins from coliphages T2, T3, and T4 in comparison with representative figures for the amino acid composition of the proteins of *Escherichia coli* B.

TABLE I  
AMINO ACID COMPOSITION OF COLIPHAGE AND *E. coli* PROTEIN<sup>a</sup>

Strain	T2 <sup>b</sup>	T3 <sup>b</sup>	T4 <sup>c</sup>	<i>E. coli</i> <sup>d</sup>
Alanine	7.6	9.4	9.4	6.7
Arginine	5.0	5.9	6.5	5.7
Aspartic acid	11.6	11.5	12.0	7.9
Cystine	(0.4)	—	—	12.2
DL Aminopimelic acid	—	—	—	5.1
Glutamic acid	11.8	11.3	12.0	9.3
Glycine	9.4	8.1	7.3	3.6
Histidine	0.9	1.7	2.6	9.0
Isoleucine	6.5	4.7	3.9	3.6
Leucine	5.9	9.4	6.5	6.3
Lysine	6.3	5.9	8.5	6.2
Methionine	2.2	1.9	1.3	3.0
Phenylalanine	5.5	3.4	4.2	3.3
Proline	3.9	4.5	5.0	3.3
Serine	5.3	4.0	4.8	3.8
Threonine	5.9	6.8	7.0	3.4
Tryptophan	(0.9-2.8)	—	—	1.3 <sup>e</sup>
Tyrosine	6.3	5.1	3.7	2.3
Valine	5.9	6.4	6.5	3.8

<sup>a</sup> Calculated from references given. Expressed as per cent of total amino acids analyzed.

<sup>b</sup> Fraser (1957).

<sup>c</sup> Polson and Wyckoff (1948).

<sup>d</sup> Roberts *et al.* (1955).

<sup>e</sup> Polson (1948).

<sup>1</sup> It is the usual experience to find that ghost preparations before treatment with DNAase contain variable amounts of DNA. Whether this represents a fragment of DNA still incorporated in its original state in the particle or the adsorption of a portion of the liberated DNA to the external surface of the ghost particle is not known.

The analyses for T2 and T3, reported by Fraser (1957), were performed on purified samples of whole phage (5 to 8 determinations), hydrolyzed in sealed tubes *in vacuo* with constant boiling HCl at 113.5°C., and analyzed by the ion exchange chromatography procedure of Moore and Stein (1948, 1951), and of Hirs and associates (1954). The values for serine, threonine, and tyrosine have been corrected for loss during hydrolysis. Determinations of tryptophan and of cysteine were not made. The bracketed values for tryptophan are from analyses of T2 ghost protein by Herriott and Barlow (1957), employing a variety of procedures; the bracketed value for cysteine was reported by Luria (1953) on a phage sample of unspecified purity, using a microbiological technique.

The values for T4 (Polson and Wyckoff, 1948) were obtained several years ago by paper chromatography after HCl hydrolysis and may be less accurate than the values for T2 and T3. The values for the amino acid content of the proteins of *E. coli* were obtained by chromatographic and radioautographic analysis, after HCl hydrolysis, of isotopically labeled amino acids from cells grown with C<sup>14</sup> glucose as the sole carbon source, with the exception of the values for cysteine and methionine (in which S<sup>35</sup> was the isotope used), and the value for isoleucine (based on the incorporation of C<sup>14</sup>O<sub>2</sub>) (Roberts *et al.*, 1955).

Fraser and Jerrell (1953) studied the amino acid composition of the protein portion of T3 under a variety of conditions and found that alterations in the nature and amount of the carbon and nitrogen sources of the host bacteria were without effect on the quantitative amino acid composition of the viral protein. It is generally assumed that the chemical composition of the other coliphages is also independent of the nutritional state of the host cell.

While the various coliphage strains show significant differences in amino acid composition as compared with each other and with the host cell, the general pattern of amino acid distribution in bacterial cell and virus is quite similar. In view of the fact that bacterial protein is not appreciably used for the synthesis of viral protein (Siddiqi *et al.*, 1952), the latter being manufactured *de novo* from the nutrient materials of the external medium, one might expect differences in protein composition of a considerable degree. However, the values of Table I represent averages for the great variety of protein molecules (enzymes, cell wall components, etc.) present in *E. coli* and, since there is increasing evidence for the physiological heterogeneity of viral protein (see Section B), it is possible that analysis of the individual proteins of host cell and virus (when this can be done) will exhibit specialized features in structure as well as function. The general similarities in amino acid composition between total bacterial and viral proteins may reflect the fact that it is the enzymic machinery of the host cell that must synthesize viral protein.

### *B. The Heterogeneity of Viral Protein*

Study of the process of replication in the bacterial viruses has led to an increasing amount of information indicating different physiological functions for various portions of the viral protein coat. It is not certain whether these can be correlated with discrete molecular entities, that is, whether the protein coat consists of a number of individual proteins held together by hydrogen bonding and van der Waals' forces, or whether the protein sheet is a continuous structure held together by the usual covalent bonds and exhibiting specific physiological properties in localized areas. Perhaps both alternatives exist; certainly in the case of the distal fragment of the tail protein of T2, the covalent linkage of the thiolester bond is the mode of attachment to the proximal portion of the particle.

At the present time, there is visual evidence for the existence of at least five components of the protein sheath, although none of these, as yet, has been isolated and chemically characterized completely. Further, it should be emphasized that evidence in this respect has been obtained primarily from the study of T2, with the major findings confirmed with T4. However, as yet, none of the other coliphages has been studied; one might expect important differences with the odd-numbered T phages. At the moment, we have evidence for the following distinguishable protein fractions:

1. The protein sheath of the head.
2. A protein of the proximal portion of the tail, having possible contractile functions in viral invasion (and differing from 3).
3. A protein of the proximal portion of the tail, possessing lytic activity for bacterial cell walls.
4. The protein of the distal portion of the tail, serving as the agent for the initial attachment of the virus to the bacterial host.
5. The protein of the so-called "core structure" of the tail of the viral particle.

Evidence for the existence of each of these components is as follows:

1. Lanni and Lanni (1953) were able to show by serological techniques the existence of at least two viral proteins, one associated with the head and another with the tail of the T2 particle. By separating the noninfectious donuts and rodlike structures present in prematurely lysed infected cells or in infected cells treated with proflavin, they were able to show that an antigen giving rise to neutralizing antibodies was localized in the rodlike structures, that is, presumably the viral tail, while another antigen, recognized by complement fixation tests, was localized in the phage heads (donuts).

There is also evidence of the differential susceptibility of the head protein to various reagents. For example, treatment of T2 with 0.1–0.2 M arginine leads to alteration of the virus head, but leaves the proximal tail intact

(Kozloff and Lute, 1957). Similarly, the head protein is fragmented by treatment with alkali (pH 10, 4 hours, 37°) (Kozloff, 1957) and by prolonged treatment with hydrogen peroxide (Kellenberger and Arber, 1955). Conversely, other reagents, such as the zinc-cyanide complexes, iodoacetate, or hydroxylamine, affecting other proteins of the viral structure, are without demonstrable effect on the head protein (Kozloff *et al.*, 1957).

From approximate calculations based on the phosphorus content of T2 and the ratio of P to protein of the virus, the particle weight of the whole protein sheath of T2 is estimated to be about 150,000,000. On the basis of relative dimensions seen in electron micrographs, the tail protein would have a particle weight of about 18,000,000, so that the value for the head protein would be in the neighbourhood of 130,000,000 (Kozloff *et al.*, 1957). Van Vunakis and Barlow (1956), using the dinitrofluorobenzene technique of Sanger, have made an end-group analysis of the protein ghosts obtained from T2 and find that alanine is the only amino acid present in the *N*-terminal position. Assuming uniform distribution of the alanine molecules, they find one per chain length of molecular weight of 80,000. Since 80 % of the viral protein is present in the head, these figures would apply primarily to that structure, unless there is a highly asymmetric distribution of alanine.

2. Treatment of T2 or of T4 with reagents or procedures that lead to the splitting-off of the distal tail (see below) gives rise to virus particles in which the proximal portion of the tail appears contracted. A study of this phenomenon by Kozloff (1957) has led to the suggestion that the proximal protein, or at least a portion of it, has contractile properties. Comparison of the behaviour of the proximal protein on treatment with alkali, ethylene diamine-tetraacetic acid, and a number of monovalent inorganic ions with that of myosin preparations under similar treatment shows remarkably parallel behaviour (Kozloff, 1957). While these results cannot be regarded as more than suggestive, they do indicate a line of investigation for further work.

3. The presence of a lytic agent acting on bacterial cell wall and localized in the proximal portion of the tail protein has been indicated by a number of observations. Weidel (1951) was the first to show that the treatment of preparations of bacterial cell wall from susceptible host cell by bacterial viruses caused dissolution of a large portion of the membrane. Barrington and Kozloff (1954, 1956) and Brown and Kozloff (1957) have studied this process in considerable detail. Of particular interest are their experiments in which they study the degree of lysis of bacterial cell walls brought about by various phage strains and preparations. With intact T2 and T4, lysis is found to occur, although with the latter strain tryptophan must be present in the medium as a cofactor. The amino acid is presumably required for the initial attachment between the virus particle and the host cell wall. When the distal protein of the two phage strains is removed by appropriate methods,

one finds that the lytic activity has been greatly increased and that with T4 tryptophan is no longer required. Further, if one studies the interaction between T2 and cell walls prepared from strain *E. coli* B/2, which is not attacked by T2, it is found that while the cell membranes are not attacked by the intact T2 virus, the usual lytic process does occur with the modified particle obtained after removal of the distal portion of the tail. All of these experiments indicate that removal of the distal portion of the viral tail exposes an area capable of lytic activity. Recently Koch and Jordan (1957) have described in T2 lysates a lytic agent which has a mode of action similar to that of the proximal protein. This material, however, is relatively small in size and has a molecular weight in the neighbourhood of 20,000. Although the identity of this lytic material with that in the proximal tail remains to be established, it is quite possible that there exist a number of lytic proteins, either attached to or imbedded in the contractile portion of the proximal protein.

4. The removal of the distal portion of the tail of T2 can be demonstrated using a variety of reagents and reactions (Kozloff *et al.*, 1957), for example, treatment with  $Zn^{++}$  or  $Cd^{++}$  cyanide complexes, hydrogen peroxide, papain, freezing and thawing (Williams and Fraser, 1956). A study of this phenomenon by Kozloff and his associates (1957) has led to the conclusion that in every case there occurs a splitting or hydrolysis of thiolester bonds, which presumably attach the distal portion of the protein tail to the proximal part. Electron micrographs taken during the course of treatment with a number of these reagents suggest that the tail protein is uncoiled into five distinct fibers prior to their complete removal from the proximal tail (Kellenberger and Arber, 1955; Williams and Fraser, 1956; Kellenberger and Sechaud, 1957). The fact that DNAase does not affect the fibers supports the view that they are protein. Estimates of size of the fibers suggest that the whole of the distal protein has a particle weight of around 1,000,000. After the distal portion of the tail has been removed, Kozloff *et al.* (1957) found that 8 % of the cysteine sulfur of the total phage protein was present in the supernatant. The interpretation is made uncertain, since the supernatant fraction also contains a variable number of viral tail "cores" (see next paragraph) whose sulfur content is unknown. But the facts suggest that the distal protein may be relatively rich in sulfur. This would support the conclusion that the splitting-off of the distal portion of the protein involves hydrolysis of a thiolester bond.

5. The presence of viral tail cores can be demonstrated in phage preparations after a variety of experimental procedures. By freezing and thawing (Williams and Fraser, 1956), by treatment with hydrogen peroxide (Kellenberger and Arber, 1955) or the cyanide complexes of  $Cd^{++}$  or  $Zn^{++}$  (Kozloff *et al.*, 1957), one obtains preparations in which most virus particles show a

core or spike protruding from the proximal portion of the tail, especially when the latter is contracted, while a number of particles would seem by their appearance and size to be free cores. The cores are not attacked by DNAase (Williams and Fraser, 1956), suggesting that they are protein in nature. Estimates of size indicate a particle weight in the neighbourhood of 4,500,000 (Kozloff *et al.*, 1957). It is possible that the core is identical with the nonsedimentable protein found by Hershey (1955) in T2 shockates. This nonsedimentable protein appears to be an authentic component of the phage particle, although it does not possess the antigenic properties of the whole phage particle and is not adsorbed to the host bacterial cell.

#### IV. NUCLEIC ACID COMPONENTS OF COLIPHAGE

A variety of techniques exists for the isolation of the DNA of the bacterial viruses. In the procedures of Mayers and Spizizen (1954), the phage concentrate is dissolved at room temperature in a 1% solution of commercial sodium lauryl sulfate (Duponol C) at pH 7. An equal volume of saturated sodium acetate is then added, and the solution held at 60°C. for fifteen minutes and then cooled to 5°C. After centrifuging, the supernatant is poured into 2.8 volumes of alcohol acidified with HCl. The nucleic acid precipitates out as a stringy mass which can be transferred to a Buchner funnel by a glass hook, washed with absolute alcohol and ether, and dried at room temperature.

In another procedure (Wyatt and Cohen, 1953) the viral particle is disrupted by urea (3.6 gm. per 10 ml. of virus suspension) and deproteinized in 1 *M* sodium chloride solution by chloroform-octanol (8 to 1). After centrifuging off the protein, the nucleic acid can be precipitated by 4 volumes of cold ethanol, washed in 80, 90%, and absolute ethanol and ether, and dried *in vacuo*.

The procedure of Hershey *et al.* (1953) involves treating the phage suspension directly with 1/10 volume of 3 *M* trichloroacetic acid (TCA) after the addition of 2 ml. of 1% serum albumin to improve the packing quality of the precipitate. The precipitate is centrifuged off, dissolved in 0.1 *N* NaOH, and reprecipitated by TCA. The acid-insoluble precipitate is warmed for 15–18 hours at 37°C. in *N* NaOH and precipitated in the cold with HCl and TCA. This precipitate is extracted with cold 0.3 *M* TCA, and then with 0.3 *M* TCA at 90°C. The extract is heated at 100°C. to decompose most of the TCA, and evaporated to dryness by further heating in a current of air, followed by storage in a vacuum desiccator.

In the recent report of Jesaites (1957) the viral structure was disrupted by repeated freezing and thawing of an aqueous suspension. After addition of sufficient sodium chloride to give a concentration of 1 *M*, the suspension is

deproteinized by stirring with chloroform-octanol (9 to 1), and the protein centrifuged off. The nucleic acid can then be precipitated from the supernatant by the addition of 2 volumes of cold 95 % ethanol. After subsequent dialysis and lyophilization, 80 to 90 % of the original nucleic acid can be recovered in this manner.

It is also possible to separate viral DNA in the T-even series by osmotic shock and removal of the ghost particles by centrifugation. The nucleic acid is subsequently precipitated with ethanol. With those phages in which the distal protein can be removed by the  $Cd^{++}$ -cyanide complex, complete liberation of the viral nucleic acid into the supernatant can be effected by the addition of the amino acid lysine. After centrifuging down the protein fragments, the DNA in the supernatant can be further purified by the procedures listed above.

Since the DNA content of T2 is about  $2 \times 10^{-16}$  gm. of nucleic acid per particle, the molecular weight of the DNA would be around 120,000,000 if it existed as a single molecule. Measurement of the DNA liberated by the urea treatment (Cohen, 1947) gives values of around 25,000,000.

#### A. ANALYSIS OF COMPONENTS OF VIRAL NUCLEIC ACID

The further analysis of viral DNA involves acid or enzymatic hydrolysis, and separation and determination of the liberated nucleic acid components, either by chromatographic procedures or specific reagents. Recognition of the fact that perchloric acid hydrolysis led to destruction of the characteristic pyrimidine, 5-hydroxymethyl cytosine (5-HMC) has induced the use of either 6 N HCl for 3 hours at 100°C. under  $CO_2$  in sealed tubes (the latter precaution is omitted by some workers), or 88 % formic acid (0.5 ml. per 1.5 mg. of virus) in a sealed tube for 30 minutes at 75°C. The further separation and determination of the purine and pyrimidine bases is made usually by paper chromatography with elution and determination of the characteristic ultraviolet (UV) absorption of the individual bases. One- and two-dimensional chromatographs are used, propanol-HCl and propanol- $NH_4OH$  being the most common solvents (Wyatt and Cohen, 1953; Hershey *et al.*, 1953). The bases can also be separated and determined by ion exchange chromatography (Jesaites, 1957). Table II is a summary of values from the literature for the purine, pyrimidine, and glucose content of the T series of phages, the temperate phage  $\lambda$  which lysogenizes *E. coli* strain, and the coliphage C16 (Burnet, 1933). This represents the extent of our quantitative knowledge of the nucleic acid components of the bacterial viruses.

The most striking feature of the DNA of the even-numbered coliphages and phage C16 is the presence of the pyrimidine, 5-hydroxymethyl cytosine, (Fig. 2) which apparently replaces cytosine (since the latter is not found in

the nucleic acid of the T-even series), together with varying quantities of glucose, presumably attached to the 5-HMC. This pyrimidine, which has not

TABLE II  
COMPOSITION OF COLIPHAGE DNA

Virus	Adenine	Thymine	Guanine	Cytosine	5-Hydroxymethyl cytosine	Glucose
	(moles/100 moles estimated bases)					
T2r <sup>a</sup>	32.5	32.6	18.2	—	16.7	13.6
T2l <sup>a</sup>	32.4	32.4	18.3	—	17.0	—
T6r <sup>a</sup>	32.5	32.5	18.3	—	16.7	26.9
T5 <sup>a</sup>	30.3	30.8	19.5	19.5	—	—
T4r <sup>b</sup>	32.8	35.8	18.0	—	13.5	17.6
T3 <sup>b</sup>	23.7	23.5	26.2	27.7	—	—
λ <sup>c</sup>	21.3	28.6	22.9	27.1	—	—
C16 <sup>d</sup>	27.9	36.0	12.9	—	22.8	—
T7 <sup>e</sup>	26.0	26.0	24.0	24.0	—	—
A1 <sup>e</sup>	23.4	33.3	18.8	24.6	—	—

<sup>a</sup> Wyatt and Cohen (1953).

<sup>b</sup> Knight and Fraser (Knight, 1954).

<sup>c</sup> Lwoff (1953).

<sup>d</sup> Mackal and Meyer (1957).

<sup>e</sup> Lunan and Sinsheimer (1956).

been found in any other naturally occurring material, is not present in the odd-numbered T phages and, so far as is known, these do not contain any

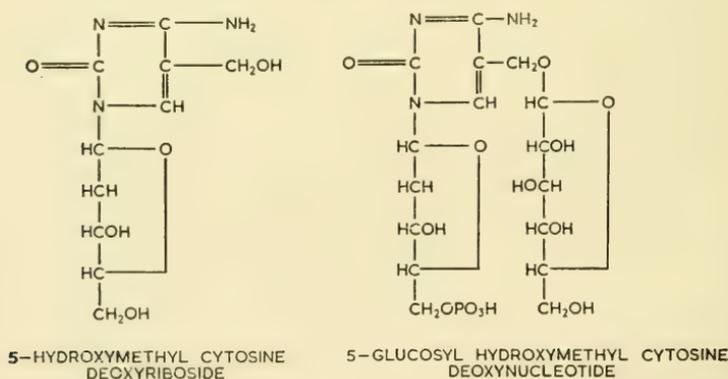


FIG. 2.

comparable purine or pyrimidine bases. The presence of 5-HMC was first discovered by Wyatt and Cohen in 1952. The nature of the compound was demonstrated by its isolation in sufficient quantities for analysis and by direct

comparison with the synthetically prepared compound (Wyatt and Cohen, 1953; Miller, 1955).<sup>1</sup>

The presence of glucose was first reported by Jesaitis in T4 (1954), and was discovered independently in T2 by Sinsheimer (1954), and in T6 by Volkin (1954). The identity of the compound was established by its behavior on chromatographic analysis. As indicated in Table II the amount of glucose present varies with the coliphage strain. Cohen (1956) has reported data for the glucose content of mutant strains of the coliphages and has suggested on the basis of his figures that the difference between T2r<sup>+</sup> and T2r, for example, can be associated with the presence of an increased amount of glucose in the DNA of the latter. Sinsheimer (1956), on the basis of analytical figures for other strains, does not concur.

While enzymatic degradation with DNAase and diesterase from snake venom causes a practically quantitative breakdown of DNA to the mononucleotide stage with T7 (Lunan and Sinsheimer, 1956), and presumably with T3 and other odd-numbered phages, this is not the case with those coliphages containing 5-HMC and glucose. With these, enzymatic degradation yields only about 60–70 % of the total phosphorus in the form of the mononucleotide. In T6, for example, while 60 % of the total thymydylic, deoxyadenylic, and deoxyguanylic acids is found as mononucleotide, only 23 % of the hydroxymethylcytidylic acid is liberated as mononucleotide (Jesaitis, 1957). In T2 (Sinsheimer, 1954) and T4 (Volkin, 1954) the quantity of 5-HMC nucleotide is somewhat higher (about 17 %), the percentage of the other mononucleotides remaining the same. It will be noted that the molar proportion of glucose to 5-HMC is below 1 in the case of T2, about 1 in T4, and is almost 2 in the case of T6. These facts are of interest in connection with the conclusion that the point of attachment of glucose is via the hydroxyl group of 5-HMC. In the case of the hydroxymethyl cytidylic acid from T2 (Sinsheimer, 1954), somewhat less than half was present as the free mononucleotide, with the rest present as the monoglucose derivative. With T4 (Volkin, 1954), all of the hydroxymethyl cytidylic acid liberated was in the form of the monoglucoside. With T6 (Jesaitis, 1957), on the other hand, somewhat more than 20 % of the liberated hydroxymethyl cytidylic acid is present in the unsubstituted form, the remainder being found as a diglucoside. While the exact structure of the latter is not known, it seems very probable that the two molecules of the hexose are linked as a disaccharide to the 5-hydroxymethyl group of the pyrimidine. Whether the major portion of the glucose present in the enzyme-resistant polynucleotide residue is attached in the same fashion or not is uncertain. The observation that the monoglucoside of hydroxymethyl

<sup>1</sup> A synthesis permitting the incorporation of C<sup>14</sup> into the 2 position has been recently described (Weygand and Swoboda, 1956).

cytidylic acid is resistant to phosphatase action while the unsubstituted mononucleotide is readily split suggests that the presence of the glucose substituent may be responsible for the lack of complete enzymic hydrolysis of viral DNA in the T-even phages.

## B. HETEROGENEITY OF VIRAL DNA

The principal chemical evidence for the heterogeneity of viral DNA is derived from the work of Brown and Martin (1955). These workers prepared DNA from purified preparations of T2 after precipitation with 95 % ethanol, resuspending the precipitate in 0.2 *M* NaCl, and agitating with chloroform in a blender and centrifuging to remove denatured protein. After three such treatments with chloroform, the process was repeated with a 9 : 1 chloroform-octanol mixture until no further denatured protein precipitate was formed. The DNA was then precipitated in 95 % alcohol, washed in 75 % alcohol, and stored in 75 % alcohol at 4°C. until required. By combining fractionated histone with cellulose by diazotization, adsorbing the nucleic acid on the cellulose histone, and then eluting with NaCl, they were able to obtain elution patterns showing two large peaks. One of these contained about 30 % of the total DNA phosphorus, with the ratio adenine + thymine/guanine + 5-HMC being approximately 1.9; the remaining 70 % had a ratio of about 2.15. Measurement of the glucose content of the two fractions by anthrone indicated that the quantity of glucose in the first fraction was molecularly equivalent to 65 % of the 5-HMC, while with the second fraction there was complete equivalence between 5-HMC and glucose. As it is unlikely that these distinct fractions would be produced by degradation of a larger single molecule by the preparative methods used, it appears that the T2r phage particle contains at least two different species of DNA molecule which differ in their ease of dissociation from protein and in their content of purine and pyrimidine bases.

These chemical observations are of interest in connection with the findings of a number of investigators over the last six years, which show that about half of the parental DNA of an infecting virus particle is transferred to the viral progeny in big pieces, the remainder being more widely distributed. (See Stent, Chapter III under Bacterial Viruses in Volume II for a full discussion.) In experiments in which the distribution has been traced into the second generation, the large pieces do not undergo any further breakdown. Levinthal (Levinthal, 1955, 1956; Levinthal and Thomas, 1957) has recently developed a sensitive radioautographic technique that allows him to measure the radioactivity of a single virus particle labeled with P<sup>32</sup>, if its atoms emit more than 10  $\beta$  particles per month. This involves surrounding the particle under study with a photographic emulsion sufficiently sensitive so that fast

electrons leave a visible track which can be observed directly under the microscope. Using  $P^{32}$ -labeled T2, Levinthal has followed individual phage particles through three infectious cycles involving phage adsorption, multiplication, and liberation. Since he could detect significantly labeled DNA in the progeny, despite the formation of more than a thousand viral progeny, he concluded that a large fragment of viral DNA (about 40 % of the size of the parental DNA) was maintained and transmitted in an intact state. In the second generation, derived from the progeny of the first replication, individual virus particles contained 20 % of the original parental radioactivity; however, in subsequent generations, the 20 % fragment was maintained as a unit. Levinthal has suggested that the original 40 % fragment is the duplicate viral chromosome, dividing into its component parts on replication and is the agent responsible for the transmission of genetic information.

When  $P^{32}$ -labeled DNA from osmotically shocked T2 was examined by the method of Brown and Martin (Brown and Simons, 1957), and the two fractions examined by the radioautographic technique of Levinthal, it appeared that fraction 1 (adenine + thymine/guanine + 5-hydroxymethyl cytosine = 1.9) was identical with Levinthal's large DNA fragment. However, Cohen (1957) reports that physical examination of the DNA isolated by the urea method does not reveal two grossly different classes of polymer, and the experiments of Stent, Sato, and Jerme (see Delbrück and Stent, 1957) are in conflict with the view that the big piece of DNA is the phage chromosome.

## V. OTHER VIRAL SPECIFIC PRODUCTS

Inasmuch as viral replication involves a redirection of the metabolic activities of the host cell with the synthesis of characteristic protein and, in the case of the T-even phages, the synthesis of 5-HMC, one might expect to find, in the infected cell, specific viral compounds that are intermediate or corollary to the synthesis of phage. The presence of particles, presumably representing empty phage heads, tails, and tail cores in lysates from infected cells or from infected cells treated with proflavin has been described (see Section III, B). Burton (1955) has presented evidence for the necessity of a brief period of protein synthesis prior to the synthesis of viral DNA. This early formed protein may represent the special enzymes needed to synthesize 5-HMC, but definite information with respect to either the chemical composition or function of this material is lacking. While there are promising hints with respect to the biosynthetic paths leading to 5-HMC in infected cells (see Cohen and Barner, 1957), here, also, the final answer awaits further investigation.

## ADDENDUM

Since the completion of this review, several additional and important observations concerning the chemistry of the bacterial viruses have been reported. Ames *et al.* (1958) have shown the presence in phage T4 of the polyamines, putrescine, and spermidine in quantities sufficient to neutralize much of the viral DNA. However, with the salmonella phages PLT-22 and 98, spermine was present with little or no putrescine and spermidine. Also, Kozloff and Lute (1958) have demonstrated the presence of small quantities of ATP and deoxyATP in phage T2, presumably in association with the contractile protein described in Section III, B, 2.

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## Chapter VIII

### The Comparative Chemistry of Infective Virus Particles and of other Virus-Specific Products : Animal Viruses \*

WERNER SCHÄFER

*Max Planck Institut für Virusforschung, Tübingen, Germany*

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#### I. INTRODUCTION

Although the first animal virus was detected nearly sixty years ago by Loeffler and Frosch (1898), our knowledge of the chemical properties of animal viruses is still comparatively inadequate. Well-verified observations are available for only a small number of these agents; the virus of foot-and-mouth disease, studied by Loeffler and Frosch (1898), is not yet in this class.

The chief obstacle to extensive progress in this field was the difficulty of preparing sufficient quantities of pure virus material. Workers on plant and bacterial viruses were in a much more favorable position. At present, however, the prospects of chemical investigations on animal viruses can be viewed more optimistically, since the methods of their cultivation, assay, and purification, as well as the techniques of chemical analysis, have been greatly improved.

\* The survey of literature pertaining to this chapter was completed in October 1957. The author is greatly indebted to Dr. R. M. Franklin for the translation of the manuscript.

This chapter will give a critical survey of our present knowledge concerning the chemical composition of the different virus-specific units, and will summarize briefly the origin and function of the chemical components. Only those animal viruses that can be regarded with certainty as true viruses will be discussed; therefore, the agents of the psittacosis-lymphogranuloma group and the rickettsiae are not included.

## II. DEFINITION OF THE VARIOUS VIRUS-SPECIFIC UNITS

The most detailed chemical analyses were generally made on the *infective particles*—the virus-specific units that are fully capable of reproducing new copies in the cells.

Other substances have been found which are noninfectious and demonstrable as virus-specific products only by seroimmunological reactions. They were first demonstrated with vaccinia (Craigie, 1932), and later in many other virus infections (see Smadel, 1952a). Since they are generally smaller than the corresponding infective particles, or at least sediment slower by ultracentrifugation, they have been designated as *soluble antigen* (S antigen), although this designation is not very meaningful. It has been noted in many cases that these S antigens are less specific than the superficial antigens of the infective particles.

The *hemagglutinins*, found with viruses of the pox group (Nagler, 1942; see Burnet, 1955a), are also noninfectious. Like the S antigens, they sediment slower than the infective particles and possess a virus-specific antigen, but they are mainly characterized by their ability to agglutinate red blood cells.

Of special interest are those hemagglutinating units which accompany the myxoviruses, and are usually designated as *incomplete forms* (von Magnus, 1947; see 1954). In most cases these can be distinguished physically from the infective particles by their lower sedimentation velocity. In comparison with infective particles, the ratio of infectivity to hemagglutination ( $ID_{50}/HA$ ) is smaller in preparations of incomplete forms. It has not been determined whether the infectivity, which has always been demonstrated in such preparations, is due merely to the presence of some infective particles or whether the incomplete particles themselves have a low, but constant, capacity to initiate infection.

One can regularly find incomplete forms in extracts of chick chorioallantoic membranes infected with influenza, fowl plague, or Newcastle disease virus, regardless of the virus dose used for infection (Granoff *et al.*, 1950; Granoff, 1955; Henle *et al.*, 1956; Schäfer and Munk, 1952b; Schäfer *et al.*, 1954). Only in the case of influenza could appreciable amounts of incomplete particles be detected in the supernatant fluids, e.g., allantoic fluid. According to Henle and co-workers (1956), incomplete forms are found outside when the

host cells initially adsorbed several virus particles within a short time interval. The adsorbed particles can be either fully infectious or partly non-infectious. Allantoic fluid of very low infectivity to hemagglutination ratio can be obtained by serial passages of undiluted infectious allantoic fluid (von Magnus, 1947). Units that resemble incomplete forms have also been obtained when mice are inoculated intracerebrally with non-neurotropic influenza strains (Schlesinger, 1950), and by the infection of HeLa cells with influenza (Henle *et al.*, 1955).

The incomplete forms produced under the various conditions are not identical. From studies of Granoff (1955), the influenza incomplete forms, obtained by undiluted passages, differ in both biological and physical properties from those found in the allantoic membrane after infection with small doses of virus. Furthermore, two types of influenza incomplete particles have been observed in the electron microscope. Those obtained from allantoic fluid after serial undiluted passage have nearly the same appearance as the infective particle (see von Magnus, 1954); whereas flat, membrane-like structures, with a rough surface architecture and average diameters larger than those of the infective particles (Werner and Schlesinger, 1954), were isolated from extracts of some infected cells. The latter type is very similar to the incomplete forms of fowl plague (Schäfer *et al.*, 1954).

In all likelihood the various particles now designated as incomplete forms will have to be reclassified after further investigations. Until such classification is available, one should always indicate how the 'incomplete forms' in question were obtained.

*Filamentous forms*, also found with several representatives of the myxovirus group (influenza, fowl plague, and Newcastle disease) (Mosley and Wyckoff, 1946; see Schlesinger, 1953), are so large that they can be observed in the light microscope. They can attain lengths up to several microns and have a width of 60 to 80  $\mu$ . It has been suggested occasionally that they are subdivided into spherical-shaped particles, but more frequently one observes a single spherical particle at the end of a filament. It has not yet been definitely decided whether the filamentous forms are infectious, since they have not been isolated. They are able to hemagglutinate and they possess virus-specific antigen (Chu *et al.*, 1949; Bang and Isaacs, 1957). Since the ultrasonic disruption of these structures tends to increase the hemagglutinating activity (Donald and Isaacs, 1954a), one can assume that they contain a larger number of hemagglutinating subunits.

There is no clear understanding of the function of the S antigens, the hemagglutinins of the pox viruses, or of the incomplete forms of the myxoviruses. Three hypotheses have been formulated. The first claims that they are necessary stages in the synthesis of new virus particles. Second, they

could be breakdown products of infective particles; and third, they may be side products of abnormal virus synthesis.

On the other hand, it is well known that the filamentous forms arise as surface protrusions of the infected cells. Some diversity of opinion exists, however, as to whether or not the spherical infective particles are produced from them.

### III. PROBLEMS AND METHODS OF CHEMICAL ANALYSIS

The chemical studies of the various particles are directed, first, toward a qualitative and quantitative determination of the main constituents, followed by a more precise analysis of their chemical composition, e.g., amino acid analysis of the proteins.

This can now be accomplished with relatively small amounts of virus material, by using several sensitive and accurate analytic methods, such as spectrophotometry and paper chromatography. A discussion of the various analytical procedures is not within the scope of this chapter. In carrying out such studies it should be kept in mind that some fractions may consist of more than one component. Thus, the protein fraction can be composed of different proteins, as suggested by the fact that some animal viruses possess several antigens. A similar situation may hold for the nucleic acid of the larger viruses.

Only a few virus particles are needed for a method developed by Dawson and McFarlane (1948) to study their chemical composition. In this procedure, the virus is incubated with enzymes after some appropriate pretreatment and then is observed in the electron microscope to see if any degradation has occurred.

The main application of this method is to localize the individual chemical components in the virus particle. Frequently, serological studies can determine whether an antigenically active component is located on the surface of the particle. X-ray crystallographic analyses can also be made, since two animal viruses have been crystallized (Schaffer and Schwerdt, 1955a; Mattern and Du Buy, 1956). One of the most important advances in this direction would be obtained with the development of electron optical staining techniques.

In looking for biochemical activities associated with the virus particle, one is especially interested in enzymes. Here, it should be borne in mind that virus enzymes can possess activities not previously known; one good example is the enzyme found in influenza virus (see Gottschalk, 1957). Actually, only one animal virus has been extensively studied with respect to different sorts of enzymes (see Smadel and Hoagland, 1942).

When the chemical components of the various virus-specific particles have been identified, the way is prepared for studies concerning their origin and

function. Labeling with radioactive isotopes can be of great value, but this procedure has not yet been fully exploited in the animal virus field.

#### IV. CHEMICAL COMPOSITION OF INFECTIVE PARTICLES AND OTHER VIRUS-SPECIFIC PRODUCTS

The value of the results of chemical analyses depends mainly on the purity of the preparations under study. This particular point brings us to one of the great difficulties in carrying out chemical studies on animal viruses.

Impurities, such as normal components of the host cell or virus-specific products of a type other than the particles in question, are often present. They are sometimes adsorbed to the latter and, therefore, are difficult to remove. In other cases, they are not bound to the particles in question, but are so similar to them in their physical and chemical properties that they cannot be separated, and are recognized only with difficulty.

With this situation in mind, the following discussion will include a brief summary of the particular purification procedure and tests for purity and identification employed, in order to facilitate a critical judgment of the available data.

##### A. *Smaller Viruses*

###### 1. *Poliomyelitis Virus*

The purest animal virus preparation now available is obtained with the infective particles of poliovirus. These particles are uniform and spherical, approximately 27  $m\mu$  in diameter, with a particle weight of  $\sim 6.7 \times 10^6$  (Schwerdt *et al.*, 1954; Schwerdt, 1957a).

The purification (Schwerdt and Schaffer, 1956; Schwerdt, 1957a) procedure involves the use of the supernatant fluid of infected tissue cultures. Only the most important steps in the procedure are given here: precipitation of the virus with methanol at pH 4 and elution of the precipitate in molar NaCl at pH 9. This is followed by two extractions with *n*-butanol, then precipitation and elution as previously, but without the methanol. Further treatment includes one cycle of high- and low-speed centrifugation, treatment with ribonuclease (RNAase) and desoxyribonuclease (DNAase), followed by a second cycle of high- and low-speed centrifugation. In this manner, one can obtain approximately 0.2 mg. protein from one liter of tissue culture fluid. Impurities still present are chiefly removed by sedimentation in a sucrose density gradient, by which four fractions, sedimenting with different velocities, could be separated. The virus contained in the fastest fraction, is now so pure that it can be crystallized into small tetragonal prisms with pyramidal ends. Recrystallization leads to no loss in infectivity. Ultracentrifugal studies and electron microscopy, as well as serological tests with antiserum to normal host-cell antigens, further confirmed the purity of the preparations

obtained. The ratio of physical particles to plaque-forming units is about 30, making it probable that the spherical particles are identical with the infective unit.

In such highly purified preparations of infective particles only nucleic acid and protein were found (Schwerdt and Schaffer, 1955; Schaffer and Schwerdt, 1955b; Schwerdt, 1957a). The quantity of carbohydrate determined by the anthrone test corresponded to the amount expected from the nucleic acid content. The high, dry-weight density (1.56–1.62), as well as the fact that the virus infectivity was not affected by organic solvents, suggested that lipids are absent.

The nucleic acid present is ribonucleic acid (RNA), most elegantly shown by the chromatographic demonstration of the appropriate bases (Table I). The purified virus possesses 22–30 % RNA, as determined by the quantitative orcinol test. This amount corresponds to a single unit of RNA, if one assumes that the biologically active RNA molecule has a weight of  $\sim 2 \times 10^6$ , as found for tobacco mosaic virus (Gierer, 1957).

The amino acid composition of the protein fraction has not yet been analyzed.

Incubation of the virus with RNAase during the purification procedure does not affect the virus infectivity, suggesting that the RNA is surrounded by protein.

In addition to the infective particle, a slower sedimenting, noninfectious unit recently has been isolated (Schwerdt, 1957a; Schwerdt, 1957b; Mayer *et al.*, 1957; Mayer, 1957). Its virus specificity is revealed only by serological tests and, therefore, it can be considered as an S antigen, according to our definition. The antigenic behavior of at least a fraction of these units is different from that of the infective particles.

This S antigen can be isolated in the last stage of the purification process—the ultracentrifugation in the sucrose density gradient—from the layer above the virus particles. Appropriate serological tests showed that preparations obtained by this method were free of host-cell antigen. By electron microscopy the S antigen appears as a round, flattened, low-contrast particle, of somewhat larger diameter than the infective particle. In contrast to the latter it contains little or no nucleic acid. It seems to be composed essentially of protein.

The composition of poliovirus brings to mind that of the spherical plant viruses. The resemblance between the noninfectious, nucleic acid-free particle of turnip yellow mosaic virus (Markham, 1951) and the S antigen of poliovirus is striking.

## 2. Rabbit Papilloma Virus

The spherical particles of rabbit papilloma virus are larger than the infective particles of poliovirus, but are nevertheless comparatively uniform,

with a diameter of  $45 \mu$  (Williams, 1953) and a particle weight of  $\sim 47 \times 10^6$  (Neurath *et al.*, 1941).

These units are obtainable from extracts of the keratinized masses of papillomas (see Beard *et al.*, 1955). About 1 mg. of virus material can be isolated from 1 gm. of papilloma material through purification by alternate high- and low-speed centrifugation. Earlier ultracentrifuge, diffusion, electron microscope, and electrophoretic studies suggested that such virus preparations were comparatively pure (Neurath *et al.*, 1941; Sharp *et al.*, 1942a,b). But later it was shown that they still contained an impurity of unknown chemical composition (Schachman, 1951). Since the infectivity test available is relatively insensitive, it is difficult to determine whether all of the characteristic particles are infective.

Chemical studies (Taylor *et al.*, 1942; see Beard *et al.*, 1955) on such material showed that essentially only nucleic acid and protein were present. Treatment with lipid solvents led to the extraction of some 1.5 % lipid material, possibly an impurity. There was no evidence of carbohydrate in excess of that expected from the nucleic acid.

The nucleic acid content is about 8.7 % and, in contrast to poliovirus, is exclusively of the deoxyribose type (DNA). From the DNA content and the particle weight of  $47 \times 10^6$ , one can conclude that the entire nucleic acid of the papilloma virus is represented by a single DNA molecule, assuming a molecular weight of  $4 \times 10^6$  (see Jordan, 1955). There has been no detailed study of this DNA. Nothing is known concerning the localization of the DNA in the virus particle.

Studies on the amino acid composition of the protein fraction have been made on two preparations, using microbiological assays (Knight, 1950; see Knight, 1954). Only the L-isomers of the amino acids were found. In contrast to the protamines and histones, which are coupled to sperm DNA and contain a large proportion of basic amino acids, a preponderance of the acidic amino acids was detected in the protein of papilloma virus.

Substances such as the S antigens, which could be distinguished from the above-described units, have not been observed in papillomatosis.

### 3. *Equine Encephalomyelitis Virus*

The spherical infective particles of equine encephalomyelitis are about  $40\text{--}50 \mu$  in diameter (Sharp *et al.*, 1943; see Beard, 1948). A particle weight of  $152 \times 10^6$  has been calculated from physicochemical measurements (Taylor *et al.*, 1943) but this value is difficult to bring into agreement with the diameter, as determined by electron microscopy. A value of about  $50 \times 10^6$  would seem to be a better approximation for a virus particle of the observed size.

The chemical studies were made on virus material, which was obtained from extracts of infected chick embryos by several cycles of high- and low-speed centrifugation (Taylor *et al.*, 1943). About 0.5 mg. of purified end-product was obtained from one gm. of embryo tissue. Although this product seemed to be homogeneous in the electron microscope and the analytical ultracentrifuge, its purity is questionable, since one infective dose ( $JD_{50}$  in mice) corresponded to an amount of protein equivalent to about 250 particles of  $152 \times 10^6$  particle weight. Furthermore, the high frictional ratio ( $f/f_0$ ) of 2.3 does not agree with the spherical form of the particles observed in the electron microscope, but suggests that some impurity of higher viscosity was present. According to the serological studies (Engel and Randall, 1947), this seems to be a normal component of the tissue.

Chemical investigations (Sharp *et al.*, 1940; Taylor *et al.*, 1943; see Beard, 1948) showed that these preparations of infective particles of equine encephalomyelitis contained protein, nucleic acid, lipids, and a small fraction of carbohydrate.

The nucleic acid is of the ribose type and comprises about 4.4 % of the particle. Assuming that the impurities represent a relatively insignificant fraction, the nucleic acid found corresponds to one RNA molecule (Gierer, 1957), on the assumption of a weight of  $50 \times 10^6$  for the virus particle. The high lipid content of 54 % is striking, and is composed mainly of phospholipid (35 % of the whole virus). Cholesterol and neutral fats are also present. The loss of infectivity after treatment of the equine encephalomyelitis virus particles with ether (Birch, 1941; Sulkin and Zarafonitis, 1947), suggests that lipid is an integral part of these units.

On the basis of a dextrose standard the total carbohydrate content, including the nucleic acid ribose, was only 4 %.

There is no mention in the literature of the existence of a S antigen in encephalomyelitis.

No enzymes have been reported to be associated with the equine encephalomyelitis virus or with any of the other small viruses described above.

## B. Viruses of Medium Size

### 1. Influenza and Fowl Plague Viruses

Most interest in the medium-sized viruses has been centered around the myxovirus group. All the representatives of this group are capable of agglutinating red blood cells. The infective particles are not as uniform in size as those of the smaller viruses. Influenza and fowl plague are the myxoviruses that have been analyzed chemically most extensively. Since they are serologically related (Schäfer, 1955b; Nitzschke, 1956; Schäfer, 1957b), they will be considered together.

In both cases the infective particles are spherical, with an average diameter of  $\sim 70$  m $\mu$  for fowl plague (Schäfer *et al.*, 1952), and  $\sim 80$  m $\mu$  for influenza virus (Williams, 1953). The particle weight of the fowl plague virus is  $150 \times 10^6$  (Schäfer *et al.*, 1952) and for type A of influenza virus, the type chemically analyzed in most detail, about  $280 \times 10^6$  (see Schramm, 1954a).

The infective particles of both viruses contain at least two types of subunits (Hoyle, 1952; Hoyle *et al.*, 1953; Schäfer and Zillig, 1954; Schäfer, 1957b). One of these is the hemagglutinin and the other the internal S antigen or "gebundenes" (G) antigen. In order to avoid confusion with free hemagglutinins and the external S antigens the two subunits of the infective particles will be designated as virus-hemagglutinin and G antigen in the following discussion. Virus-hemagglutinin and G antigen may be obtained by treating the virus particles with ether. They are not infectious, either separated or in a mixture.

The virus-hemagglutinin has a diameter of 30 m $\mu$ , as determined for the fowl plague virus. It is able to agglutinate red cells and corresponds serologically to the superficial virus antigen (V antigen) of the infective particles. The G antigen is isolated from the fowl plague virus in chainlike arrays, with individual spherical components, each having a diameter of approximately 15 m $\mu$ . It can be characterized as a virus-specific unit only by its serological behavior. In the case of fowl plague, where the purest preparations of subunits were obtained, there is no cross reaction between G antigen and virus-hemagglutinin. The above-mentioned serological relationship between the infective particles of fowl plague and influenza seems to extend only to the G antigens, which was demonstrated using the Rostock strain of fowl plague and the FM1 strain of influenza (Schäfer, 1955b; Schäfer, 1957b).

For chemical investigations, highly purified infective particle preparations of fowl plague and influenza viruses are usually obtained from the allantoic fluids of infected chick embryos. The virus particles are first adsorbed on erythrocytes, the cell/virus-complex is thoroughly washed in the cold, and the virus then eluted from the cells at 25-37°C. This is followed by one or several cycles of high- and low-speed centrifugation (Taylor, 1944; Ada and Perry, 1954b; Zillig *et al.*, 1955). In the case of fowl plague virus, 7 mg. of protein per liter of starting material is obtained by such a procedure.

There are considerable difficulties associated with the tests for purity for influenza and fowl plague infective particles. Preparations that are homogeneous with respect to their electrophoretic and ultracentrifugal behavior, as well as by electron microscopy, still contain an antigenic moiety characteristic of the host (Knight, 1946; Munk and Schäfer, 1951; Schäfer *et al.*, 1952). This antigen, designated as normal component, must somehow be tightly bound to the virus, since it cannot be eliminated, even with the aid of specific

antibody. The question arose whether this is an integral part of the infective particle; Smith and co-workers (Smith *et al.*, 1953, 1955) believe it is, since the normal host-cell antigen and the 'normal' component of the influenza virus are slightly different immunologically and also possess different thermal stabilities. Furthermore they could show that there are also differences between the 'normal' components of influenza A and B viruses.

From quantitative studies, it has been determined that about 10 spherical particles correspond to one  $JD_{50}$  in eggs (Isaacs and Donald, 1955; Donald and Isaacs, 1954b). Since one can never completely prevent the inactivation of infective particles by external influences and since the infectivity tests are not so sensitive that every particle can cause an infection, one can safely assume that at least a large majority of the spherical units is identical with the infective particles.

According to our present knowledge, the purest preparations of infective particles of influenza and fowl plague contain four main constituents: nucleic acid, protein, lipid, and carbohydrate.

Very probably RNA is the only nucleic acid present (Ada and Perry, 1954b; Frommhagen and Knight, 1956; Frisch-Niggemeyer and Hoyle, 1956; Ada, 1957; Zillig *et al.*, 1955; Schäfer, 1957b). The small amount of DNA (0.1 %) that recent studies, using microbiological tests (Miller, 1956), demonstrated in influenza virus preparations may be due to impurities, although Miller (1956) is not of this opinion. Reports on the RNA content of influenza virus vary between 0.7 and 1 %, and for fowl plague virus between 1.8 (von Zahn-Ullmann, unpublished) and 4 %, corresponding to 1 molecule of M.W.  $2 \times 10^6$  (Gierer, 1957) per infective particle of influenza and 1-3 molecules per fowl plague virus. Chromatographic studies of the bases of different strains of influenza virus have shown that differences exist in the base ratios (Table I) of the A and B type viruses (Ada and Perry, 1955b, 1956); and they are considered characteristic by Ada (1957). Thymine, the pyrimidine base characteristic of DNA, was not found in any case.

Up to this point the discussion has referred to virus preparations obtained from infectious allantoic fluid. Ada (1957) has also studied the RNA composition of influenza virus preparations obtained from lungs of infected chick embryos, where different base ratios were found. However, one must carefully test the possibility that these differences are due to host material impurities, which could be present in larger quantities when tissue extracts are employed as the starting material. The lower specific activity (hemagglutinating units per milligram dry weight) of the virus obtained from lung material, as compared with the virus obtained from allantoic fluid, suggests such an interpretation.

The major percentage of the infective particles is protein in both viruses (60-70 %) (Taylor, 1944; see Beard, 1948; Zillig *et al.*, 1955). Amino acid

analyses have been made only of the influenza virus (Knight, 1947b). As with the base ratios, some differences were also observed here between the A and B types, specifically in the amino acids arginine, glutamic acid, lysine, tryptophan, and tyrosine. In further work it will be necessary to differentiate between the proteins of G antigen and virus-hemagglutinin.

TABLE I  
PROPORTION OF BASES IN THE NUCLEIC ACID OF ANIMAL VIRUSES

Source of nucleic acid	Number of analyses	Bases (moles/100 moles)				
		Adenine	Guanine	Cytosine	Uracil	Thymine
Poliomyelitis						
Infective particle						
Mahoney	5	30.5	25.5	19.2	24.8	—
Influenza						
Infective particle						
A (PR8)	5	23.1	20.1	24.0	32.8	—
A (MEL)	2	23.0	19.7	25.3	32.0	—
A (WSE)	2	22.6	20.1	24.1	33.2	—
A (Swine)	2	22.7	20.4	24.5	32.4	—
A (CAM)	2	22.7	19.3	24.5	33.5	—
Incomplete forms						
A (PR8) <sup>a</sup>						
ID <sub>50</sub> /HA = 4.5	1	23.6	20.0	24.1	32.3	—
A (PR8) <sup>a</sup>						
ID <sub>50</sub> /HA = 3.9	1	23.0	20.3	24.4	32.3	—
Infective particle						
B (LEE)	4	23.0	18.3	23.1	35.6	—
B (MIL)	3	22.8	17.5	23.7	36.0	—
B (ROB)	2	22.5	18.6	23.4	35.5	—
Vaccinia						
Infective particle	1	29.5	20.6	20.0	—	29.9

<sup>a</sup> Undiluted passage virus from allantoic fluid.

The lipid of the influenza and fowl plague infective particles is  $\sim 25\%$  and consists chiefly of phospholipid and cholesterol (Taylor, 1944; see Beard, 1948; Zillig *et al.*, 1955). The amount of neutral fat seems to be negligible according to recent investigations with influenza virus (Frommhagen *et al.*, 1958). The treatment of the virus particles with ether results in their disintegration and in a loss of infectivity (Hoyle, 1952; Schäfer and Zillig, 1954). Hence it would appear that the ether-soluble lipids are necessary for the maintenance of the structure of the particles and are not impurities that are difficult to remove.

\* Ada and Perry (1954b) claim that PR8-influenza virus contains 44% lipid.

Several carbohydrate compounds have been demonstrated in influenza virus (Taylor, 1944, Knight, 1947a). Besides the ribose from RNA, galactose, mannose, fucose, and amino sugar have been identified by chromatographic means in recent investigations (Ada and Gottschalk, 1956; Frommhagen and Knight, 1956). Ada and Gottschalk (1956) as well as Frommhagen and Knight (1956) have reported that glucosamine is present. The total percentage of carbohydrate, excluding the RNA sugar, is approximately 3%, according to Frisch-Niggemeyer and Hoyle (1956), or 5–8%, according to Ada and Gottschalk (1956), and older studies of Knight (1947a). Since heteropolysaccharides of similar composition are always bound to protein, it is suggested that mucoprotein also occurs in the influenza virus particle (Ada and Gottschalk, 1956). The amount of carbohydrate in fowl plague virus seems to be higher than in influenza (Zillig *et al.*, 1955); there has not yet been an analysis of the individual carbohydrates.

In the earlier studies on the hemagglutination phenomenon made by Hirst (1942), it was observed that influenza virus particles adsorbed onto the surface of red cells eluted spontaneously when the complex was incubated at 37°C. Thereafter the cells were no longer capable of adsorbing virus particles and hence could not be agglutinated. On the other hand, the eluted virus was completely intact, functionally. Thus it was assumed that a virus enzyme had destroyed the cell receptors responsible for adsorption of the virus particle. Other viruses of the myxovirus group were later found to behave similarly (see Hirst, 1952). A more extensive biochemical investigation of the postulated enzyme became possible when it was found that mucins from different sources can inhibit the hemagglutinating effect of the virus particles and that this inhibitor effect can be overcome by incubating the mucin-virus mixture at 37°C (see Gottschalk, 1957). From these observations the concept emerged that the particular mucins and the cellular receptors of the myxoviruses have a common chemical grouping that can be attacked by the virus enzyme.

Through extensive investigations on influenza virus it was recently proved that the enzyme has the character of a neuraminidase (see Gottschalk, 1957).

This must be a specific part of the virus particle, because it does not occur in the fluids from which the virus is obtained, in the uninfected host cells, or other animal cells. Only some microorganisms e.g. *Vibrio cholerae* produce a substance with similar enzymatic activity, referred to as receptor-destroying enzyme (RDE) (Burnet and Stone, 1947). The substrate and the action of the neuraminidase will be treated in detail in another volume (vol. III, chap. 4) of this book.

The neuraminidase in the infective particles seems to be located in or on the virus-hemagglutinin (Hoyle, 1952; Schäfer, 1957b). Since this contains only protein and carbohydrate (Frisch-Niggemeyer and Hoyle, 1956; Zillig

*et al.*, 1955), it seems likely that the mucoprotein of the virus particle (Ada and Gottschalk, 1956) is also situated here. Owing to a lack of sufficient material, it has not yet been possible to study the sugars in detail; however, by the use of superimposed absorption curves, it was suggested that both galactose and mannose are present in the virus-hemagglutinin of influenza (Frisch-Niggemeyer and Hoyle, 1956).

In contrast to virus-hemagglutinin the G antigen, of influenza and fowl plague virus, consists of protein and RNA. The G antigen of influenza virus contains 5.3 % RNA (Frisch-Niggemeyer and Hoyle, 1956; Ada, 1957) and that of fowl plague virus 10–15 %, possibly corresponding to the higher amount of RNA that is found in its infective particle (Zillig *et al.*, 1955; Schäfer, 1957b). In both virus types the G antigen seems to carry all the nucleic acid (Zillig *et al.*, 1955; Hoyle *et al.*, 1954; Paucker *et al.*, 1956). Thus one should not expect any differences in the nucleic acid composition of the infective particle and the G antigen. This has been demonstrated in influenza virus, where the proportion of the bases in the nucleic acid of the infective particle and of the G antigen are the same (Ada, 1957).

There is some evidence concerning the localization of the two subunits in the infective particle. The virus-hemagglutinin must be part of the surface, since it possesses the biological surface characteristics of the infective particle. These characteristics are the hemagglutinating and enzymatic activities, as well as the virus (V) antigen. On the other hand in highly purified preparations of infective particles the ribonucleoprotein antigenic component can only be detected in appreciable amounts after the particles are carefully disrupted. Thus, the G antigen and the RNA seem to be located in the interior of the infective unit (Schäfer and Zillig, 1954; Schäfer, 1957b; Lief and Henle, 1956a,b). Further evidence favoring this structure has been obtained by degrading the infective particle with various enzymes and studying the results in the electron microscope. There is an external shell of protein and an internal nucleoprotein ring having the RNA on its external surface (Valentine and Isaacs, 1957a,b).

Virus-specific products appearing along with the infective particles of influenza and fowl plague are the S antigen, the incomplete and the filamentous forms.

In both cases the S antigen cannot be serologically differentiated from the G antigen; in the case of fowl plague, where the S antigen has been isolated in a relatively pure form, the physical properties of the two antigens were also found to be similar.

The S antigen of fowl plague was purified from extracts of infected chorio-allantoic membranes (Schäfer and Munk, 1952a; Schäfer *et al.*, 1956). The first step was the removal of the infective particles, as well as the incomplete and filamentous forms, by adsorption onto red blood cells. This was followed by precipitation of the antigen at pH 4.5, shaking with butanol and ether,

precipitation with ammonium sulfate (40 %), and high- and low-speed centrifugation. Such preparations were free from impurities, as demonstrated by ultracentrifugation, electrophoresis, electron microscopy, and also by serological investigation with antiserum to normal component.

Chemical studies on the S antigen strengthened the concept that it is very closely related to the G antigen. S antigen also proved to be a ribonucleoprotein (Schäfer *et al.*, 1956; Schäfer, 1957b). But the RNA content of individual S antigen preparations varied to a greater degree. This is not unexpected, since the S antigen is not protected from degradation during the purification procedure as is the G antigen, which is within the virus particle. The RNA content of the fowl plague S antigen was found to be between 6 and 14 %. Studies of the composition of the RNA and the protein, which would be useful in a further clarification of the relationship between the two antigens of fowl plague, have not yet been made.

Attempts to purify influenza S antigen from chick embryo lung extracts were carried out by methanol precipitation and treatment with chloroform, or by ultracentrifugation in a sucrose density gradient (Ada *et al.*, 1952). It is difficult to judge the purity of the preparations obtained since extensive tests of purity have not been made.

As far as one can judge from studies on such preparations and on precipitates of these by specific antiserum to S antigen (Ada and Perry, 1954a), the influenza S antigen also seems to be a ribonucleoprotein. It contains about 6 % RNA. This corresponds approximately to the RNA content of the G antigen of the influenza virus.

In an S antigen preparation from infected chorioallantoic membranes, only 0.7 % RNA was found (Ada, 1957). This antigen had been isolated by ultracentrifugation and extraction with ether. The reason for this discrepancy is not clear.

The incomplete forms of influenza and fowl plague virus may be purified by cycles of adsorption on and elution from red blood cells, since they possess hemagglutinating and enzymatic activities like the infective particles (Schäfer *et al.*, 1954; Ada and Perry, 1956; Paucker *et al.*, 1956). This can be followed by several cycles of high- and low-speed centrifugation, which are chiefly useful in the removal of the infective particles still present, resulting in preparations quite pure physically (ultracentrifuge, electrophoresis, and electron microscope) (Schäfer *et al.*, 1954; Pye *et al.*, 1956). However in the case of fowl plague, a normal component could still be demonstrated serologically (Schäfer, 1955c).

Chemical studies have been made on influenza incomplete forms obtained from allantoic fluid after serial undiluted passage or infection with heat-inactivated (37°C.) standard virus. Reduction in the infectivity/hemagglutination ratio was accompanied by decrease in the nucleic acid content

(Ada and Perry, 1955a, 1956; Paucker *et al.*, 1956). The content of G antigen in this material behaved in a similar fashion; this was to be expected, since G antigen is the carrier of the RNA in the infective particle (Lief and Henle, 1956c). According to Ada and Perry (1956), the RNA content of preparations of incomplete forms can decrease to about 0.3 % (dry weight percentage). From his results, Ada (1957) came to the conclusion that the incomplete forms of influenza isolated from allantoic fluid are not uniform with respect to their nucleic acid content. No differences in the nucleic acid composition, compared with that of the infective particles, were detectable (Ada and Perry, 1956) (see Table I). In contrast to the nucleic acid content, the amount of lipids is higher in the incomplete forms than in the infective particles. This was suggested by experiments using P<sup>32</sup>-labeled material (Paucker *et al.*, 1956). The label in the alcohol-soluble fraction increased with increasing degrees of incompleteness. Uhler and Gard (1954) showed that substances soluble in ethanol and ethyl ether amounted to 54 % in incomplete forms from allantoic fluid.

Incomplete forms from infected tissues were investigated in fowl plague. Some 50–60 % lipid was found in preparations which possessed a high degree of purity (Schäfer, 1955a). Thus, it seems likely that a common characteristic of the incomplete forms of influenza and fowl plague is a relatively high lipid content, up to twice the percentage found in the infective particles. Glucose-6-phosphatase has also been demonstrated to be associated with preparations of the incomplete forms of fowl plague virus, but it is not yet clear whether this is an intrinsic component (Schäfer, 1957a).

The filamentous forms of influenza and fowl plague have scarcely been studied chemically. By treatment with enzymes, however, it has been shown that the filamentous particle of influenza is mostly trypsin-sensitive (Valentine and Isaacs, 1957a); consequently, it would seem to consist mainly of protein. Most agents capable of lysing red blood cells destroy the filaments. This led Burnet (1956) to conclude that the surface of filamentous forms has properties similar to those of the cell surface.

## 2. Newcastle Disease Virus

The Newcastle disease virus (NDV), which also belongs to the myxovirus group, is considerably larger than influenza and fowl plague virus. The flattened round particles, seen only under appropriate conditions in the electron microscope, have a highly variable diameter with an average value of 150 to 190 m $\mu$  (Elford *et al.*, 1948; Bang, 1948; Schäfer *et al.*, 1949). From 5 to 7 particles of this sort are needed for one ID<sub>50</sub> in eggs (Bang, 1948; Isaacs and Donald, 1955). The particles change their form in solutions of higher salt concentration, without loss in infectivity. After such treatment

one sees mostly extended forms. The techniques already described for influenza and fowl plague are also suitable for the purification of this agent.

The chemical composition of the infective particles of NDV seems to be similar to that of the myxoviruses previously discussed. Older studies (Cunha *et al.*, 1947; see Beard, 1948) have demonstrated the presence of  $\sim 65\%$  protein,  $\sim 4\%$  nucleic acid,  $\sim 7\%$  carbohydrate glucose equivalents, and  $\sim 27\%$  lipid, mostly phospholipid;  $3.5\%$  RNA was found, as well as  $0.61\%$  DNA. However, these investigations were carried out on preparations purified solely by ultracentrifugation and which, according to serological investigations, contain a large quantity of normal component (Munk and Schäfer, 1951). Recently, studies have been made on small amounts of carefully purified NDV which had been labeled with  $P^{32}$  (Franklin *et al.*, 1957). These showed that probably only one nucleic acid type is present in NDV, namely, RNA. The RNA is arranged on a ringlike structure that is surrounded by a trypsin-sensitive shell (Valentine and Isaacs, 1957b). In contrast, the phospholipid seems to be superficially situated, since it can be degraded by treatment with phospholipase. Since the degradation is associated with a loss of infectivity this fraction must be necessary to the particle (Franklin *et al.* 1957).

NDV possesses not only a receptor-destroying activity but also the ability to lyse red cells (Traub and Miehler, 1946; Kilham, 1949; Burnet and Lind, 1950). Burnet (1955b) considers that "hemolysis is due to enzymatic action going beyond the normal type of receptor destruction." Detailed studies are not available.

It is not yet known whether an S antigen occurs in NDV-infected tissues. Incomplete and filamentous forms are present but have not been chemically analysed.

### 3. *Virus of Avian Myeloblastic Leukosis*

The agent of avian myeloblastic leukosis, is morphologically very similar to NDV (see Beard *et al.*, 1955; see Beard, 1956). Large quantities of the virus ( $\sim 1.5$  mg./ml.) are present in the plasma of certain diseased chickens.

Although detailed chemical studies have not been made on this agent, it is mentioned here because an adenosinetriphosphatase (ATPase) is associated with it. The following observations suggest that the enzyme is coupled with the virus particle: (a) the enzyme and the specific particle appear progressively in the chick plasma with the development of the disease; (b) they cannot be separated from each other by electrophoresis or by ultracentrifugation; (c) they are precipitated together by antiviral serum.

But this behavior does not prove conclusively that the ATPase is actually an intrinsic part of the virus, because it could also be due to an enzyme

already adsorbed to the particles in the host cell. It is well known from studies on vaccinia virus how difficult it is to remove enzymes from the surface of virus particles once they have been adsorbed.

### C. Larger Viruses

#### 1. *Vaccinia Virus*

Vaccinia was the first animal virus obtained in a highly purified state (see Smadel and Hoagland, 1942). It is bricklike in shape, with dimensions  $\sim 280 \times 220 \times 220 \mu$  (Williams, 1954; Peters, 1956) and a particle dry weight  $\sim 3.2 \times 10^7$  (Smadel *et al.*, 1939; see Schramm, 1954b). Associated with these particles is a series of antigens: an NP (nucleoprotein) antigen, an LS antigen, composed of a heat-labile (L) and a heat-stable (S) component, an agglutinin X, and perhaps another antigen responsible for the formation of neutralizing antibody (see Smadel and Hoagland, 1942). The situation became even more complicated when a hemagglutinating principle was found in extracts of vaccinia infected tissue (hemagglutinin) (Nagler, 1942; see Burnet, 1955a), which also is said to have a specific antigenic structure (Chu, 1948b; Mayr, 1956). Indications of their chemical nature have been obtained only for NP and LS antigens and for the hemagglutinin.

There is no doubt that the NP antigen is an intrinsic component of the infective particle of vaccinia, since it is not found separate from the virus in tissue extracts (Smadel *et al.*, 1942). This is not the case for the LS antigen and the hemagglutinin, which are present in relatively large amounts, along with the infective particle, and hence may only be adsorbed to the latter. However the available evidence has suggested to some authors that the LS antigen is a part of the virus surface (see Smadel, 1952b). The hemagglutinin has been practically completely separated from the virus in several cases (Burnet and Stone, 1946; Chu, 1948a).

The crude material for the purification of infective particles of vaccinia is obtained from scrapings of infected rabbit skin. The virus particles were isolated from such material by repeated cycles of high- and low-speed centrifugation. In this manner some 8–10 mg. of highly purified virus particles were obtained from several grams of rabbit pulp. Such virus preparations had a single boundary in the analytical ultracentrifuge and by electrophoresis. The analytical chemical data on different lots were very constant, and did not change after further washing and centrifugation of the preparations (see Smadel and Hoagland, 1942; see Smadel, 1952b). Overman and Tamm (1956) recently showed that a single characteristic brick-shaped particle is able to initiate an infection on the chorioallantoic membrane of the egg.

The elementary analysis of this type of preparations yielded the following values: 33.7 % C; 15.3 % N; 0.57 % P; and 0.05 % Cu. A total of 2.8 % reducing sugar was found. Most of this, as well as the phosphorus present, is from nucleic acid, which comprises 5.6 % of the total. The major fraction of this virus, amounting to  $\sim 89$  %, is protein. About 5.7 % of the total consists of lipid (Hoagland *et al.*, 1940a,b, 1941a; see Smadel and Hoagland, 1942; see Hoagland, 1943).

The nucleic acid of vaccinia virus, as well as that of papilloma virus, has been demonstrated with some certainty to be exclusively DNA, but vaccinia virus does not contain just one molecule of DNA of  $4 \times 10^6$ . On the basis of a particle weight of  $\sim 3.2 \times 10^9$  it contains well over 40 such DNA molecules. Paper chromatographic analysis of the bases showed that there is no uracil and that 5-hydroxymethyleytosine, present in the DNA of several coliphages, cannot be detected in the nucleic acid of vaccinia virus (Wyatt and Cohen, 1953).

Detailed studies on the protein fraction, particularly as to its amino acid composition, have not yet been carried out.

The lipid fraction of the purified preparations is composed of 1.4 % cholesterol, 2.2 % phospholipid, and 2.2 % neutral fat. Since the cholesterol could be extracted by ether without impairing the infectivity of such a virus preparation, it was not considered an essential part of the infective particles (Hoagland *et al.*, 1940b). But as Beard (1948) has previously mentioned, this conclusion does not necessarily follow from the particular experiment, since the extracted cholesterol may have originated from previously denatured virus particles rather than from intact particles. Since only about 1.5 % of the particles in the preparation studied were still infectious before the ether treatment, this latter theory appears likely.

Tightly bound to the virus particle is a riboflavin, which could be identified as flavine-adenine dinucleotide (FAD) with the aid of the specific apoenzyme of *D*-amino acid oxidase from pig kidney (Hoagland *et al.*, 1941b). This component could not be separated from the virus by electro dialysis, ultrafiltration, or by washing the virus particles with buffers ranging from pH 6 to pH 8. The separation was possible only after denaturation of the virus protein. Adsorption of extraneous FAD can be widely excluded since control experiments showed that the virus particle does not take up additional amounts of this coenzyme from dilute solutions (Hoagland *et al.*, 1942). Using microbiological assays with *Lactobacillus casei* E, it was shown that 100 gm. of highly purified virus material contains between 1.1 and 1.5 mg. of riboflavin.

Although this coenzyme is present, no dehydrogenase activity was detectable. Further, neither cytochrome nor cytochromoxidase could be detected by spectroscopic or biochemical methods.

Emission spectra of purified vaccinia virus preparations have shown that the only metal present in the virus is Cu (Hoagland *et al.*, 1941a). The copper could not be separated from the virus particle by repeated washings, ultrafiltration, or electro dialysis. In contrast, copper ions added to the virus preparations could very easily be removed again. The copper concentration increased during the purification of the virus and was present in approximately the same concentration in all purified preparations. These findings strongly suggest that copper is an intrinsic part of the virus. The presence of copper was first noticed after observation of a relatively enormous uptake of oxygen in the presence of cysteine. Further investigations showed that the copper containing material did not correspond to any of the known copper oxidases.

It is not clear whether catalase, also found in virus preparations, is essential to the infective particles. In contrast to FAD and the copper ions, this enzyme can be so strongly bound from solutions that it cannot be removed by extensive washings. The same is true for hydrolytic enzymes, like phosphatase and lipase, which are also associated with the infective particles (McFarlane and Salaman, 1938; Hoagland *et al.*, 1942). There is no question that one must be exceptionally critical concerning the presence in virus preparations of enzymes that are normally present in the host cells. Unfortunately, suitable methods to differentiate between true virus components and adsorbed cell material of this sort are not available.

The biotin found (Hoagland *et al.*, 1940c), using microbiological methods, seems to be a true component of the virus, since it is not adsorbed from solution in appreciable amount (Hoagland *et al.*, 1942), and because infective particles release biotin during hydrolysis.

The NP antigen amounts to about 50 % of the infective particle. It may be extracted from the infective particles with the aid of dilute alkali. From chemical studies, the NP antigen is found to be a nucleoprotein containing some 6 % DNA (Smadel *et al.*, 1942).

The seroimmunological behavior of the infective particles suggests that at least the antigen portion of this nucleoprotein is located on the surface of the virus particle (see Smadel, 1952b). This finding is difficult to correlate with degradation experiments controlled by electron microscopy. These studies show that the DNA of the virus, which is to a large extent a fraction of the NP antigen, is located in the interior of the particle. After the earlier work of Dawson and McFarlane (1948), Peters and his co-workers (Peters and Nasemann, 1953; Peters and Stoeckenius, 1954; Stoeckenius and Peters, 1955) made extensive studies, using this method. On the basis of these investigations and of studies on ultrathin-sections Peters (1956) has proposed the structure of vaccinia virus illustrated in Fig. 1. In the interior of the particle is a structure (a), which appears dumbbell-shaped

in vertical section and approximately rectangular in horizontal section. This inner body consists of DNA and protein. It is surrounded by a protein coat (b). A further structural element (c) is located in a central position above and below this protein coat, and is claimed to be protein. The entire infective particle is bounded by a membrane, which consists of two layers and probably contains lipid, as well as other components. The nucleoprotein inner body, which can only be seen clearly after treatment of the virus particle with pepsin, varied considerably in size and form in the studies of Peters and Nasemann (1953). Along with various transitional stages, some

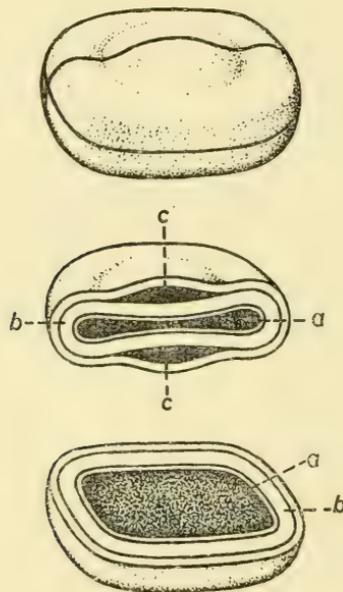


FIG. 1.—Structure of the infective particle of vaccinia (Peters, 1956).

particles were observed that no longer had any pepsin-resistant central body. These particles were not characterized biologically. They bring to mind the nucleic acid-deficient S antigen of poliovirus. Since they are of lower mass, they are probably not present in the preparations purified by ultracentrifugation and studied by the other chemical methods.

Of the virus-specific products which accompany the infective particles the LS antigen has been most completely studied. It has been isolated from virus-free filtrates obtained from the extracts of infected dermal pulp of rabbits. It can be highly purified by precipitation at a suitable pH (isoelectric point, pH 4.8) (Craigie and Wishart, 1936; Shedlovsky and Smadel, 1942). Such

preparations were homogeneous by electrophoretical and ultracentrifugal analysis (Shedlovsky *et al.*, 1943). After degradation of the S component with chymotrypsin, the antigen formed needle-shaped crystalloids (Smadel *et al.*, 1943), providing further evidence for the high degree of purity. The molecular weight of the intact LS antigen, as determined by sedimentation and diffusion, is 214,000; the axial ratio, 1 : 20 (Shedlovsky *et al.*, 1943).

Chemical analyses have shown that the LS antigen is a protein, having an elementary composition of 15.8 % N and 50.6 % C. Tests for lipid, phosphorus, nucleic acid, and glucosamine were all negative. The antigen is attacked by proteolytic enzymes. Both the L and S parts may be destroyed by papain, but chymotrypsin under suitable conditions, will attack only the S fraction, as already mentioned (Smadel *et al.*, 1943).

The hemagglutinin, according to studies with the preparative ultracentrifuge, is smaller than the infective particle, but larger than the LS antigen (Chu, 1948a; Mayr, 1956). Assuming that it is approximately spherical, the diameter is said to be 65  $\mu$ , a density of 1.1 was estimated (Chu, 1948a). Sufficient material for a detailed chemical analysis has not yet been prepared. It is known, however, that the hemagglutinin can be precipitated with half-saturated ammonium sulfate (Burnet, 1946), and destroyed by the  $\alpha$ -toxin of *Clostridium welchii*, as well as by cobra snake venom, both known to contain lecithinase (Stone, 1946). These and some other observations have led Burnet (1955a) to ascribe to it the character of a phospholipid-protein complex. No enzyme corresponding to the neuraminidase of influenza virus has been found associated with the vaccinia hemagglutinin.

#### D. Summary (cf. Tables I and II)

The chemical composition of the infective particles of animal viruses seems to increase in complexity as the size of the particles increases. The smallest particles contain only nucleic acid and protein. With increasing size, one can find in addition lipid and sometimes carbohydrate; in the largest, the vaccinia virus, one even finds copper, biotin, and a flavin-adenine-dinucleotide, i.e., a coenzyme of the respiratory chain.

The nucleic acid is sometimes RNA and sometimes DNA, but true animal viruses unequivocally containing both types of nucleic acid are not known. Past claims that both types are present in the same virus can probably be attributed to impurities. A correlation between the size of the infective particle and the type of nucleic acid does not exist. Thus, DNA is found in the relatively small papilloma virus, as well as in the larger vaccinia virus. Poliovirus, one of the smallest viruses known, and the much larger Newcastle disease virus both possess RNA. However, differences are observed in the

TABLE II  
CHEMICAL CONSTITUENTS OF INFECTIVE PARTICLES AND OTHER VIRUS SPECIFIC PRODUCTS

Virus	Virus unit	Nucleic acid	Protein	Lipid	(Carbohydrate, excluding sugar of nucleic acid)	Enzymes and miscellaneous constituents
Poliomyelitis	Infective particle	22-30 % RNA	70-78 %	—	—	—
	S Antigen	Little or none	+	Probably none	—	—
Rabbit papilloma	Infective particle	8.7 % DNA	~ 90 %	1.5 % (?)	—	—
Equine encephalomyelitis	Infective particle	4.4 % RNA	~ 40 %	54 % Phospholipid, cholesterol, neutral fat	~ 3 %	—
Influenza	Infective particle	0.7-1 % RNA	60-70 %	~ 25 % Phospholipid, cholesterol	3-8 % Galactose, mannose, fucose, glucosamine	Neuraminidase
	Virus-hemagglutinin	—	+	—	4.2 % (?) Galactose (?) Mannose (?)	Probably neuraminidase
	G Antigen	5.3 % RNA	+	—	—	—
	S Antigen	0.7-6 % RNA	+	—	—	—
	Incomplete forms (from allantoic fluid, undiluted passage)	RNA down to 0.3 %	+	Up to 54 %	—	Probably neuraminidase
	Filamentous forms	—	+	+	+	(?)

Fowl plague	Infective particle	1.8-4 % RNA	~ 60 %	~ 25 % Phospholipid, cholesterol	+	Neuraminidase
	Virus-hemagglutinin	—	+	—	+	Neuraminidase
	G Antigen	10-15 % RNA	+	—	—	
	S Antigen	6-14 % RNA	+	—	Probably none	
	'Incomplete forms' (from cell homogenates)		+	50-60 %		Probably neuraminidase and glucose-6-phosphatase
Newcastle disease	Infective particle	Probably only RNA	~ 65 % (?)	~ 27 % (?) Phospholipid, cholesterol	~ 6 % (?)	Receptor-destroying and hemolytical (?) enzyme
Vaccinia	Infective particle	5.6 % DNA	~ 89 %	5.7 % Phospholipid, neutral fat, cholesterol (?)	Little or none	Flavine-adenine-dinucleotide (~0.001 %), biotin, Cu (0.05 %); enzymes found are probably impurities
	NP Antigen	6 % DNA	+			
	Hemagglutinin		+	(?) Phospholipids (?)		
	LS Antigen	—	~ 100 %	—	—	

absolute amounts of nucleic acid. The smaller and some medium sized infective particles seem to have only one molecule, but the vaccinia virus contains more, if one assumes a molecular weight of  $\sim 2 \times 10^6$  for RNA (Gierer, 1957) and  $\sim 4 \times 10^6$  for DNA (see Jordan, 1955). No unusual purine and pyrimidine bases have as yet been found in animal virus nucleic acids.

The information concerning the composition of protein from infective particles is too scanty and uncertain to enable one to make generalizations.

As for the lipids, it has been reported that cholesterol, phospholipids, and neutral fats are found with the equine encephalomyelitis and certain larger viruses, but it is not always clear which of these lipids are integral parts of the particles.

The presence of carbohydrates has been proved with some certainty for fowl plague and influenza virus, where they apparently are a part of a mucoprotein.

The same viruses and probably the other viruses of the myxovirus group also possess a special enzyme with the properties of a neuraminidase. Other enzymatic activities associated with vaccinia virus are suspected to come from adsorbed impurities. It should be mentioned, however, that the investigation of enzymatic activities is quite incomplete for most animal viruses. The presence of flavine-adenine-dinucleotide, copper, and biotin in the infective particle of vaccinia suggests that this agent has its place on the border between virus and higher organized biological units.

As far as one can see from the results at hand, the nucleic acid of infective particles seems to be localized generally in their interior, surrounded by protein. Lipids were found to be a part of the infective particle surface, in those cases where there is some evidence about their localization. The neuraminidase of the myxoviruses is also situated here.

The virus-specific products designated as S antigens are not a homogeneous group from the chemical point of view. Some are proteins, like the LS antigen of vaccinia and, probably, the S antigen of poliomyelitis; others are nucleoproteins, such as the S antigen of fowl plague and influenza. It is noteworthy that in the latter cases there exists a close relationship between the S antigen and a particular component enclosed in the respective infective particles. This observation somewhat limits speculation concerning the functions of these S antigens. It is not probable that a side product of virus multiplication would exhibit such behavior.

Our knowledge concerning the chemical composition of the hemagglutinin of pox viruses and the filamentous forms of myxoviruses is still very incomplete. But there are some indications that both contain protein and lipids.

An extremely high lipid content seems to be a characteristic of the various incomplete forms of the myxoviruses. One must still ascertain whether all

these units have a lower RNA content than the infective particles. At the moment, this has only been proved for the incomplete forms of influenza obtained from allantoic fluid. Further investigations are also needed on the glucose-6-phosphatase observed to be associated with the incomplete forms from fowl plague.

#### V. ORIGIN AND FUNCTION OF THE CHEMICAL CONSTITUENTS OF ANIMAL VIRUSES

Considerations concerning the origin and function of the various components unfortunately must remain speculative, because of insufficient information. Nevertheless, such a discussion will conclude this chapter in order to suggest and stimulate further experiments.

The lipids apparently maintain the integrity of the infective particles and protect them. When they are removed from some myxoviruses with ether, the particles disintegrate and the viral subunits are released. Studies using  $P^{32}$  for labeling fowl plague virus have brought some insight as to the origin of the lipids (Wecker, 1957). When the tissue cultures are incubated with  $P^{32}$  prior to infection, the specific activity of the lipid fraction of the new virus particles is significantly higher. It increases with the time of preincubation with constant amounts of the isotope. This increased activity suggests that the cellular phospholipid, as such, is transferred to the virus particle. It is quite likely that this lipid comes from the cell membrane, since ultrahistological studies show that the infective particle of fowl plague virus appears as an unified organized unit only at the cell membrane (Hotz and Schäfer, 1955).

It is possible that the lipid fraction of some incomplete forms has an origin other than that of the infective particles. The hypothesis was advanced that the 'incomplete forms' of fowl plague are microsomes of the host cells loaded with virus-specific material (Schäfer, 1957a). This hypothesis is strengthened by the observation that these incomplete forms and the microsomes of normal cells are morphologically very similar. Further, there is some evidence that these incomplete forms are situated in the cytoplasm (Breitenfeld and Schäfer, 1957) and that they contain glucose-6-phosphatase, an enzyme known to be located in the microsomes.

The carbohydrates present in the infective particles of myxoviruses seem to be a part of a mucoprotein. The composition of the virus mucoprotein resembles a normal mucoprotein of the host (Ada and Gottschalk, 1956). This fact and the observation that influenza and fowl plague virus always contain a non-removable, normal component have led to the suggestion that the presence of mucoproteins in these virus particles is a necessary concomitant of the process by which the myxoviruses mentioned are formed (Ada and Gottschalk, 1956).

The neuraminidase found in the myxovirus infective particles is most likely newly formed in the host cell as a virus-specific product. This must be assumed, since such a component cannot be demonstrated in a normal, noninfected animal cell. In regard to the function of the neuraminidase, the following three possibilities come to mind: (1) the enzyme can play a role in the penetration of the virus into the host cell, (2) it could mediate the release of the newly formed virus particle, (3) it could mediate both the penetration and release of the infective particles.

The presence of copper, flavin, and biotin in vaccinia virus would indicate as Smadel and Hoagland (1942) mention, that some rudimentary system of respiration may exist for this relatively highly organized virus "although no substrate capable of sustained activation by these catalytic substances has been found."

As mentioned above nucleic acid and protein are found in all animal viruses investigated to date.

The paths of formation of the virus nucleic acid in the host cell has been scarcely studied for the animal viruses. But the one study, carried out with  $P^{32}$  labeling in fowl plague virus (Wecker, 1957), suggests that P-containing material of the host cell is not directly used in large amounts for the synthesis of that virus nucleic acid.

From studies of other types of virus (tobacco mosaic virus and phages) a reasonable working hypothesis for the animal viruses is that the nucleic acid is the most essential component for the multiplication. In accord with this theory is the fact that the S antigen of poliovirus and the LS antigen of vaccinia virus, both non-infectious, contain no nucleic acid. Further, preparations of the incomplete forms of influenza, having a lower probability of causing infection, seem to contain less RNA than the infective particles. But there are also virus-specific products, like the S and G antigens of fowl plague and influenza virus, with a relatively high percentage of nucleic acid, with which it has not yet been possible to induce infection. However, one has to consider that these particles may be too small to contain a full RNA molecule of  $\sim 2 \times 10^6$  molecular weight, which Gierer (1957) has shown to be essential for the infectivity in tobacco mosaic virus. If we assume, as Ada and co-workers (1952) claim for the S antigen of influenza, a particle weight of  $1.5 \times 10^6$  and an RNA content of about 10 %, then the molecular weight of the RNA from these units is only 150,000. One has to bear in mind that the G antigen obtained by ether treatment of infective particles is probably a degradation product derived from a ringlike structure that contains the intact nucleic acid necessary for multiplication.

Initiated by the work with tobacco mosaic virus (Gierer and Schramm, 1956; Fraenkel-Conrat, 1956) some investigations have recently been done with animal viruses to show that isolated RNA from these sources is also

able to induce the production of new infective particles in suitable cells. By treatment with phenol, fractions have been obtained from tissues infected with Mengo-encephalitis- (Colter *et al.*, 1957) and equine encephalomyelitis-virus (Wecker and Schäfer, 1957). These behaved, according to some tests, like RNA and were infectious, but the stability of such fractions was relatively low.

The main function of the virus protein components seems to be to protect and stabilize the labile genetic material. Furthermore, they can be important for the penetration of the virus into the host cell or for the release of new virus. They can carry out these functions most efficiently by forming a coat around the nucleic acid; it has been shown that this is actually the case for several animal viruses. There are recent experiments that localize the sites of formation of some of the virus proteins, but the mechanism of their formation is unknown.

The evaluation of the chemical interactions between viruses and their host cells will require a great deal of further painstaking work. In the long run, this work will be more rewarding using animal viruses rather than plant or bacterial viruses, since the animal cells possess a well-defined inner structure and can now be easily cultured *in vitro* under specified conditions.

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## Chapter IX

### Biochemistry of Insect Viruses\*

G. H. BERGOLD

*Laboratory of Insect Pathology, Sault Ste. Marie, Ontario, Canada*

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Considerable information is available on the morphology and physicochemical properties of insect viruses and their inclusion body proteins (Bergold, 1957, and Sections II and III following). Although to date few concrete results have been obtained on biochemical changes in infected host insects it is hoped that the following summary of data will stimulate further investigations using recently developed biochemical techniques.

#### I. THE CHEMICAL CHANGES IN THE INSECT HOST DURING VIRUS INFECTION

Komarek and Breindl (1924) and Breindl (1938) investigated the metabolism of *Lymantria monacha* (L.) larvae that were suffering from nuclear polyhedrosis. They found a decrease of the Feulgenpositive chromatin in infected nuclei during the formation of polyhedra, which suggests that chromatin material is perhaps converted into polyhedra. Similar results were obtained by Gratia *et al.* (1945), who observed, two days after infection, an increase of deoxyribonucleic acid (DNA) in the chromatin and of ribonucleic acid (RNA) in the cytoplasm and nucleoli in cells of *Bombyx mori* (L.). The increase continues until the fourth day, when DNA appears in the "ring zone," but the nucleoli disappear during the formation of polyhedra. An increase of DNA and RNA in diseased *B. mori* larvae was also found with histochemical methods by Semenova (1951). Extraction experiments have shown an increase of DNA and a decrease of RNA in diseased *B. mori*

\* Contribution No. 438 Division of Forest Biology, Science Service, Department of Agriculture, Ottawa, Canada.

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larvae (Yamafuji *et al.*, 1954). No difference between the oxygen consumption of dissected tissue of infected and healthy *B. mori* larvae was found by Gratia *et al.* (1945), but Akune (1951a) observed an increase in the respiratory and a decrease of the catalase activity. Similarly Gershenson (1956a) found a higher  $O_2$  concentration in infected *Antherea pernyi* Guer. pupae, which, however, decreased at the time of death to the level of healthy pupae. Ishimori and Osawa (1951) found, after an initial drop, a rapid increase of the catalase activity upon appearance of polyhedra in the blood cells. The inhibition of endogenous respiration of a homogenate of *B. mori* larvae infected with cytoplasmic polyhedrosis by 0.01 *M* EDTA is less than in healthy larvae but can be restored by the addition of  $Mg^{++}$ ,  $Mn^{++}$ ,  $Co^{++}$ , and  $Fe^{+++}$ , but not with  $Cu^{++}$ . The rate of recovery is greater in diseased than in healthy larvae, but no difference was found in the activities of cytochrome oxidase and succinic dehydrogenase in mitochondria. In homogenate and mitochondria the oxidation of succinate, malate, and glutamate was decreased in the diseased larvae, and in mitochondria the activity of glutamate oxidation by DPN and succinate oxidation by ATP was also less in diseased than in healthy larvae (Ishikawa, 1958). The hexokinase activity on glucose and fructose in the fat body of *B. mori* larvae appears to be decreased at the onset of symptoms of polyhedrosis (Shigematsu, 1958), but the dehydrogenase activity in larval blood and pupal homogenate is increased in diseased *B. mori* (Murai and Aizawa, 1957).

Investigations of the amino acid metabolism revealed that there were no changes in the hemolymph of infected *B. mori* larvae until the disease had well progressed (Drilhon 1951; Drilhon *et al.*, 1951, 1952). However, an increase in histidine and a decrease in aspartic acid, cysteine, glutamic acid, glutamine, threonine, tyrosine, and valine were observed in another investigation (Ishimori and Muto, 1951). Alkaline extraction (0.05 *N* NaOH) of diseased and healthy *B. mori* larvae with acetone show no difference (Yoshihara, 1952). The activity of a dipeptidase and of a peptone-decomposing enzyme appears to be increased (Yoshihara 1956a,b), as well as that of a trans-oximase, which could also be boosted by feeding  $NaNO_3$ ,  $KNO_2$ , and  $(NH_4)_2CO_3$  (Yamafuji *et al.*, 1953b). The  $K_2O$  content of diseased *B. mori* larvae appears to be decreased (Akune, 1951b). Feeding *B. mori* larvae with young mulberry leaves leads to a rise in the acidity of body fluids (over 300 mg. % of ammonium N in excreta), which provokes polyhedrosis. Insufficient amounts of K in the oak leaves favors the outbreak of polyhedrosis in *A. pernyi* larvae (Arseniev and Bromley, 1951). The addition of cobalt nitrate and cobalt sulfate (0.05 %) to the food of *B. mori* larvae decreases frequency of "spontaneous" polyhedrosis, particularly if  $CaCl_2$  (1 %) is added (Gershenson, 1958).

The concentration of protein is higher (5.4 %) in diseased larvae than in

healthy ones (3.4 %); this is probably due to an increase of a protein component with a sedimentation constant of 6.2 Svedberg (Bergold and Friedrich-Freksa, 1947). About 7 % of the dry weight of infected *B. mori* larvae consists of virus and polyhedra, which contain 2.5 % of the total P, 13 % of the nucleo-protein P, and 10.6 % of the acid-soluble residual fraction P. The total N of the diseased larvae is increased and the N of the virus nucleoprotein forms 24 % of the N protein fraction. Polyhedron-diseased *B. mori* larvae are deficient in P, but not in N (Tarasevich, 1952). In diseased larvae the amount of P (chiefly the acid soluble components) is increased. The increase of protein fraction is less than in the other fractions. Application of diaminopurine and 4-aminopterine increased the number of polyhedrosis cases and the P content of the protein fraction; whereas dinitrophenol, hydroxylamine, acridine, and aminomethylphosphoric acid boosted the P content of fat tissue (Tarasevich, 1953). The amount of total nucleic acid and protein in the fat body and body fluid is also increased in the later stages of nuclear polyhedrosis of *B. mori* larvae (Shigematsu and Takeshita, 1958). The activity of tyrosinase in diseased larvae is only 20 % of that of healthy ones, and in diseased larvae the amount of DNA is increased and that of RNA is decreased (Tarasevich, 1954).

The uptake of  $P^{32}$  into polyhedra from a standard dose of  $0.6 \mu\text{C}/\text{larva}$  depends on the time elapsing after infection with the virus before the injection of  $P^{32}$ . If the interval is three days the amount (about 22 c.p.m./mg. polyhedra) is three times as high as with an interval of one day (Yamafuji and Omura, 1954). Polyhedral bodies obtained from dead *B. mori* that were previously injected with  $C^{14}$ -labeled alanine and glycine (about  $10 \mu\text{C}/\text{larva}$ ) show an activity of about 1300 c.p.m./mg. However, virus particles isolated from such polyhedra have 3800 c.p.m./mg. To explain this, one can assume preferential incorporation of  $C^{14}$  into the virus particles, or that polyhedra develop later than the virus particles (Bergold, 1954). The rate of incorporation of glycine- $C^{14}$  into blood protein is not different in healthy and diseased *B. mori* larvae (Faulkner, unpublished), although injection of  $C^{14}$ -labeled alanine and glycine (about  $10 \mu\text{C}$  per larva) appears to inhibit somewhat the virus multiplication, enabling the larvae to develop to adults (Bergold, 1954).

## II. PHYSICOCHEMICAL PROPERTIES AND CHEMICAL COMPOSITION OF INCLUSION BODIES

### A. Physicochemical Properties

Nuclear and cytoplasmic polyhedroses and granuloses are characterized by the formation of the so-called inclusion bodies. Two main types can be distinguished: regular- or irregular-shaped polyhedra, which are about  $0.5\text{-}15 \mu$  in diameter, and ellipsoidal capsules with dimensions of about  $200 \times 500 \text{ m}\mu$ . Bolle (1893) was the first to investigate polyhedra; he found that they are

insoluble in hot and cold water, alcohol, ether, chloroform, benzol, acetone, etc. They are heavier than water: for example, *B. mori* polyhedra have a density of 1.268 and *Cacoecia murinana* (Hbn.) capsules, 1.279 (Bergold, 1957). Polyhedra are clearly transparent, highly refractive, but not doubly refringent, and, when dried, remain unchanged for years. Polyhedra and capsules are not destroyed by any of the natural putrefaction processes, but dissolve in aqueous solutions of NaOH, KOH, NH<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub>, and CH<sub>3</sub>COOH. Staining is, therefore, greatly facilitated by pretreatment with acids (Escherich and Miyajima, 1911). In heat fixed smears *B. mori* polyhedra are gram-positive, but they become gram-negative after 2 hours treatment with glycolic acid at 60° C. Polyhedra of *B. mori* are not digested by papain (at pH 8.3), trypsin (at pH 6.8), or pepsin (at pH 3.3–4.0), but they are by pepsin at pH 2.0–2.9 and by trypsin and papain after alkali treatment: the addition of cystein prevents inactivation by trypsin and papain of alkali treated polyhedra (Zalmanzon, 1949, 1952). *B. mori* polyhedra are insoluble in hemolymph of *B. mori* larvae (Bergold, 1943; Ishimori and Osawa, 1952; Gershenson, 1956d), but seem to lyse in *B. mori* pupal lymph (Roegner-Aust, 1949) and in prepupae of *Neodiprion sertifer* (Geoffr.) (Krieg, 1955), which might be due to an enzymatic mechanism rather than to an alkali.

Verson (1872) first suggested the crystalline nature of polyhedra and found that the *B. mori* polyhedra are mostly rhombododecahedra, but that cube-shaped crystals occur, too. Polyhedra of *L. monacha* are usually tetrahedra and those of *Porthetria dispar* (L.) are of irregular shape. The crystalline nature of polyhedra was confirmed by preliminary X-ray investigations (Bergold and Brill, 1942) and by direct demonstration of the macromolecular, paracrystalline lattice, by electron microscopy (Morgan *et al.*, 1955, 1956; Day *et al.*, 1956). Measurements taken from such electron micrographs of thin-sectioned *B. mori* polyhedra suggested an ellipsoidal cross section of the polyhedron protein molecule with major and minor axes of 79 × 52 Å (Morgan *et al.*, 1955). However, an intensive investigation of a great number of thin sections (Fernández-Morán and Bergold, 1958) could not confirm this finding but revealed a circular cross section with a diameter of about 70 Å. Considering the molecular weight, the length of the molecule should be 180 Å. The molecules are not in a hexagonal but in a simple cubic packing. The cubic packing seems to be characteristic for all inclusion body proteins, independent of the shape of the corresponding crystals. Since the cubic packing is not the closest possible, one might assume differentiations on the surface of the molecules with preferred spots of attraction. It is interesting that the ellipsoidal capsules also show a crystalline lattice (Morgan *et al.*, 1955; Fernández-Morán and Bergold, 1958), and the examination of a great number of capsules revealed that some capsules have developed one or several sharp corners giving them an appearance similar to that of small polyhedra.

When inclusion body proteins are dissolved, according to the standard method for the liberation of virus particles (Bergold, 1947, 1957) in weak solutions of alkali (0.005 *M*–0.03 *M* Na<sub>2</sub>CO<sub>3</sub> + 0.05 *M* NaCl for 1 to 3 hr.), a yellow, almost clear solution of the inclusion body protein is obtained after the sedimentation of virus particles at 10,000–12,000 *g*. In order to remove a few remaining virus particles and virus membranes, the solution is ultracentrifuged for 30 min. at about 25,000 *g*. The resulting clear solution represents the inclusion body protein, which can be precipitated by lowering the pH with HCl or CH<sub>3</sub>COOH, or by dialysis against distilled water. The precipitate can be washed with slightly alkaline water and dissolved again in dilute alkalis. When this process is repeated several times, one can obtain a very pure preparation of inclusion body protein. Such protein solutions are, under certain salt and pH conditions, very homogeneous and yield molecular weights of about 276,000–378,000 (Bergold, 1947, 1948). Panebianco (1895) claims to have recrystallized polyhedra by adding H<sub>2</sub>SO<sub>4</sub>, but all attempts by the reviewer had no success, although some more or less regular patterns are found when polyhedron protein solutions are allowed to dry.

Apart from being insoluble in water, the inclusion body proteins behave quite unusually. They are very sensitive to any salts; for instance, 0.01 *M* NaCl at pH 8 causes aggregation and increases the sedimentation constant from about 12.5 to 18.0 Svedberg (double molecules). The main molecule of *B. mori* polyhedron protein, with a molecular weight of 378,000, dissociates reversibly into its first split component with a molecular weight of about 60,500, or sixths (theoretically 63,000). Further addition of alkali causes the sixths to split irreversibly into the second split component, or eighteenths, with a molecular weight of 20,300 (theoretically 21,000) (Bergold, 1947). In agreement with this finding, Kratky (1943, cited in Bergold, 1947) found in small angle X-ray investigations a molecular weight of 22,000 for the smallest elementary cell, with dimensions of 45.3 × 28 × 20.4 Å. The second split component is not destroyed by boiling briefly in 0.5 *M* NaOH and it crystallizes in bodies up to 5 μ in diameter and similar in appearance to polyhedra (Glaser and Chapman, 1916; Bergold, 1947). It was recently found that a major part of *B. mori* polyhedra (about 70 %) dissolves in alcohol after pretreatment with trichloroacetic acid (Eto, 1956a, b). This might be due to a partial degradation into the second split component (18ths). Storage of *B. mori* polyhedra for thirty-seven years in a desiccator over CaCl<sub>2</sub> does not change their solubility in Na<sub>2</sub>CO<sub>3</sub> (Aizawa, 1953, 1954).

Undissolved *B. mori* polyhedra migrate in an electrical field to the positive pole (von Prowazek, 1913; Dikasova, 1949) with an isoelectric point at pH 5.2 (Tarasevich, 1945). Polyhedron protein solutions move homogeneously but with different mobilities. They are completely insoluble at their isoelectric points, which are at pH 5.7 for *P. dispar* and between pH 5.3 and 5.6 for

*B. mori* and *L. monacha* polyhedron protein (Bergold and Schramm, 1942). In nonpurified polyhedron solutions two components with different mobilities were found (Yamafuji *et al.*, 1953a). Polyhedron protein seems not to migrate on filter paper at pH 8.6 (Aizawa, 1955). Polyhedron proteins spread readily on water surfaces and can be loaded twice as much (55 dynes/cm.) as films of casein or egg albumen. *B. mori* polyhedron protein has a maximum area at pH 5 and those of *P. dispar* and *L. monacha* at pH 4. Between pH 1 and 7 the thickness of the films varies (Bergold and Brill, 1942).

### *B. Chemical Composition of Inclusion Body Proteins*

Bolle (1893) first analyzed polyhedra and found that they consist of protein and contain no lipids. A quantitative analysis of slightly impure *B. mori* polyhedra revealed 14 % N, 0.79 % P, and 0.79 % S (Manunta, 1940). More recent analyses of polyhedra and capsules and purified polyhedron and capsule proteins are summarized in Table I. In comparing the results in Table I, it should be realized that polyhedra and capsules consist of essentially two components: the polyhedron or capsule protein that constitutes about 95 % of the total weight and the virus particles, about 5 % (Bergold, 1947, 1948). Therefore, analyses of polyhedra and capsules are, in effect, analyses of polyhedron and capsule protein plus those of virus particles. The N content of polyhedra, capsules, and purified protein preparations of them, from different insect hosts, does not vary much and is about 14–15 %. The P content of polyhedra varies with the preparation and is much smaller in the purified polyhedron protein. The small amount of P (about 0.05 %) that cannot be removed in spite of repeated precipitations and washings may not even belong to the protein molecule and may be only adsorbed. If it does belong, it can be calculated that each molecule of polyhedron protein of *P. dispar*, *L. monacha*, and *B. mori* has about 4, 4, and 8 P atoms, respectively. Furthermore, not all split molecules—sixths and eightieths—can possess one P atom. The difference of 50 to 60 % in the P content between polyhedra and polyhedron protein is due to free dialyzable phosphate, liberated by the alkaline treatment (Desnuelle *et al.*, 1943; Bergold, 1947).

A search for metals has revealed only 0.005 % Fe in *B. mori* polyhedra and polyhedron protein (Holoway and Bergold, 1953), 0.083 % Mg in polyhedra, and no Mg in polyhedron protein (Holoway and Bergold, 1955).

Desnuelle *et al.* (1943) and Desnuelle and Chang (1943) were the first to determine the amino acid content of *B. mori* polyhedra and found (expressed as % of protein N): cysteine and cystine, 0.4 %; alanine, 4.6 %; tyrosine, 5.2 %; histidine, 4.7 %; arginine, 12.1 %; phenylalanine, 3.8 %; tryptophan, 3.00; ammonia, 6.4 %; and humin N, 3.8 %. An extensive investigation of the

TABLE I

COMPOSITION OF INCLUSION BODIES, INCLUSION BODY PROTEINS, AND VIRUSES  
(Values given are in per cent.)

Substance	Polyhedra	Capsules	Protein		Virus	
			Polyhedral	Capsular	Polyhedral	Capsular
	<i>Bm</i> *	<i>Cf</i> *	<i>Bm</i>	<i>Cf</i>	<i>Bm</i>	<i>Cf</i>
N	14.73 <sup>a</sup> -15.5 <sup>b</sup>		15.16 <sup>a</sup>		13.92	
	14.5 <sup>d</sup> , 14.29 <sup>e</sup>		15.0 <sup>d</sup>		13.9	
	14.88-15.11 <sup>f</sup>		14.53-15.81 <sup>f</sup>			
	14.9-15.2 <sup>g</sup>					
P	0.191-0.243 <sup>a</sup>		0.062 <sup>a</sup> , 0.064 <sup>b</sup>		0.915	
	0.243 <sup>d</sup> , 0.32 <sup>e</sup>		0.00-0.08 <sup>f</sup>			
	0.22-0.35 <sup>g</sup>					
	0.21 <sup>f</sup>					
DNA-P†	0.00-0.03 <sup>g</sup>		0.01-0.02 <sup>g</sup>			
RNA-P†	0.05-0.12 <sup>g</sup>		0.00-0.04 <sup>g</sup>			
P†	0.07-0.14 <sup>g</sup>		0.02-0.10 <sup>g</sup>			
P‡	0.10-0.17 <sup>g</sup>		0.07-0.14 <sup>g</sup>			
DNA-P‡	0.07-0.10 <sup>g</sup>		0.06-0.11 <sup>g</sup>			
RNA-P‡	0.00-0.09 <sup>g</sup>		0.00-0.04 <sup>g</sup>			
N/P	60-77 <sup>a</sup>	54 <sup>c</sup>	245 <sup>a</sup>	290 <sup>c</sup>	15.2 <sup>b</sup>	16 <sup>c</sup>
C	54.37 <sup>e</sup>		51.67-52.33 <sup>f</sup>			
	40.98-50.78 <sup>f</sup>					
H	7.02 <sup>e</sup>		6.81-7.31 <sup>f</sup>			
	6.54-6.85 <sup>f</sup>					
S	1.48, 0.92 <sup>f</sup>		0.83 <sup>d</sup>			
Cl	0.075 <sup>e</sup>					
Ash	1.51 <sup>e</sup>		0.67-0.98 <sup>f</sup>			
	0.26-0.31 <sup>f</sup>					

\* *Bm* = *B. mori*; *Cf* = *C. fumiferana*

† Nucleic acid fraction.

<sup>b</sup> Bergold and Wellington (1954).

<sup>d</sup> Desnuelle *et al.* (1943).

<sup>f</sup> Ikeda (1946; 1951 not available).

‡ Acid soluble fraction.

<sup>a</sup> Bergold (1947).

<sup>c</sup> Bergold (1951).

<sup>e</sup> Glaser and Stanley (1943).

<sup>g</sup> Yagi *et al.* (1951).

TABLE II  
 AMINO ACID COMPOSITION OF INCLUSION BODY PROTEINS <sup>a, b</sup>

Amino acid	Polyhedral type <sup>c</sup>						Capsule type <sup>c</sup>
	<i>Pd</i>	<i>Cf</i>	<i>Ma</i>	<i>Md</i>	<i>Em</i>	<i>Cpe</i>	
No. of hydrolyzates analyzed	3	3	2	2	5	2	3
Aspartic acid	11.1 ± 0.35 <sup>d</sup>	12.2 ± 0.27	13.5	13.6	12.5 ± 0.29	13.3	11.7 ± 0.30
Glutamic acid	13.2 ± 0.30	12.2 ± 0.33	12.4	11.7	12.7 ± 0.30	12.8	12.9 ± 0.33
Histidine	3.7 ± 0.44	3.2 ± 0.23	2.8	2.2	2.8 ± 0.18	2.8	1.7 ± 0.17
Lysine	8.5 ± 0.29	9.8 ± 0.41	9.4	6.3	10.5 ± 0.25	8.7	6.3 ± 0.21
Arginine	9.2 ± 0.26	7.9 ± 0.29	9.7	9.4	6.8 ± 0.33	10.1	10.1 ± 0.41
Glycine	3.1 ± 0.12	3.0 ± 0.15	2.7	2.8	3.3 ± 0.09	3.0	2.8 ± 0.17
Alanine	3.3 ± 0.26	2.8 ± 0.18	2.9	2.8	2.9 ± 0.05	2.8	2.8 ± 0.12
Valine	6.0 ± 0.24	7.2 ± 0.04	6.9	5.8	5.7 ± 0.16	7.0	5.2 ± 0.21
Leucine and/or isoleucine	13.0 ± 0.46	12.3 ± 0.38	14.3	13.4	13.3 ± 0.20	13.2	14.9 ± 0.20
Proline	4.9	5.7	8.2	6.0	6.0	8.3	5.4
Phenylalanine	7.5 ± 0.66	7.9 ± 0.20	7.0	7.1	6.9 ± 0.20	6.4	9.4 ± 0.52
Tyrosine	10.0 ± 0.23	9.4 ± 0.27	9.6	10.1	10.9 ± 0.21	7.6	7.2 ± 0.38
Serine	3.8 ± 0.07	3.4 ± 0.07	3.6	4.3	3.3 ± 0.07	3.6	3.4 ± 0.15
Threonine	4.7 ± 0.15	3.3 ± 0.10	3.1	4.6	3.8 ± 0.14	3.0	6.0 ± 0.09
Cysteine and/or cystine	1.1 ± 0.00	1.3 ± 0.07	1.3	1.1	0.6 ± 0.08	1.3	1.3 ± 0.00
Methionine	1.7 ± 0.18	3.5 ± 0.12	2.4	2.3	3.0 ± 0.09	2.3	3.4 ± 0.04
Total	104.8	105.1	109.8	103.5	105.0	106.2	104.5
Total calculated as amino acid residues/100 gm. protein	91.2	91.2	95.2	89.7	90.5	90.60	92.1
							92.7

<sup>a</sup> From Wellington, 1954.

<sup>b</sup> Estimated by paper chromatography of acid hydrolyzates, and expressed as gm. amino acid/100 gm. material analysed.

<sup>c</sup> Abbreviations for host insects: *Pd*, *Porthetria dispar* (L.), gypsy moth; *Cf*, *Choristoneura fumiferana* (Clem.), spruce budworm

(this insect is the host of both polyhedral and capsule type viruses; the inclusion protein of the former and the virus of the latter were analyzed); *Ma*, *Malacosoma americanum* (F.), eastern tent caterpillar; *Md*, *Malacosoma disstria* Hbn., forest tent caterpillar; *Em*, *Bombyx mori* L., silkworm; *Cpe*, *Colias philodice eurytheme* Bavl., alfalfa butterfly; *Ns*, *Neodiprion sertifer* (Geoffr.), pines awfly; *Cm*, *Cacoecia murinana* Hb.

<sup>d</sup> Mean value and its standard error.

amino acid composition of purified polyhedron and capsule protein from different insect viruses was carried out by Wellington (1951, 1954). The results, which are summarized in Table II, reveal that all the inclusion body proteins analyzed have a very similar amino acid composition, although some are from nuclear polyhedra of lepidoptera, one from polyhedra of a hymenopteron (*N. sertifer*), and one from capsules of a lepidopteron (*C. murinana*) that develop in the cytoplasm. However, comparing any two of these proteins, significant differences ( $P \leq 0.01$ ) can be found in the quantity of amino acids. An analysis of the membranes of *P. dispar* polyhedra showed no qualitative differences of the amino acids between them and the polyhedron protein (Wyatt, 1950).

Treatment of *B. mori* polyhedra with anhydrous methanol and dried hydrogen chloride gas converts all carboxyl groups to methyl esters (111 groups) and the basic groups to hydrochlorides (67 groups) (Desnuelle and Chang, 1945). No analyses are available of other kinds of inclusion bodies, such as cytoplasmic polyhedra and those characteristic of the polyhedrosis of *Tipula paludosa* (Meigen).

### III. CHEMICAL COMPOSITION OF VIRUS PARTICLES

Breindl and Jirovec (1936) were the first to report that *P. dispar* polyhedra give a positive Feulgen reaction. As we know now, this is due to the DNA content of the enclosed virus particles. Ultraviolet absorption spectra of alkaline polyhedra solutions also revealed nucleic acid (Dannenbergh, cited in Bergold and Schramm, 1942), which was confirmed by chemical tests but contain no uronic acid and no free carbohydrates (Tarasevich, 1946). Quantitative determinations show 0.84 % DNA, but no RNA in *B. mori* polyhedra (Gratia *et al.*, 1945); 13 % DNA in purified virus particles of *B. mori*; and 16 % in those of *P. dispar* (Bergold, 1947; Bergold and Pister, 1948a). Intensive analyses of purified suspensions of different viruses causing nuclear polyhedroses gave no indication of RNA (Wyatt, 1952a,b). A reinvestigation of highly purified *B. mori* virus particles revealed that they consist of about 7.9 % DNA and 0.915 % P, of which only 87 % is bound in the DNA (Table III) (Bergold and Wellington, 1954). This is in good agreement with the ratio of DNA bases to total P (Wyatt, 1952b). The remaining 13 % P could come from the surrounding virus membranes, which contain about 0.45 % P. There is about half as much nondialyzable P in the membranes as in the virus. The kind of bond is unknown. About 9 % DNA, but again no RNA was found in a purified preparation of the rod-shaped nuclear virus of *Aporia crataegi* L. (Krieg, 1956). Investigations of the N and P content of different virus particles and virus membranes, summarized in Tables I and III indicate that the virus membranes obtained by alkaline

treatment of the virus particles and by ultracentrifugation, have less N (12.5 %) than the virus particles (13.9 %).

TABLE III

NITROGEN, PHOSPHORUS, AND DEOXYRIBONUCLEIC ACID (DNA) CONTENT OF VIRUS MEMBRANES, VIRUS, AND POLYHEDRAL PROTEIN<sup>a</sup>

	Polyhedral protien (%)	Virus (%)	Virus membrane (%)
N	15.5	13.9	12.5
P <sup>b</sup>	0.064	0.915	0.45
DNA <sup>c</sup>	—	7.9	0.8 <sup>d</sup>

<sup>a</sup> After Bergold and Wellington, 1954.

<sup>b</sup> Determined by the strychnine phosphomolybdate method (Bergold and Pister, 1948b).

<sup>c</sup> Determined by the indole method (Ceriotti, 1952).

<sup>d</sup> Probably due to contamination by virus to the extent of about 10 %.

The first report of the presence of purine bases in *B. mori* polyhedra came from Manunta (1940); intensive investigations (Smith and Wyatt, 1951; Wyatt 1952a,b) of the bases of several insect viruses are summarized in Tables IV and V. The rod-shaped nuclear insect viruses contain only the purines (adenine and guanine) and the pyrimidines (cytosine and thymine), but no 5-methyl-cytosine or uracil. It is interesting that the ratios of adenine to thymine and guanine to cytosine are almost constant, but the ratio of adenine + thymine to guanine + cytosine is variable. The differences are apparently not at random but occur in steps (0.7; 0.95–1.10; 1.34–1.36; 1.67; and 1.87), representing groups of similar or identical ratios (see Table V). The significance of these steps is not known. As expected, polyhedron and capsule virus of closely related hosts (*Malacosoma disstria* Hbn. and *Malacosoma americanum* (F.), and *Choristoneura fumiferana* (Clem.) and *C. murinana* (Hbn.)) have similar ratios. It is surprising, however, that polyhedron viruses of widely separated lepidopterous hosts, a polyhedron virus from a hymenopteron (*N. sertifer*), and a capsule virus of a lepidopteron (*C. murinana*) have similar AT/GC ratios. This may suggest that not all DNA is of genetic specificity. It is noteworthy that representatives of two different groups of polyhedron and capsule viruses have entirely different ratios, although they occur in the same host (*C. fumiferana*).

Little is known about the chemical composition of cytoplasmic polyhedra. A preliminary test of such polyhedra from *C. fumiferana* was positive for DNA (Bird and Whalen, 1954). However, an analysis of purified spherical

TABLE IV  
PURINE AND PYRIMIDINE COMPOSITION OF DNA OF INSECT VIRUSES<sup>a</sup>

Host species	Host order and family	No. of analyses <sup>b</sup>	Moles per 100 moles total bases <sup>c</sup>				Percentage of total P accounted for
			Adenine	Thymine	Guanine	Cytosine	
<b>Polyhedral viruses</b>							
<i>Lepidoptera</i>							
<i>Porthetria dispar</i> (L.)	Lymantriidae	4	21.2 ± 0.15 <sup>c</sup>	20.05 ± 0.18	30.5 ± 0.11	28.25 ± 0.09	92
<i>Lymantria monacha</i> L.	Lymantriidae	1	24.6	23.8	26.8	24.7	—
<i>Choristoneura fumiferana</i> (Clem.)	Tortricidae	3	24.8 ± 0.12	24.0 ± 0.09	26.7 ± 0.14	24.5 ± 0.14	86
<i>Ptychopoda seriata</i> Schrk.	Geometridae	2	26.7	25.7	24.4	23.2	87
<i>Malacosoma americanum</i> (F.)	Lasiocampidae	3	29.2 ± 0.22	28.0 ± 0.34	22.5 ± 0.19	20.2 ± 0.11	93
<i>Malacosoma disstria</i> Hbn.	Lasiocampidae	3	29.2 ± 0.23	28.5 ± 0.37	21.9 ± 0.19	20.3 ± 0.07	86
<i>Bombyx mori</i> (L.)	Bombycidae	3	29.3 ± 0.25	28.0 ± 0.33	22.5 ± 0.05	20.2 ± 0.13	88
<i>Colias philodice eurytheme</i> Bdv1.	Pieridae	4	29.9 ± 0.35	27.6 ± 0.08	22.4 ± 0.15	20.1 ± 0.22	90
<b>Hymenoptera</b>							
<i>Neodiprion sertifer</i> (Geoffr.)	Tenthredinidae	2	32.3	30.3	19.5	17.8	—
<b>Capsule viruses</b>							
<i>Lepidoptera</i>							
<i>Cacoecia muricana</i> Hb.	Tortricidae	3	32.1 ± 0.14	30.5 ± 0.44	19.7 ± 0.35	17.9 ± 0.29	84
<i>Choristoneura fumiferana</i> (Clem.)	Tortricidae	1	32.8	32.4	18.4	16.4	—

<sup>a</sup> From Wyatt, 1952a.

<sup>b</sup> Independent analyses performed on different preparations of virus.

<sup>c</sup> Mean value and its standard error.

TABLE V  
MOLAR PROPORTIONS OF THE BASES IN DNA'S OF INSECT VIRUSES<sup>a</sup>

Host order and family	Virus host	Adenine Thymine	Guanine Cytosine	Purines Pyrimidines	Adenine + Thymine Guanine + Cytosine
Polyhedral viruses					
Lepidoptera					
Lymantriidae	<i>Porthetria dispar</i>	1.06	1.08	1.07	0.71
Lymantriidae	<i>Lymantria monacha</i>	1.03	1.08	1.06	0.94
Tortricidae	<i>Choristoneura fumiferana</i>	1.03	1.09	1.06	0.95
Geometridae	<i>Ptychopoda seriala</i>	1.04	1.05	1.04	1.10
Lasiocampidae	<i>Malacosoma americanum</i>	1.04	1.11	1.07	1.34
Lasiocampidae	<i>Malacosoma disstria</i>	1.02	1.08	1.05	1.36
Bombycidae	<i>Bombyx mori</i>	1.04	1.11	1.07	1.34
Pieridae	<i>Colias philodice eurytheme</i>	1.08	1.11	1.09	1.35
Hymenoptera					
Tenthredinidae	<i>Neodiprion sertifer</i>	1.07	1.09	1.07	1.67
Capsule viruses					
Lepidoptera					
Tortricidae	<i>Cacoecia murinana</i>	1.05	1.11	1.07	1.67
Tortricidae	<i>Choristoneura fumiferana</i>	1.01	1.12	1.05	1.87
	Ox spleen DNA <sup>b</sup>	1.04	1.02	1.03	1.22

<sup>a</sup> From Wyatt, 1952b.

<sup>b</sup> Included for comparison.

virus particles isolated from the cytoplasmic polyhedra of *Dasychira pudibunda* L. revealed that all P could be accounted for as RNA, amounting to about 7 % by weight of the virus (Krieg, 1956). According to unpublished results of Markham and Xeros, the cytoplasmic polyhedra of *Sphinx populi* L. also contain about 0.9 % RNA but no DNA (Xeros, 1956). The fact that these virus particles have RNA instead of DNA is of great significance, suggesting fundamental differences in the chemical structure and hereditary mechanism between the rod-shaped nuclear and the spherical cytoplasmic insect viruses. The difference in the type of nucleic acid is also of diagnostic importance. It is interesting that the cytoplasmic noninclusion virus of *T. paludosa* contains DNA (Williams and Smith, 1957).

Spectrographic and analytical investigations have revealed that Fe (0.015 %) and Mg (0.33 %) are the only metals present in appreciable quantities in virus particles of *B. mori* (Holoway and Bergold, 1953, 1955). It is probably not advisable to interpret the significance of such small quantities, but it is interesting that there are three times as much Fe and four times as much Mg in the virus particles as in the surrounding polyhedron protein.

Intensive investigations of the amino acid composition of several viruses were carried out by Wellington (1951, 1954). The results, which are summarized in Table VI, show that the five different polyhedron viruses and two capsule viruses analyzed all have a similar pattern of amino acid composition which differs markedly from the pattern of the surrounding inclusion body proteins. The virus contains proportionally twice as much arginine and serine, only half as much lysine and tyrosine, and shows a greater variability than the inclusion body protein. There are significant differences between any two viruses which tend to parallel the morphological classification rather than the grouping based on nucleic acid analyses. For example, the two morphologically similar capsule viruses of *C. murinana* and *C. fumiferana* do not differ much in their amino acids, but vary considerably in their nucleic acid composition; whereas the morphologically different viruses of *B. mori*, *M. disstria*, and *M. americanum* are similar in their nucleic acid, but not in their amino acids, with smaller differences between the two *Malacosoma* viruses. However, the *P. dispar* virus appears to be different from all others with respect to amino and nucleic acid composition, as well as in morphological characteristics. The chemical composition of the virus membranes was analyzed in another investigation and compared with that of the virus and polyhedron protein (see Table VII) (Bergold and Wellington, 1954). It was found that the membranes dissolve completely in 0.01 *M* alkali but not in alcohol, ether, or cold HCl of any concentration. Quantitative analyses of acid hydrolyzates (6 *N* HCl, 16 hr., 110°C.) revealed that the virus membranes contain more aspartic acid and much less arginine than the virus. The amino acids account for only 57.8 % of the weight of the virus membranes,

TABLE VI  
AMINO ACID CONTENTS OF INSECT VIRUSES <sup>a, b</sup>

Amino acid	Polyhedral type <sup>c</sup>					Capsule type <sup>c</sup>	
	<i>Pd</i>	<i>Ma</i>	<i>Md</i>	<i>Bm</i>	<i>Cpe</i>	<i>Cm</i>	<i>Cf</i>
Number of hydrolyzates analyzed:	2	2	1	2	2	1	2
Aspartic acid	12.0	11.0	11.0	13.3	13.2	14.0	13.2
Glutamic acid	8.5	9.3	10.0	6.9	7.6	7.0	6.9
Histidine	0.9	1.5	1.1	0.7	1.2	1.9	1.6
Lysine	2.1	3.5	3.5	3.3	2.2	3.4	3.1
Arginine	19.8	16.1	15.5	11.1	19.5	16.4	16.3
Glycine	6.1	4.0	4.9	8.1	3.5	4.6	5.0
Alanine	5.9	3.9	3.6	4.5	3.5	3.6	3.5
Valine	4.4	3.6	3.1	3.8	3.7	3.6	3.9
Leucine and/or isoleucine	11.0	11.8	12.7	11.2	11.4	13.0	11.7
Proline	6.4	6.3	5.2	4.9	4.5	5.3	7.6
Phenylalanine	4.5	4.7	5.5	4.6	4.2	5.6	5.4
Tyrosine	2.8	4.8	6.1	6.2	6.3	5.0	4.7
Serine	9.4	10.5	9.0	8.5	9.2	9.8	9.2
Threonine	4.4	6.0	6.4	9.6	6.7	3.8	5.0
Cysteine and/or cystine	0.9	0.8	0.6	0.7	1.1	0.6	0.6
Methionine	1.0	2.4	2.4	2.8	1.8	2.8	2.4
Gm. amino acid recovered/100 gm. virus	57.5	87.1	83.7	75.9	77.4	—	—

<sup>a</sup> From Wellington, 1954.

<sup>b</sup> Expressed as percentage of total recovered amino acids. The presence or absence of tryptophan could not be established.

<sup>c</sup> Species are abbreviated as in Table III.

64.6 % of that of the virus, and 94.5 % of that of the polyhedron protein. On a nitrogen basis, the analyses account for 94.4 % of N (nitrogen) in the membranes, 86.1 % of N in the virus, and 95.2 % of N (nitrogen) in the polyhedron protein. These figures would be even higher if corrections for

TABLE VII

AMINO ACID COMPOSITION OF THE VIRUS MEMBRANES, VIRUS, AND POLYHEDRON PROTEIN ON A NITROGEN BASIS<sup>a</sup>

Amino acid	Amino acid N <sub>2</sub> as per cent. of sample N <sub>2</sub>		
	Membranes	Virus	Polyhedron protein
Cystine and/or cysteine	0.6	0.4	0.5
Aspartic	10.1	7.6	8.5
Glutamic	4.1	3.5	7.8
Serine	6.9	6.2	2.8
Glycine	10.1	6.8*	4.0
Threonine	4.7	6.2	2.9
Alanine	3.4	3.9	3.0
Tyrosine	1.8	2.6	5.4
Methionine	1.3	1.4	1.8
Histidine	0.6	0.9	4.9
Lysine	4.1	3.4	13.6
Arginine	11.2	19.3	14.1
Valine	2.6	2.5	4.4
Proline	2.7	3.2	4.7
Leucine + isoleucine	7.2	6.5	9.2
Phenylalanine	2.4	2.1	4.5
Tryptophan**	?	?	3.1
Humin N <sub>2</sub>	19.6	?	?
Nucleic acid N <sub>2</sub>	1.0	9.6	0
Totals (% N <sub>2</sub> accounted for)	94.4	86.1	95.2

<sup>a</sup> From Bergold and Wellington, 1954.

\* Corrected for glycine produced by hydrolysis of nucleic acid.

\*\* Determined by the method of Gordon and Mitchell (1949).

ammonia, tryptophan, and humin could have been applied. The differences between the amino acid nitrogen and total nitrogen are due to nitrogenous compounds and decomposition products not recovered. Tryptophan could not be determined in the virus because of interference of DNA in the color reaction nor in the membranes because of lack of material. Considering the

yields of amino acids (on a nitrogen and a weight basis) and the total N content, one can conclude that the virus membranes consist of about 80 % and the virus of about 85 % nitrogenous compounds. About 1.3 and 0.2 % lipids can be extracted with boiling petrol ether from virus membranes and from the virus particles respectively, and a maximum of 7.5 % with a mixture of chloroform and methanol (4 : 1, 1 hr. at 50–55°C., and overnight at 20° C.) from the virus particles. About half of the 7.5 % extractable material of the virus can be accounted for by paper chromatography as total lipids. With the anthrone reaction about 1.2 % carbohydrates were found in the virus. Considering all the analyses, one can calculate that about 9 % of the weight of the virus and an even higher percentage of the virus membranes are still unaccounted for. It is not likely that much water was retained in the virus because it was dried over P<sub>2</sub>O<sub>5</sub> for about one week in high vacuum at room temperature and under the same conditions for 4 hr. at 110°C. (Bergold, 1957).

Gershenson (1956b,c) isolated protein-free DNA with Na Cl and DNA-free protein from *A. peryni* polyhedra. Neither preparation was infectious when injected singly or in succession into *A. peryni* pupae. However, when the DNA and protein preparations were mixed and left together for 7 hr. (4 hr. at 4° C. and 3 hr. at room temperature) before injection, 40 % died of polyhedrosis when about 1 mg. DNA and 5 mg. protein per pupa was used. Bergold (1958a,b) isolated DNA from purified *B. mori* particles using para-amino salicylate and phenol. The DNA preparation was very viscous; it had an  $S_{20}$  of about 14.5 Svedberg, but contained some protein which, however, was serologically not related to virus protein. The infectivity of this DNA preparation (10<sup>-1</sup> gm./*B. mori* larvae) was only 0.0001 % of that of the same amount of untreated DNA still contained in the intact virus particle.

In a search for the presence of enzymes in insect virus particles, a catalase was reported from alkaline solutions of *B. mori* polyhedra, which was more active at pH 8.9 than at pH 7.6 (Yamafuji *et al.*, 1941) as well as a proteinase (Yamafuji *et al.*, 1957). However, in other investigations no peptidase or phosphatase could be found (Duspiva and Bergold, 1942), and no lipase, lecithinase, hexosediphosphatase, nuclease, amylase, carboxylase, phenyl-oxidase, dehydrogenase, catalase, or protease could be detected in polyhedron solutions (Kuzin and Krzhevova, 1948). A deoxyribonuclease was recently reported to be present in an alkaline solution of *B. mori* polyhedra (Yamafuji, 1956; Yamafuji *et al.*, 1957). However, a reinvestigation in which the enzyme was assayed by measuring the release of trichloroacetic acid-soluble nucleotides revealed that no significant amounts of deoxyribonuclease are present in suspensions of polyhedra, polyhedron solutions, and purified virus particles (Faulkner and Bergold, 1957).

*B. mori* virus obtained by Berkefeld filtration is adsorbed on kaolin (negatively charged) and on aluminium hydroxide (positively charged) at a pH range between 5.0 and 6.0. It can be eluted with 0.2 *M* phosphate buffer of a pH of 9.0–9.5, but loses a considerable degree of its infectivity (Watanabe, 1941a). It was found that the virus migrates in the electrical field towards the cathode at a pH of 4.5–5.7 (positively charged) and to the anode between pH 6.9–10.0 (negatively charged) but does not move between pH 5.8–6.3 (isoelectric) (Watanabe, 1941b). In precipitation experiments with polyhedron solutions, it was found that the isoelectric point of the virus is at pH 6.0–6.2 (Aizawa, 1952).

The above results indicate that some classic insect viruses are fairly well investigated, but that we know nothing or very little about the chemical composition of the other groups of insect viruses, for example, the  $\sigma$ -virus of *Drosophila* and the so-called insect-plant viruses.

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## Chapter X

### The Scope and Limitations of Immunological Methods in the Characterization and Functional Study of Viruses

F. M. BURNET

*Walter and Eliza Hall Institute, Melbourne*

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#### I. INTRODUCTION

Historically, virology was developed as an elaboration of attempts to prevent human viral infections by immunological methods. It might also be said that immunology itself derives from Jenner's discovery of vaccination. In the study of the virus diseases of man as public health and clinical problems, the production of immunity by means not involving the danger of clinical disease has remained a central theme. Almost equally important has been the development of methods for the recognition and measurement of antibody in serum as a means of diagnosing subclinical as well as clinical infection.

With the development of interest in the study of viruses as such in the laboratory, immunological methods became of much practical importance, especially for the identification of virus strains. There are also many situations in the experimental study of infection when a method is needed that will

inactivate a certain fraction of virus, while leaving another fraction active, and that will have no harmful effect on the host cells in the system. For such purposes specific immune serum, used with due regard to its powers and limitations, is the standard reagent.

In the last analysis, all such uses depend on the immunological specificity of viral antigens, which in some instances, and possibly in all, are of protein character. Only indirect attention has so far been paid to the functional significance of viral proteins, but as such studies develop it is inevitable that much use will need to be made of immunological methods. In the animal viruses, at least, immunological specificity has a vital part in determining survival of a virus strain; it is possible that in all viruses specific protein patterns, enzymatic or other, play a part in the initiation of infection. At the present time, the immunological approach provides an excellent experimental technique directly applicable to the finer pattern differences between proteins and it will often provide information in both the modern technical and the trivial senses beyond anything obtainable by other chemical means.

The present contribution is concerned, not with the significance of immunity in viral disease, but with immunological procedures that have been used for the characterization of viruses and for the study of the virus-host cell interaction. As far as possible, the approach will be made as applicable to plant and bacterial viruses (where immunological phenomena are of no significance in nature) as to animal viruses (where they may dominate the ecological situation).

As in so many other current aspects of virology, knowledge of the antigenic structure of viruses is incomplete and patchy. Reasonably pure and well-characterized protein antigens have been obtained from some of the better-studied plant viruses (Schramm, 1943) and one antigen (LS) of limited significance from vaccinia virus (Smadel *et al.*, 1942). From the great majority of virus types no attempt has been made to separate antigenic fractions from the virus particle.

This does not, however, detract from the value of the studies made at a more superficial level. As examples may be mentioned the use that has been made of appropriate antisera in analyzing the relation of protein and nucleic acid in plant viruses, in correlating details of morphology with function in the bacterial viruses, and in demonstrating the nature of filamentous forms of influenza virus. In the future we can reasonably expect that elaboration of work on the soluble complement-fixing antigens of animal viruses will throw much light on the process by which virus-specific protein is synthesized in the infected host cell.

There is still some question as to the nature of the process by which an animal becomes immune to a virus disease after clinical or subclinical infection. Many would consider that cellular immunity of some type must be

involved to account, for instance, for the relatively normal course of viral infections in children with agammaglobulinemia (Good and Varco, 1955). In all probability the immune process is a complex one and involves more than a simple production of circulating antibody in the form of a modified  $\gamma$ -globulin. There is no doubt, however, that classic circulating antibody does play an important part; in all the applications of serology to be discussed, serum from animals that have either been infected with a virus or appropriately inoculated with a suitable preparation of virus particles is used. In most instances, the rabbit is the animal used, both because of its availability and convenience and because of the specific and reactive type of antibody it produces. With some animal viruses much more satisfactory sera are obtained if the animal is susceptible to infection by the virus than if a nonsusceptible animal is used. This holds particularly if antibody reacting with soluble complement fixing antigen is required. On the other hand, Gard *et al.* (1956) have considered that for such a standard task as assessing the antigenic potency of a killed virus polio vaccine, there are positive advantages in using a nonsusceptible animal, in their case the guinea pig. The absence of antigenic damage during inactivation can then be verified by showing that the antibody response is equivalent to that given by fully viable virus.

In the present context the production of antibody can be regarded as a simple response to the entry of foreign antigen into the lymph and blood stream of the rabbit, or whatever other vertebrate is being used. We are concerned only with the reaction of virus particles or virus products with already formed classic antibody. No attempt will therefore be made to discuss either the general problem of antibody production or the controversial question of the persistence of virus in the immune host.

As is found with most other types of antibody, that produced in mammals, either following infection with live virus or by immunization with inactivated material, is present in the  $\gamma$ -globulin fraction (Koprowski *et al.*, 1947).

For our present purpose, antibody may be characterized as specifically patterned  $\gamma$ -globulin molecules which, in virtue of that pattern, unite preferentially with antigenic determinants forming part of the chemical structure of the virus used for immunization. The various sections of this chapter will be concerned essentially with the nature of the union between antibody and virus, and with the physical and functional results of that union.

## II. THE CONCEPTS OF IMMUNOLOGICAL SPECIFICITY

The development of concepts of immunological specificity, due largely to Landsteiner (1946), has been based almost wholly on the use of precipitin or complement fixation reactions, both of which are essentially aggregation reactions in which a complex of antigen and antibody units either builds

up into a visible aggregate or by its newly developed surface qualities adsorbs complement. Most neutralization tests with viruses do not necessarily involve aggregation but, when concentrations and conditions are appropriate, classic aggregation reactions can probably be demonstrated with any virus. There are a number of instances where virus-neutralizing antibody can be shown to act also as a precipitin or complement-fixing antibody and, with qualifications based on the possible presence of a variety of antigens on the surface of some viruses, most workers believe that the two types of antibody are identical.

The classic theory of the precipitin reaction, due to Heidelberger and Kendall (1935) and influenced much by Marrack's (1934) ideas on lattice formation, may be used as a basis for the discussion of virus-antibody reactions as for any type of antigen-antibody reaction.

In this view, reaction takes place between determinant groups, of which there are usually multiple examples on each antigenic molecule or particle, and complementarily patterned groupings on antibody molecules. There is evidence to suggest that there are two such specifically patterned areas on standard antibody globulin molecules (Marrack *et al.*, 1950; Singer and Campbell, 1951). In a recent review of the nature of antigen-antibody aggregates, Marrack (1955) considers that the approach of Goldberg (1952) provides the most physically satisfactory picture of the process. According to this view, all reactions are reversible and union will take place according to the principle of maximal entropy. The primary aggregates with a soluble protein antigen will be mostly in the form of branched chains with little formation of cyclic (lattice) structures. Further aggregation is dependent on ionic content and varies according to the species furnishing the antiserum and other factors. With excess of antigen, precipitation fails to occur and with toxin-horse antitoxin systems, precipitation also fails in the presence of excess of serum.

Union between two complementary configurations on antigen and antibody seems unlikely to be expressible in simple mass action terms. For union to occur an appropriate steric orientation will be required and it may be that a provisional ionic union of some sort is needed to allow the definitive specific union to take place. Nothing is really known about the "goodness of fit" between a given antibody and antigen, but there is much experimental and theoretical reason to believe that wide variations in this quality must exist. Where antigenic molecules are embedded in the complex environment of a particle surface, as in a virus, there must be a still greater range of opportunity for various degrees of partial union. In the classic antigen-antibody reaction, the globulin of the antibody is denatured and rendered insoluble. The conditions under which union of an antibody globulin molecule to an antigenic molecule or surface results in its denaturation have not been

reported. The process appears to take time and to result eventually in an irreversible union.

In the opinion of one important group, whose spokesman is Boyd (1947), the lattice theory is inappropriate, the essence of the precipitin and allied aggregation reactions being that the deposition of a spread-out and denatured molecule or mosaic of molecules of antibody on a molecule or particle of antigen will give it relatively hydrophobic qualities, with resultant loss of solution or suspension stability in the usual ionic environment.

The question of specificity is also controversial, but from the point of view of relevance to the phenomena encountered in immunological work with viruses, the most important feature is the heterogeneity of all antibody populations. If one has two similar but not identical antigens of any degree of complexity, such as purified serum albumin of human and bovine origin, red cells of mouse and rat or two influenza A strains, appropriate antisera against each member of the pair can be prepared. Each will show a high degree of cross reaction. In each case, however, absorption of a serum with the heterologous antigen of the pair will remove its reactivity with that antigen but leave the homologous activity hardly altered. Where, as is often the case with a group of related bacteria, many heterologous strains will react with a given serum, it is possible by appropriate absorptions to produce a widely different set of reagents, each necessarily corresponding to a reassortment of the molecular species in the population of antibody molecules. When absorbed with homologous antigen, however, all types of activity, homologous and heterologous, are removed in parallel.

Analysis of the significance of this phenomenon has been virtually confined to bacterial antigens. Both Furth and Landsteiner (1929) working with salmonellas, and Burnet (1934) with dysentery bacilli, showed that there was no evidence for the presence of a mosaic of antigens on the bacterial surface. Soluble preparations were precipitated as a whole, not in fractions. The diversity is in the antibody population, not in the antigen population. The suggestion previously put forward (Burnet, 1934) can still be supported. This is that any antigenic molecule carries a variety of potential antigenic determinants, e.g., ABCD, but that any antiserum will contain antibody molecules carrying complementary groupings that are not representative of the whole antigen. If we simplify the conditions by assuming that each antibody molecule carries active patches corresponding to two of the four antigenic determinants, then the population of antibody will be composed of ten molecular species, aa, ab, ac, etc. All of these will react with antigen ABCD, but only a proportion with antigens AEFB or BFGH.

If this is true, it has important implications for one of the classic immunological problems in virology, the significance of serological differences in influenza A viruses. Hirst (1952) found that by appropriate absorption of

immune sera with heterologous strains he could obtain monovalent reagents, which allowed the recognition of a series of new antigens emerging irregularly over the years. In some sense this is undoubtedly an artifact. The important question is not whether there is change in antigenic structure, but whether there is any intrinsic regularity in the changes. If the above hypothesis is correct, it should be possible by appropriate heterologous absorptions to make the number of steps as many or as few as one desires. From the lack of concordance among those who have used the method, it seems very probable that this is in fact the case.

Another point that it is desirable to keep in mind is the varying antigenic potency of antigens and, by implication, of determinant groups. Some proteins, like gelatin, are virtually nonantigenic; some bacterial viruses, like C16 and T2, produce high titer antisera with the greatest ease; others, like SS, produce minimal titers only (Burnet, 1933b). Landsteiner (1946) found some of his azoproteins much more effective in producing specific antibody than others. Although it is hard to envisage how an experimental test of the point might be made, it seems certain that antibody molecules can differ in their effectiveness of union with the corresponding antigenic determinant. The simplest explanation of incomplete (or monovalent) antibody is that such molecules can make only a weak, highly reversible union unless they are trapped, as it were, in the lattice formed by antibody molecules of full avidity. The sum of these contentions is that the complexity of the situation when antiserum reacts with antigenic material is far too great to make it justifiable to do more than look cautiously for broad analogies with simple chemical systems to which the elementary laws of physical chemistry can be applied. In any system of significance for virology, we are concerned with antigenic molecules embedded in a complex particulate system. The system that is effectively concerned with antibody production, i.e., the virus particle presumably in the process of disintegration in cells of the antibody-producing system of the host, is also different from the virus particle with which the antiserum reacts in any experimental test. This may be responsible for certain asymmetrical antigenic relations between virus strains. Strain A may produce antibody which reacts more actively with strain B than with itself, as in van der Veen and Mulder's (1950) P-Q phenomenon in influenza viruses.

### III. AGGREGATION REACTIONS IN VIROLOGY

Aggregation reactions with qualities intermediate between the specific precipitation of a soluble antigen and immune agglutination of bacteria can be observed whenever preparations of sufficiently high virus content and active antisera are available for test. Except in the plant virus field they have not been extensively used. Complement fixation has, however, become

progressively more important for diagnostic work in animal virology in recent years.

### A. *Precipitin Reactions*

#### 1. *Bacterial Viruses*

Macroscopic agglutination (or precipitation) of viruses of the C16 - T2 group was demonstrated by Schlesinger (1933) and Burnet (1933a). The latter found that the range of viruses agglutinated corresponded to the group defined on the basis of neutralization reactions. An indirect type of aggregation reaction with bacterial viruses is by the agglutination of formalin-killed bacteria to which phage has been specifically adsorbed at high multiplicity (Burnet, 1933c). This has been confirmed by Tolmach (1957), but little or no use has been made of either method in recent years.

#### 2. *Plant Viruses*

Precipitin reactions with rabbit antisera are used as a standard method in all laboratories working with plant viruses and the reaction has been examined in detail by Kleczkowski (1941), Schramm and Friedrich-Freksa (1941), and Malkiel and Stanley (1947). In general, the results correspond to those of other typical antigens, the quantitative relations at the equivalence point being what would be expected in relation to the size of the virus particle. With tobacco mosaic virus, Malkiel and Stanley calculated that about 60 molecules of antibody were attached to each virus rod at the equivalence ratio.

The specificity relationships between related but not identical virus types conform to what has been found with bacteria or animal viruses. Precipitation with a heterologous antiserum is less complete than with homologous. Less antibody is precipitated with heterologous virus than with homologous. Absorption with homologous virus removes all antibody while heterologous absorption leaves most of the activity against the homologous virus. Such relations hold irrespective of the host plant on which the virus was grown (Malkiel, 1947). Complement fixation reactions can be shown with tobacco mosaic virus as antigen by standard technique (Chester, 1935).

More recently, agar gel precipitation methods (Ouchterlony, 1949) have been extensively used, the most recent account being that by Kleczkowski (1957). Most workers have been concerned with tobacco mosaic virus (Sang and Sobey, 1954), and, specifically, with the relationship of the antigenically related proteins free of nucleic acid, which are either found in infected tissues (Takahashi and Ishii, 1952; Commoner and Rodenburg, 1955; Commoner and Yamada, 1955) or produced by the action of alkali on purified tobacco mosaic virus (Schramm, 1943). The results reported by Kleczkowski, which are concordant with those of Jeener *et al.* (1954) indicate that more than one line-forming component is present in tobacco mosaic virus. If rabbits are given a

relatively prolonged course of immunization, they provide an antiserum that will react with all determinants of tobacco mosaic virus, the x proteins present in sap from infected plants and the A proteins resulting from mild chemical treatment of the virus. The conditions of line production in gels are probably too complex to allow any precise statement beyond the indication that disaggregated or altered fragments of protein are present in x and A. Whether these components are present as such in the virus itself and whether they play a part in the synthesis of the virus is unknown.

Electron micrographs indicate clumping of the virus of tobacco mosaic by antiserum in a fashion resembling the agglutination of bacteria (Anderson and Stanley, 1941; Malkiel and Stanley, 1947). Using two spherical viruses, Southern bean and bushy stunt, Black *et al.* (1946) observed principally a failure of the aggregated particles to form the regular arrays seen without antibody, and some evidence of an increased separation between the particles. Bawden and Pirie (1938) drew attention to the characteristically different macroscopic appearance of the precipitates with a spherical virus (bushy stunt) and a filamentary one (TMV); the former being compact and granular, the latter loosely flocculent, resembling closely the O and H types of agglutination seen with bacteria.

### 3. *Animal Viruses*

In this field, aggregation reactions involving virus particles which had been concentrated and at least partially purified have been reported for vaccinia viruses, influenza viruses, and polio viruses. There is little doubt that they will be equally demonstrable with any virus which can be obtained in purified form and for which a high titer immune serum is available. Relatively little work has been reported and the results have not significantly influenced the general trend of virological research.

Immunological work with purified vaccinia virus was initiated by Craigie and Tulloch (1931), and subsequently elaborated by Craigie (1932) and by the group associated with Rivers at the Rockefeller Institute. Two soluble antigens, L—S protein (Shedlovsky and Smadel, 1942) and NP, nucleoprotein, were obtained from the elementary particles; both the corresponding antisera were shown to agglutinate particle suspensions (Smadel *et al.*, 1942). Neither of these antisera, however, showed any power to neutralize the infectivity of the virus. The power of a hyperimmune serum to inactivate the virus may be related to a residual agglutinin x, which remains after absorption with the two chemically characterized antigens (Smadel and Hoagland, 1942).

Detailed study of the precipitin reactions of purified influenza virus by Knight (1946) was principally concerned to demonstrate the presence in or on the virus surface of antigenic groups with host rather than viral specificity. Virus prepared from infected allantoic fluid showed chick antigenic qualities,

in contrast to mouse lung virus, which reacted with antiserum to normal mouse tissues. In both instances, specific aggregation by antiviral sera could also be demonstrated and Knight's conclusion was that perhaps 20-30 % of the surface of the virus particle carried host antigen patterns. Influenza virus in the filamentary form is agglutinable by immune serum, producing woolly aggregates easily visible by dark ground microscopy (Burnet and Lind, 1957). Belyavin (1955, 1956) has suggested the use of the agglutination reaction in routine serological work with influenza virus strains. The hemagglutinin inhibition reaction, however, is so convenient that it is not likely to be supplanted by another test giving essentially the same information.

Smith *et al.* (1956a) have shown that concentrated polio virus preparations are flocculated specifically by immune rabbit sera. The reaction can be read either macroscopically or by dark ground examination under the microscope (Smith *et al.*, 1956b). It has been observed with human convalescent serum.

### *B. Complement Fixation*

Despite extensive recent work on the nature of the four components of complement, complement fixation still remains essentially an empirical means of indicating that an antigen-antibody reaction has occurred. Orthodox interpretation is that the forming aggregates of an antigen-antibody reaction actively adsorb complement, rendering it unavailable to hemolyze the sensitized red cells added as indicator. The conditions for optimal fixation of complement vary with the system and empirical adjustments will usually be needed to obtain the most useful results. Reactions with both soluble and particulate antigens will fix complement; in most of the earlier work with viruses no distinction was made between reactions due to virus particles and those due to soluble products of virus multiplication.

For obvious reasons, complement fixation has been much more extensively applied to the study of animal viruses than with plant or bacterial viruses, but some applications have been reported in both the latter fields. A subject of major interest at the present time is the significance of soluble protein with viral specificity that is produced in the course of infection. Such material can often be detected by complement fixation reactions after particulate virus is removed by centrifugation, adsorption to red cells, or other appropriate method. Extensive discussion of this theme in relation to influenza virus will be found in Chapter III(b) of Volume 3. If the findings with influenza can be generalized, it appears that soluble complement-fixing antigen is produced early in the course of infection, that the corresponding antibody is stimulated only by infection, and that it is of no significance in conferring protection against reinfection. Positive complement fixation tests following infection tend to become negative much sooner than positive neutralization

reactions and for much epidemiological work the complement fixation test can be taken as a useful index of recent infection.

### *C. Fluorescent Antibody Techniques*

An immunological technique which is rapidly extending its usefulness in virology is the use of fluorescent antibody as a histochemical reagent. This was introduced by Coons and has been applied extensively by his group to the examination of experimental lesions of influenza and mumps virus infections (Coons *et al.*, 1950; Watson and Coons, 1954). There is evidence that both soluble complement fixation antigen and somatic viral antigen can be located with this method; one of the most interesting findings has been the demonstration claimed by Watson and Coons that the first antigen to appear in the infected cell is of the soluble complement fixation type and is located in the nucleus. Another interesting development has been its use to detect a production of viral material in cells resisting infection in the ordinary sense. Prince and Ginsberg (1957) have shown that Ehrlich ascites tumor cells infected *in vitro* with Newcastle disease virus and then returned to the mouse peritoneal cavity failed to develop infectivity, hemagglutinin, or complement fixation antigen, but did develop strong cytoplasmic fluorescence when stained with treated anti-Newcastle disease virus serum. Wecker and Schafer (1957), using separate fluorescent sera for soluble complement fixation antigen and hemagglutinin of fowl plague virus, found that the S antigen begins to appear in the nucleus at three hours, and an hour later hemagglutinin antigen is found in the cytoplasm. More applications of this versatile technique to studies of this general type can be expected in the future.

## IV. THE PROCESS OF VIRUS NEUTRALIZATION

### *A. Neutralization of Bacterial Viruses*

Bacterial viruses, particularly those of the large particle C16-T2 group (Adams, 1952) are readily neutralized by immune sera; a great deal of work has been devoted to the elucidation of the reaction. In recent years, discussion of the process has largely centered on the finding that union of phage particle to the susceptible bacterial cell takes place by the tip of the phage tail (Anderson, 1953). At the present time, there is much to suggest that the agent that actually attaches to the bacterial cell wall is contained in fibrils, which are probably wound spirally around an inactive core, and frayed ends of which can often be seen in electron micrographs at the tip of the tail (Williams and Frazer, 1956; Evans, 1956).

There are at least two distinct antigens on the surface of phage T2 (Lanni and Lanni, 1953). One, which is present on the "donuts" developed in proflavin-treated bacteria as well as on normal phage, provokes complement-fixing and aggregating antibody but not neutralizing antibody. The other produces neutralizing antibody, is not present in donuts, but is liberated as soluble material on bacterial lysis (Burnet, 1933a), as well as being present on the virus surface, presumably in the terminal filaments of the tail.

If we confine ourselves to phages of the C16-T2 group, except where other phages are specifically mentioned, and to active antiserum produced by an adequate course of immunization, the main features of the reaction between phage and neutralizing antibody can be summarized in a few paragraphs.

1. The antigens concerned in neutralization reactions are relatively specific, but there is sufficient range of cross reaction to make serological relationship the most satisfactory basis for the classification of bacterial viruses (Burnet, 1933b; Adams, 1952). When two related phages, e.g., C16 and D29 (Burnet, 1933c), or T2 and T4 (Hershey, 1946) are studied by cross absorption of immune serum, the usual relation holds. Heterologous antigen removes neutralizing activity against itself but leaves homologous activity little reduced.

2. The reaction proceeds logarithmically, with some evidence of a slight initial lag and some slowing in the later phases (Burnet *et al.*, 1937; Andrewes and Elford, 1933; Hershey *et al.*, 1943). The activity of neutralization in any given system is most conveniently expressed as the first order velocity constant  $K$  (Adams, 1950). Very little evidence for the attainment of an equilibrium state has been presented but, in suitable experiments, it has been shown that addition of soluble antigen can reactivate a proportion of neutralized phage (Burnet, 1933a). This finding suggests that long exposure with dilute reagents might allow the demonstration of an equilibrium state. The kinetics of the reaction indicate that the rate-limiting process is the combination of one antibody molecule with one phage particle. Minor discrepancies, such as the initial lag, are probably due to "inhibited" phage in which adsorption of bacterial products hinders free attachment to the host (Cann and Clark, 1954).

3. Virus inactivated by immune serum can be reactivated by papain digestion (Kalmanson and Bronfenbrenner, 1943) or sonic treatment (Anderson and Doermann, 1952). This points clearly to the denatured antibody globulin acting by interference with the interaction of virus and host and not by any directly destructive effect.

4. Despite the indication from the kinetics of the standard neutralization process that neutralization normally results from the union of a single antibody molecule, there is adequate evidence for the existence of partly neutralized phage. The apparent rate of neutralization in the same phage-serum

mixture may differ according to the host strain on which titrations are made (Kalmanson and Bronfenbrenner, 1942).

Partly neutralized phage will fail to pass a gradocol membrane through which the same phage will pass readily in the absence of serum (Andrewes and Elford, 1933). This may well be due to adsorption of antibody of Lanni's second type but two other phenomena described by Burnet *et al.* (1937) appear to be relevant. These are the production of very small diameter plaques by partly neutralized virus and the loss of sensitivity to inactivation by specific bacterial extracts. This phenomenon is almost the converse of that described by Cann and Clark (1954) and mentioned above.

5. Bacteria killed by formalin actively adsorb phage and are then agglutinable by antiphage sera and can absorb out neutralizing antibody (Burnet, 1933c; Tolmach, 1957). In other words, attachment to bacteria does not block completely the sites at which neutralizing antibody molecules can be adsorbed. It can also be shown (Burnet *et al.*, 1937; Hershey and Bronfenbrenner, 1952; Nagano and Mutai, 1954) that neutralized virus is still at least partially capable of attachment to bacteria.

6. Bacterial viruses can be inactivated by material extracted from the host cell. These contain polysaccharide (Gough and Burnet, 1934) and probably represent fragments or derivatives of the lipomucoproteins which make up the somatic antigen of the cell wall of intestinal bacteria (Goebel and Jesaitis, 1953). One such component active against T5 has been shown by Weidel and Kellenberger (1955) to be in the form of small spherical particles which can be seen in electron micrographs attached to the tip of the tail of inhibited phage. There is a very close resemblance in the kinetics of inactivation by phage by antiserum and by such bacterial products (Burnet *et al.*, 1937).

7. The process of attachment of phage to bacterium is complex. Morphologically it appears that the distal half of the tail sheath unwinds or frays out into fine fibrils exposing a core which appears to be the agent responsible for penetration of the cell wall and irreversible binding of the phage. It is stated by Tolmach (1957) that neutralization by specific antibody inhibits irreversible union of T2, but hardly affects the initial, reversible phase of union. Once irreversible union has been affected, subsequent addition of antiphage does not prevent infection (Delbrück, 1945).

From this summary, a relatively simple interpretation of the action of antibody on phage can be derived. Neutralization results when sufficient antibody molecules are attached to the sheath (fibrillar) protein of the distal third of the tail in such a fashion as to prevent the proper functioning of the process leading from reversible to irreversible union. One would expect (and there is nothing reported incompatible with the expectation) that a very high concentration of antibody will also block reversible union. One antibody molecule appropriately attached is apparently enough to interfere sufficiently

with the unwinding of the fibrils to prevent infection. Under such conditions, however, the phage particle could still adsorb further antibody.

In this discussion no mention has been made of the important findings of Jerne (1956; Jerne and Avengo, 1956) in regard to low-grade antibody against phage T4 (tryptophan-dependent strain) present in normal serum, or during the early stages of immunization. The topic is, however, more relevant to a discussion of the nature of antibody production than in the present context.

### *B. Neutralization of Plant Viruses*

Relatively little work has been reported on the neutralization of infectivity of plant viruses since the work of Chester (1934) more than twenty years ago. The reasons for this relative lack of interest are probably multiple. Most fundamental work has been at the biochemical level; infectivity tests have been largely limited to assessing the viability of virus treated in various fashions. Sufficient amounts of virus are available to make precipitin reactions the method of choice for immunological work and methods for estimating infectivity quantitatively by the local lesion method are of only limited applicability.

The relationship between the amount of virus applied and the number of local lesions produced deviates considerably from the linear, but this can be allowed for by the use of appropriate corrections. A more serious difficulty is that the concentration of virus particles necessary to produce a single lesion on a susceptible leaf is of the order  $10^6$ – $10^7$  per milliliter; to obtain usable results in quantitative inactivation experiments, concentrations approaching  $10^{10}$  particles per milliliter must be used (Rappaport, 1957). In contrast to the type of experiment used in studying the action of antibody on bacterial or animal viruses, this means that aggregation occurs in all virus-antiserum mixtures and no formal comparison with the results obtained with standard animal and bacterial virus neutralizations is possible.

A discussion of the older results was given by Burnet *et al.* (1937). As might be expected from the high concentration of virus antigen used in infectivity tests, the neutralizing action of serum is best shown when virus is used as highly diluted as is practicable. Mixtures of relatively concentrated reagents show a high degree of reactivation on dilution. Bawden (1943) quotes a mixture which undiluted gave an average of two lesions. Successive 5-fold dilutions of this mixture gave, respectively, 4, 9, 9, and 6 as the average numbers of lesions; the last figure would correspond to 3750 lesions undiluted if a linear relation held.

More recent experiments by Rappaport and Siegel (1955) and Rappaport (1957) are in general accord. Working with tobacco mosaic virus, their most

striking experimental result was the persistence of a considerable fraction (3-6 %) of infectivity in a zone of considerable antibody excess. After removal of aggregates and any uncombined virus by centrifugation, the supernatant fluid contained antibody excess, as tested either *in vitro* or by inactivation tests. The explanation favored, although it was recognized that others were possible, was that particles with critical sites unoccupied by antibody could be trapped within the aggregates and thus protected from antibody excess. Little use has been made of neutralization tests in the characterization and differentiation of plant viruses, but enough has been done to indicate that the method has potentialities. Spooner and Bawden (1935) showed that antisera against potato virus X failed to neutralize potato virus Y and tobacco mosaic virus. Rappaport *et al.* (1957), by making use of the shape and position of inactivation curves with a rather wide range of antiserum concentrations, were able to distinguish three serological groups among five strains of tobacco mosaic virus; they consider that the method has important potentialities. It may be of special use for the characterization by serum neutralization of viruses that are difficult to isolate in quantity.

### *C. Neutralization of Animal Viruses by Immune Serum*

Despite the extensive everyday use of neutralization tests in diagnostic virus laboratories, very little has been effectively established about the nature of the process by which infectivity of animal viruses is destroyed. A large part of this difficulty can be ascribed directly to our ignorance of the details of the process by which infection occurs. For every virus that has been studied in detail, with the possible exception of vaccinia virus (Overman and Tamm, 1956) the  $ID_{50}$ , as estimated in the most susceptible available host, corresponds to more than one morphological particle.

A table has been compiled by Isaacs (1957) to show the approximate number of morphological particles corresponding to one  $ID_{50}$  for a number of animal viruses. Some examples taken from that table and, where necessary added to as a result of more recent publications are shown in Table I.

No satisfactory explanation for these discrepancies has been provided. There is no convincing evidence even as to whether in any given case where  $n$  particles are needed to produce one initiated infection, the situation is best interpreted (a) as one active particle to  $n-1$  inactive particles, or (b) as  $n$  potentially infective particles of which only one will, on the average, initiate a continuing series of infections. Since it is almost invariably found that if two host species are compared a higher proportion of infections are initiated on one than on the other, one has a strong prejudice in favor of alternative (b), but extrapolation of the argument beyond the most susceptible available host may not yet be justifiable.

TABLE I

THE NUMBER OF MORPHOLOGICAL PARTICLES NEEDED TO  
GIVE ONE ID<sub>50</sub> FOR SOME REPRESENTATIVE VIRUSES <sup>a</sup>

Virus	Number of particles
Vaccinia	366; 4.2 1.0 <sup>b</sup>
Psittacosis group	43; 100
Myxovirus group—	
Influenza A and B	10
Newcastle disease	5
Mumps	100
Poliovirus	20,000
	35, 60, 120 <sup>c</sup>
Western equine encephalitis virus	14 <sup>d</sup>

<sup>a</sup> Isaacs, 1957.

<sup>b</sup> Overman and Tamm, 1956.

<sup>c</sup> Schwerdt and Fogh, 1957.

<sup>d</sup> Dulbecco *et al.*, 1956.

Similarly, it is found that a given virus-serum mixture that is neutralized for a moderately susceptible host may be highly infective for a more susceptible species. Working with the same strains of influenza virus at different stages of adaptation to growth in the allantoic cavity, Burnet (1943) found that neutralization by homologous immune serum was more active against the less completely adapted substrain. Von Magnus (1951) showed that antibody against Theiler's virus could not be demonstrated by intracerebral inoculation in adult mice, but with baby mice from a virus-free colony intraperitoneal inoculation gave clear-cut positive results.

### 1. Neutralization as Tested in the Experimental Animal

The crude facts are simple. If infective material is mixed with immune serum and inoculated by an appropriate route into a susceptible host, its infectivity will be less than if normal serum is used. Closer study, however, raises difficulties of interpretation in every direction. When the process by which vaccinia virus is neutralized first came under study around 1928-1935 it soon became evident that serum had no directly destructive action on the virus. A mixture judged to be fully neutralized by failure to give a lesion in intradermal inoculation in the rabbit could be reactivated by simple dilution (Andrewes, 1929, 1930). Andrewes also commented on the great variability in the results of inoculating the same serum-virus mixtures into different individual rabbits. Evidence that union between virus and neutralizing antibody took place *in vitro* was eventually obtained by Smith (1930); in 1937 Salaman showed that protective antibody could be absorbed with elementary

bodies. Even as late as 1935, however, Sabin (1935) considered that the evidence pointed strongly toward an effect of antiserum on the cell rather than on the virus.

This early work was sufficient to show, first, the complications associated with using the intact animal, in this case the rabbit, as the test object, including difference in results according to the route of inoculation, irregularity in replicate tests plus the possible complication of early active immunity and, second, the strong indication that a proportion of active virus could persist in the presence of a large excess of antibody with, as a virtual implication of this, the possibility of reactivation on dilution.

## 2. *Neutralization on the Chorioallantois*

With the development of pock-counting methods on the chorioallantois, it seemed possible that most of these disabilities could be overcome and Burnet and associates (1937) carried out a large volume of work on the neutralization of most of the viruses then known to produce discrete foci on the chorioallantois. This led to the development of a general interpretation which was summarized at the time as follows:

“(i) Virus inactivation by immune serum results primarily from union of antibody to the virus surface. This union is a reversible one; it takes place at a rate and reaches an equilibrium determined by the ordinary laws of reversible chemical unions.

(ii) Union has no intrinsic inactivating effect on the virus: the inactivation is the result of the interaction between susceptible cell and antibody-coated virus particle. Certain susceptible cells are protected against infection by lesser degrees of antibody-coating on the virus particle than are required by other types of cell.

(iii) In practice the experimental results obtained with any given virus will deviate from the theoretical values owing to the operation of one or more of the following factors:

- (a) variations in susceptibility within the virus population,
- (b) the time needed to reach equilibrium,
- (c) secondary events between time of inoculation and initiation of lesion,
- (d) secondary changes including aggregation and irreversible antibody union,
- (e) local or general passive immunity produced by antibody inoculated with the virus.”

## 3. *Application of Plaque Techniques*

The application of plaque techniques to tissue culture sheets infected with Western equine encephalomyelitis (WEE), poliovirus and other viruses by

Dulbecco (1952) provided an opportunity of applying the same principles that were used in poek counts on the chorioallantois to a more elegant and controllable system. The paper by Dulbecco and associates (1956) is the most important contribution on the theoretical side that has yet appeared. They were concerned with both WEE and poliovirus and, although the results obtained were generally similar in quality to those reported by Burnet *et al.* (1937) on the chorioallantois, their interpretation was radically different. All who had previously discussed the matter had agreed that a reversible reaction between virus and antibody must be involved. Dulbecco *et al.* (1956) concluded: (1) Neutralization is a direct consequence of the combination of the virus particle with antibody molecules; (2) the kinetics of neutralization in presence of antibody excess are of the first order; and (3) the rate of neutralization is linearly dependent on the concentration of antibody. From (2) and (3) it is deduced that attachment of a single molecule of antibody is sufficient to inactivate a particle; (4) the virus-antibody complexes formed are very stable; (5) virus particles of the types used could absorb up to 15 equivalents of antibody; and (6) the characteristics of the process are independent of the cell system used for assay.

Dulbecco's view may be summarized simply by saying that when animal viruses are handled like bacteriophages the virus-antibody reaction has the same characteristics. Most readers of their paper will, however, feel that too little attention has been given to providing an acceptable explanation of the residual infectivity which, like all other experimenters, Dulbecco *et al.* (1956) find in the presence of a high antibody concentration. With WEE the "persistent fraction" diminishes regularly with increasing serum concentration, while with poliovirus it remains approximately constant over a fairly narrow range of serum dilutions. To account for this, it is assumed (7) that each virus preparation contains a fixed fraction of unneutralizable virus particles. (The quality is not genetic and is assumed to be due to non-hereditary differences in antigenic constitution.) The further fact that addition of inactive virus to a neutralized mixture releases infective virus is rendered compatible with the claim that virus-antibody union is essentially irreversible by assuming (8) that transfer of antibody is possible only by thermal collision between inactive virus, and antibody-coated active virus particles.

Fazekas de St. Groth and associates (1957) have recently provided a serious criticism of Dulbecco's theoretical treatment, holding that the data presented in the paper by Dulbecco *et al.* (1956) is better interpreted on the assumption of fully reversible reactions. (I am indebted to Dr. Fazekas de St. Groth (1958) for the opportunity to see a prepublication draft of this paper, from which the following summary is derived.)

(a) It is assumed that dissociable complexes are formed by union between

antibody molecules and antigenic sites on virus particles, effective collisions requiring to be sterically correct. If  $A$  and  $V$  are the concentrations of antibody and virus at the beginning of reaction,  $s$  the number of reactive sites for virus particle and  $y$  the number of antigen-antibody complexes, the equilibrium condition will be:

$$\frac{dy}{dt} = \frac{k_a}{s} (sV - y)(A - y) - k_2y = 0.$$

or 
$$\frac{(\text{combined antibody})}{(\text{free sites})(\text{free antibody})} = \text{constant}$$

(b) The nature of neutralization of infectivity is obscure and a number of possible models can be selected for mathematical treatment. Matters needing consideration are the proportion of nonviable particles and the various possibilities of there being critical and noncritical sites on the virus particle or sites which differ in the *probability* that union with an antibody molecule will destroy infectivity.

(c) If an equilibrium system is involved, it becomes very difficult to allow quantitatively for the dissociation unavoidably associated with various phases of the Dulbecco technique. In particular, washing the plates to which experimental mixtures have been added introduces what might be called an asymmetric dilution of an equilibrium system.

(d) In a comparison between the implications of Dulbecco's formulation (D) and an appropriately elaborated formulation (F) of the reversible interaction hypothesis, Fazekas de St. Groth and Reid (1957) find that both will account for the kinetic and multiplicity curves given by Dulbecco *et al.* The changes associated with dilution are in accord with F, not with D. The persistent fraction is not independent of the type of antibody used, as would be expected on D. The use of Smoluchowski's equation in accounting for reactivation of neutralized virus by collision is inappropriate to a system requiring orientated collision. If the necessary modifications are made, the discrepancy with the observed result involves a factor of about  $10^6$ . On F, the results follow naturally, since the system by hypothesis is a reversible one.

It may be that a majority of experimental virologists will be skeptical of all attempts to provide too precise a quantitative formulation of the situation. It is interesting to note how, in Dulbecco's experiments, there are systematic differences in the behavior of poliovirus as compared to WEE virus, and of immune horse serum as compared with immune rabbit serum. In Burnet *et al.*'s (1937) work, every new species of virus examined show some individual deviation from what was expected to be "normal" behavior. Anyone with experience in this field also knows that there are virus strains that "behave"

better than others, and that when a "good" antiserum is obtained, it is advisable to complete a whole experimental series with this reagent. All three reagents, virus, antibody, and host cells, are intrinsically variable; the most that can be hoped for is that a system can be found in which they can all be controlled sufficiently to provide a model system on which the implications of any theoretical hypothesis can be tested.

The present tendency to use tissue culture methods for all virological procedures for which suitable cell types are available makes it desirable that Dulbecco's approach should be followed up for other virus-antibody systems. Probably the most important aspect to be clarified is in regard to the various technical manipulations involving dilution of one or both of the reagents in the original reaction mixture. This is the point at which Fazekas de St. Groth's and Dulbecco's interpretations seem to be most at variance. If the reduction in plaque count is to become the standard method for the accurate estimate of viral antibody, it is most important that technique should be standardized to give the most generally meaningful result. This is perhaps the main justification for a sophisticated mathematical analysis of the process.

#### 4. *Inhibition of Hemagglutination by Immune Serum*

Where, as with viruses of the influenza (myxovirus) group, hemagglutination is a function of the virus particles themselves, another interesting model system is available. This has not yet been studied as elaborately as it deserves.

Hirst (1942) showed that neutralization by immune serum could be conveniently measured by the extent of inhibition of hemagglutination and showed that the end point corresponded to a constant serum-virus proportion. In Melbourne, much incidental work on the nature of the reaction has been carried out over the last fifteen years, but no general account has been published.

The following findings, which are of general interest to the problem of the nature of virus-antibody interaction, may be noted:

(1) Provided time is allowed for very dilute reagents to reach equilibrium, the law of constant proportions holds to a close approximation, i.e., if in the conventional fashion the serum concentration is plotted against reduction in virus titer, a straight line at 45 degrees is obtained (Burnet and Boake, 1945).

(2) The apparent titer of an immune serum depends on the nature of the fowl cells used as indicator of hemagglutination. Anderson *et al.* (1946) found that cells from individual fowls could be placed in a sequence which was consistent in three distinct properties. At one end were cells which gave the highest hemagglutinin titer with standard virus and relatively low values for specific antiserum titers, and which did not show any inhibition by normal

serum. At the other end, the susceptibility of the cells to virus hemagglutination was lower while higher antiserum titer was indicated and there was much inhibition by normal serum. To obtain specific results in antiserum titrations with recently isolated viruses, only cells of the first type were suitable. The analogy of this model to the results obtained with neutralization tests *in vivo* when hosts of different susceptibility are used is obvious.

Isaacs and Stone (1949) found that when mixtures of serologically identical O and D virus were used to titrate homologous antiserum, the results differed greatly according to whether fowl (agglutinated only by D) or guinea pig cells (agglutinated by both O and D phases) were used. The result obtained corresponded to the amount of virus *capable of agglutinating the test red cells used*. With fowl cells, the presence of O virus had no influence on the titer obtained. In a basically similar experiment, Burnet (1955) found that virus inactivated at the lowest temperature necessary to destroy hemagglutinin had no influence on the apparent titer of a homologous serum when it was added to active virus.

(3) An interesting indication of the reversibility of both virus-cell and virus-antibody unions can be obtained by setting up a uniform series of serum dilutions  $S$  with standard amounts of virus  $V$  and red cells  $C$  in three different ways, by putting two components together for an hour and then adding the third and reshaking all tubes, i.e., (a)  $S + V - C$ , (b)  $S + C - V$ , (c)  $V + C - S$ . At the end of the first hour (c) will, of course, show uniform agglutination. On resettling, all three will give similar end points, which will come closer still if the tubes are again reshaken and allowed to settle.

The results of hemagglutination inhibition experiments indicate that in the reaction mixtures where the end-point reaction of partial agglutination is shown, there is always present considerable amounts of free antibody and that, in the early stages at least, virus-antibody union is highly reversible. There is a good deal to suggest that there is a slowly progressive development of irreversible union in all virus-antibody reactions. Many workers have shown that antihemagglutinin can be absorbed out of an immune serum by the addition of an excess of virus, followed by centrifugation at a speed sufficient to sediment the virus particles (Hirst, 1952).

##### 5. Neutralization of Influenza Virus in the Allantoic Cavity

This reaction has qualities which give it some general interest. The characteristic finding (Burnet, 1943; Walker and Horsfall, 1950) is that the effectiveness of antiserum falls off sharply with dilution. When plotted logarithmically, the neutralization curve is an approximate straight line with a slope between 3 and 5, i.e., if a certain concentration of serum  $S$  reduces the titer of virus to  $10^{-6}$  of the original,  $S/2$  may reduce it to  $10^{-4.8}$  and  $S/10$  to  $10^{-2}$ .

Two theoretical interpretations have been published to account for the empirical relationship

$$a \log S = \log V_0 - \log V_1 + b$$

where  $a$  is the constant determining the slope of the curve and  $b$  is equivalent to the reduction that would be shown by undiluted serum.

Tyrrell (1953) assumes that in the allantoic cavity a higher-order reaction is needed to convert a just unneutralized particle to an inactive one and that  $a$  is a measure of the number of molecules of antibody needed. Dulbecco *et al.* (1956), on the other hand, believe that the process cannot be defined by the initial concentration of the reagents. They believe that in the embryo where virus is just neutralized, primary interaction of virus and antibody allows infection of a proportion of cells. These liberate virus of which a high proportion is neutralized by residual antibody, but a small proportion can initiate a second cycle of cell infection. Whether or not infection results, as measured conventionally by the appearance of hemagglutinin at 3-4 days, will depend on whether successive cycles give progressively smaller yields or whether at some point a virus breakthrough occurs. Dulbecco interprets  $a$  in the formula as a measure of the average number of generations before "cut-off" in virus multiplication occurs.

Another aspect of virus neutralization exemplified by influenza viruses may be of great theoretical importance, although its implications have not been widely discussed. Partial absorption of an immune serum by virus reduces antibody as measured by some tests much more than by others. This was shown first by Burnet *et al.* (1937) who compared titrations on the chorioallantois and in the mouse lung. Using a strain of Melbourne pathogenic, both for mouse lung and for chorioallantois, it was shown that the same mixtures with absorbed antiserum would indicate a 99% removal of antibody by the chorioallantoic route, but a retention of one-third when tests were made in mice. Walker and Horsfall (1950) rather similarly showed that an absorbed antiserum could lose most of its activity as tested by mouse lung neutralization but retain it in antihemagglutinin tests.

## 6. Conclusion

The over-all impression that one receives from both the quantitative and qualitative studies of animal virus neutralization is the heterogeneity of both antibody and virus populations. Influenza studies may not be wholly relevant to those with smaller viruses but it is reasonable to expect that when equally extensive methods are available the same complications will emerge with forms like poliovirus and WEE virus. If this is so, the only useful generalizations are likely to continue to be expressed in qualitative terms.

At the present time the following statement does not seem to be invalidated by any of the recorded work:

(1) Virus neutralization by immune serum results from union of antibody molecules with the virus surface.

(2) These unions are of varying degrees of firmness depending on variations (a) in the population of antibody molecules, (b) in the disposition and accessibility of binding sites on the viral surface, and (c) on steric factors operating at the time of effective collision.

(3) Different strains of virus show various degrees of immunological relationship, i.e., a proportion of a population of antibody molecules in anti-A serum will demonstrably interact with virus of strain A<sup>1</sup> and vice versa. In general, absorption with A<sup>1</sup> will remove antibody reacting with this strain, leaving antibody against the homologous strain little altered. The nature of these "pattern" differences in antigen and antibody molecules is unknown and introduces further opportunity for variability when reactions with antisera not strictly homologous with the virus are studied.

(4) With high concentrations of reagents, firm union, presumably with denaturation of antibody globulin, takes place and, particularly when aggregation occurs or is produced by centrifugation, antibody absorption is demonstrable.

(5) Destruction of infectivity by adsorbed antibody will vary according to the susceptibility of the indicator host. It is probably not often dependent on prevention of attachment to the susceptible cell surface, but too little is known of the processes by which cell infection is initiated to allow any specific hypothesis.

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## Chapter XI

### The Reproduction of Viruses: A Comparative Survey

S. E. LURIA

*Department of Bacteriology, University of Illinois, Urbana, Illinois*

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#### I. VIRUS INFECTION AS INFECTIVE HEREDITY

##### *A. Virus Multiplication, Cell Multiplication, and Cell Growth*

The problem of virus growth has features that differ from those of growth problems in cells and in multicellular organisms. Multicellular organisms grow by fission of cells and multiply by releasing some more or less specialized cells, which give rise to new individuals. Multiplication of cells is itself, in turn, the culmination of intracellular processes, during which specific subcellular structures and molecular species increase in number. The replication of these subcellular elements represents cell growth, ultimately leading

to cell multiplication. The thesis of this chapter will be that virus multiplication as a biological process belongs on the level of the replication of subcellular elements, that is, on the level of cell growth rather than of cell multiplication.

In the same way as the morphogenesis of multicellular organisms must be interpreted in terms of the creation of organized patterns of specifically differentiated cells, so must the growth of cells be interpreted in terms of the formation and maintenance of organized patterns of specific molecules and macromolecular complexes. The morphogenesis of these patterns and the synthesis of their constituent parts are the subject matter of cytochemistry. The study of virus multiplication is a branch of cytochemistry; a remarkable branch, in fact, since it presents unique opportunities for the study of some cellular constituents in isolation in a fully native, functional, undegraded form, and of their transition from the isolated, inert state to the integrated, functional state as parts of the living protoplasm.

That virus multiplication is not a process homologous to cell multiplication is suggested immediately by the structure and composition of virus particles. All cells capable of multiplication, no matter how different their origin, size, and structure, contain certain essential chemical constituents—including proteins and nucleic acids both of the ribose (RNA) and deoxyribose (DNA) types—and certain essential organelles—nucleus, mitochondria, microsomes, cell membranes. Instead, as discussed in Chapters 3 and 6, most virus particles lack one or more of the basic chemical constituents of cells. Their composition and organization are much simpler than those of any cell.

There is great variation in these respects among different groups of viruses. Viruses are grouped together taxonomically on methodological rather than biological criteria. There is no reason to assume that they represent a naturally related group. The ability to invade living cells from outside and to multiply within them, which is a major criterion used to define viruses, may well be common to a variety of unrelated elements. The size of virus particles, another criterion used to group viruses together, ranges over a factor of 10,000 in mass and is no indication of natural relationship.

Whatever basic similarities exist among all viruses or among groups of them can be revealed only by the methods of cytochemistry, that is, by the study of the structure and composition of the virus particles and of the chemical and physiological events that their presence and multiplication produce in the cells. The relevant facts are discussed in detail in other chapters of this book. Here we are concerned only with tracing basic similarities and outstanding differences among viruses as revealed in their processes of multiplication and with deciding whether any generalizations appear justified by our present knowledge of these processes.

Four main approaches provide information on virus multiplication: (1) the *kinetic* approach, which follows the increase in numbers of virus particles by measurements of infectivity or of other specific virus properties; (2) the *cytochemical* approach, which studies the structural changes in cell organization accompanying virus production and the localization of viral materials within infected cells; (3) the *biochemical* approach, which analyzes the biosynthesis of virus constituents, their origin, fate, and continuity, and the alterations in cellular functions correlated with virus multiplication; and (4) the *genetic* approach, which traces the continuity and variation of the specific determinants of virus properties, their organization within the virus, and the interactions between viral and cellular determinants of specificity.

All these approaches must be utilized, and their results correlated, in order to obtain a complete picture of virus multiplication. Only for some bacteriophages has such a program of research been carried out to any great extent; a number of other viruses are now being studied in similar ways.

### *B. Virus as Genetic Determinant*

The results of these studies have led to what we consider as two central generalizations: the concept of virus multiplication as an altered pattern of biosyntheses in an otherwise functional cell; and the concept of the virus as contributing to the cell a set of genetic elements, which initiate and determine the new biosynthetic pattern.

We shall first elaborate these concepts; then, outline the evidence available from various areas of virology to support and specify them; and finally, discuss briefly the meaning of these concepts for the interpretation of cytomorphogenetic and pathogenic effects of viruses and of the relationship between viruses and cellular constituents.

### *C. Virus Replication and Virus Maturation*

Virus action within the host cell consists essentially in the production of abnormal or unusual cellular products as a result of exact specifications contributed by the virus itself. The unusual cell products may include virus particles, virus-related materials, and also cell constituents that have no obvious similarity to the component parts of the virus particles as observed in the free state. Virus infection can properly be considered as a form of *infective heredity*, in the sense that the essential contribution of the infecting virus is to introduce into the infected host cell a functional material, which may be only a small portion of the infectious particle, and which contains the exact specifications for the unusual syntheses that will ensue. That is, the

viral material is not simply an activator of latent potentialities of the recipient cell, but a detailed blueprint, which in the cell takes its place within the hierarchy of cellular determinants of specificity, and whose genetic functions may sometimes be compatible, sometimes incompatible with normal cell functions.

According to this view, the infectious virus particles produced by a virus-infected or virus-carrying cell are simply one product of the pattern of synthesis determined in the virus-containing cell by the genetic apparatus, which includes both viral and host determinants, and which functions as an integrated whole. The significant acts of viral multiplication involve the replication of the genetic blueprints introduced into the cell by the virus. This replication may be integrated to a greater or lesser extent with the replication of the whole genetic apparatus of the cell. Instances range all the way from almost complete integration and synchronization, as with the prophage of temperate phages in lysogenic bacteria (Lwoff, 1953), to complete incompatibility, as with the most intemperate, destructive phages and animal viruses.

The mature, infectious particles appear to be the ultimate product of virus multiplication. Some of the replicating viral elements, together with non-genetic but specific materials produced under viral control in virus-infected cells, become incorporated into mature, nonmultiplying forms—the virus particles. These are recognized by their characteristic infectivity and organization. This assembly of virus particles removes some of the viral elements from the multiplying process and makes them suitable for introduction into new cells. It is analogous to spermatogenesis, which by a complex cytomorphogenetic process transforms a haploid cell into a form suitable for introduction of its nucleus into the egg cell. We consider this “dual hypothesis,” which distinguishes two complementary and mutually exclusive processes, *replication* and *maturation*, as central to our understanding of virus biology.

Virus maturation will be a selectively advantageous process if it makes it easier for the virus to invade other hosts from without. The replicating form of a virus often appears to be noninfectious. By this we mean that, when extracted in this form, it is ineffective in initiating infection under conditions where the mature virus particle can do so. Yet, the lack of infectivity of the replicating virus may be only apparent. Under conditions that ensure protection from destructive agents and facilitate introduction into susceptible cells, we may succeed in observing initiation of infection by more or less incomplete virus particles, by their genetic components alone, or by multiplying viral elements extracted directly from cells prior to maturation. Instances of this sort will be discussed in the following sections. We shall return later to the relation between maturation and infectivity and to its significance for the general problem of infective heredity.

## II. MULTIPLICATION OF BACTERIOPHAGE

*A. The Nature of the Replicating Phage Material*

A tadpole-shaped phage particle attaches itself by the tip of its tail to the bacterial cell wall (Anderson, 1951). After a complex series of mutual interactions between phage and cell envelopes (Kellenberger and Arber, 1955; Kozloff *et al.*, 1957), the phage particle injects into the cell its DNA, together with some minor constituents (Hershey and Chase, 1952; Hershey, 1955, 1957). The protein shell is left at the surface and plays no further role in virus multiplication. This separation of the viral DNA from the protein shell, which is needed for attachment to cells, explains the "eclipse" of infectivity observed when extracts of newly infected bacteria are tested for ability to infect other cells (Doermann, 1952).

Following penetration of phage DNA, the infected cell may follow one of two paths,<sup>1</sup> depending on the genetic properties of the phage and on the environmental conditions: either the path of virus replication in "vegetative" form (Doermann, 1953), followed by virus maturation, cell lysis, and virus liberation; or the path of lysogeny (Lwoff, 1953), in which the cell multiplies, the virus persists in a noninfectious form and, as "prophage," becomes closely and persistently associated with the genetic apparatus of the bacterial cell (Jacob and Wollman, 1957). In the progeny of the lysogenic cells the prophage manifests itself occasionally by shifting to the vegetative form, which multiplies and produces mature virus and cellular lysis.

There is direct biochemical evidence that the phage material, both in its vegetative and in its prophage form, consists of DNA. The evidence concerning the vegetative form of phage derives mostly from work on coliphage T2. Isotope experiments have shown that in the cells that are going to produce phage there accumulates a pool of specific phage-precursor DNA (Hershey, 1956a,b), which is identifiable as phage DNA by its content of the unique pyrimidine (hydroxymethyl) cytosine, instead of cytosine (Wyatt and Cohen, 1952). In the pool, the phage-precursor DNA is not associated with any phage-precursor protein related to the proteins of the phage coat (Hershey and Melechen, 1957). Upon maturation, the phage-precursor DNA is removed at random from the pool and then becomes associated with phage-specific proteins. Synthesis of some protein (Cohen and Fowler, 1947; Burton, 1955; Tomizawa and Sunakawa, 1956) and, possibly, also of some specific RNA (Volkin and Astrachan, 1957) is required for the synthesis of phage DNA. These specific RNA and protein may be necessary intermediates in the replication of DNA. There may actually be a transfer of information from DNA to

<sup>1</sup> Other alternatives, such as abortive infection, or persistence of a nonmultiplying phage element in the cell, will not be considered here, insofar as they do not lead to multiplication.

non-DNA molecules, which will then carry, specified in their own chemical language, the whole specificity of the phage heredity (Delbrück and Stent, 1957). If so, some such non-DNA intermediate may be able to take over the control of DNA synthesis when the DNA itself is incapacitated, for example, by radioactive decay of  $P^{32}$  atoms in its nucleotides (Stent, 1955).

The evidence concerning the DNA nature of prophage comes from isotope experiments using coliphage  $\lambda$ . For this and other phages it has been possible to determine by bacterial crosses and by transduction (Lederberg and Lederberg, 1953; Jacob and Wollman, 1957) the presence and location of the corresponding prophages within the linear sequence of genetic determinants of the bacterial cell chromosome. The  $\lambda$  prophage can be inactivated in the lysogenic cell by the radioactive disintegration of  $P^{32}$  atoms incorporated into the cell. The rate of this inactivation is the same as the rate of inactivation of the infectivity of similarly labeled mature phage  $\lambda$  (Stent *et al.*, 1957). This provides a remarkable proof of the similarity of the content of essential DNA in the mature phage and in the  $\lambda$  prophage.

### *B. Infectious DNA from Phage Particles*

These studies make it possible to identify the genetic material of the phage in its various states—mature, vegetative, and prophage—with a specific piece of DNA, which, at least in the form introduced into the cell by the mature particle, is probably not associated with genetically significant protein. Direct evidence has been obtained with phage T2 about the existence and size of this “master piece” of DNA and about its behavior and conservation in the process of replication (Levinthal and Thomas, 1957a,b; Hershey and Burgi, 1956).

Assuming that the nongenetic components of the mature virus particle are a protective and injecting device for the essential phage DNA, it can reasonably be expected that the DNA portion, extracted either from mature particles or from infected bacteria, may be able to initiate infection, if a system is available that permits penetration of the DNA into susceptible cells. At least for DNA from mature phage, the expectation seems to have been realized by the use of “protoplasts”, that is, of cells deprived of part of their cell wall (Spizizen, 1957; Fraser and Mahler, 1957). According to these reports (which may not have excluded all possible pitfalls) the naked protoplast can be infected by disrupted phage, albeit with very low efficiency. We may recall in this connection that transformation phenomena with bacteria have established that fragments of bacterial DNA may be transmitted even to intact cells (Avery *et al.*, 1944; Hotchkiss, 1956). We may also mention here the phenomenon of zygotic induction (Jacob and Wollman, 1956), in which vegetative phage multiplication is initiated by the

penetration of some prophages into a susceptible protoplasm upon mating of a lysogenic bacterium with a nonlysogenic partner. It seems safe to assume that infection of a sensitive cell can be initiated by entry of the phage DNA in any one of its possible states. It is almost superfluous to point out that the possibility of infection of bacterial protoplasts with phage DNA promises new insight into the relation between structure and function of viral nucleic acid. Some protein component of the phage appears to play an essential role in the infection of protoplasts (Spizizen, 1957).

### *C. Kinetics of Replication of Vegetative Phage*

If phage specificity throughout its reproductive cycles is embodied in DNA elements, the question arises of the kinetics of DNA replication in the course of vegetative multiplication of virus. By what mechanism does multiplication take place? Does it consist of repeated copyings of a single template, used over and over? Or does it involve a series of reduplications, in which the newly produced individuals serve in turn as sources for replication? In other words, is multiplication linear or geometric? The second alternative is verified by genetic observations on spontaneous phage mutations (Luria, 1951). These mutations occur only during multiplication; the resulting mutant phage particles are found among normal particles in the phage yield from single bacteria. The clonal distribution of the mutants in individual cells fits a distribution predicted by the hypothesis of geometric multiplication and incompatible with the hypothesis of a linear kinetics.

Current ideas on the structure and replication of DNA are compatible with its role as a geometrically replicated genetic material (Watson and Crick, 1953). A DNA molecule consists of two complementary polynucleotide chains. Its replication must involve the formation of two new complementary chains. The four chains will then yield two indistinguishable DNA molecules, presumably equal to each other in reproductive capacity.

Phage replication must also allow an exact homologous pairing between viral elements in order to account for the observed phenomena of genetic recombination. Pairing and recombination can also be accounted for in terms of mating during DNA replication (Delbrück and Stent, 1957; Levinthal and Thomas, 1957a), although more complex schemes invoking mating between non-DNA intermediates may ultimately prove preferable (Stent, 1958). The possibility of interactions similar to recombination between genetic elements of the phage and of the host is also suggested by a number of genetic observations, as discussed in Vol. II, chaps. VII and VIII of this work.

### *D. Functions of the Phage Genome*

Viewed as functional DNA, the vegetative form and the prophage form of a bacterial virus are basically similar to fragments or portions of cellular

genetic material. Two consequences follow: First, like all genes, the phage DNA may be expected to control other cellular functions besides its own replication; second, the formation of mature phage may be considered as an expression of the specific genetic function of the phage DNA. Both predictions are supported by available evidence.

### 1. *Conversions of Cellular Properties by Phage*

A number of cell properties that are not obviously related to virus production are controlled by phage genes. Most remarkable among these is the control of the composition of the cell wall, which manifests itself by specific changes in cellular antigens upon phage infection. This phenomenon has been studied mostly in the genus *Salmonella* (Iseki and Sakai, 1953; Uetake *et al.*, 1955). In what is probably a typical instance, infection with a certain phage results in the appearance, within a few minutes, of somatic antigen 15 and in the equally prompt suppression of the production of antigen 10. This change occurs both in cells in which the phage multiplies vegetatively leading to cell lysis and in cells that survive infection and in which the phage becomes prophage (Uetake *et al.*, 1958). It occurs even in infection with a virulent phage mutant that lyses every infected cell. The reverse change, from antigen 15 to antigen 10, follows the loss or removal of the phage from the carrier cells.

Clearly, the relation between the phage DNA and the specific constituents of the somatic antigens is no more and no less obvious than the relation between the DNA of a transforming principle and the capsular polysaccharides in *Pneumococcus* (Avery *et al.*, 1944), or, for that matter, than the relation between any gene and the ultimate product of its activity in any cell.

There is a whole series of these "conversions" of cell properties by phages, ranging from the production of diphtheria toxin (Freeman, 1951) to the ability to support multiplication of other phages (S. Lederberg, 1957). It was believed at first that such new properties required the presence of an established prophage; hence the name of "lysogenic conversions" (J. Lederberg, 1955). We realize now, however, that these conversions of cell properties are expressions of heterocatalytic activities that may be exerted by all functional states of phage within a cell.

### 2. *Biosyntheses Related to Phage Replication*

It seems reasonable to attribute to the heterocatalytic functions of phage also the appearance in phage-infected cells of new enzyme activities related to the needs for synthesis of phage DNA. The most remarkable instance is the appearance of an enzyme that catalyzes the hydroxymethylation of deoxyctyidylic acid (Flaks and Cohen, 1957) in bacteria infected with the

coliphages of the T2 group, which contain the hydroxymethylated nucleotide (Wyatt and Cohen, 1952). The enzyme is clearly required for synthesis of phage DNA itself. Although the enzyme may conceivably be present in inactive form in the bacteria and be activated by phage infection, as in the case of a bacterial deoxyribonuclease (Pardee and Williams, 1952; Kozloff, 1953), it seems more probable that the enzyme is synthesized anew under the genetic control of the incoming phage DNA. Similar mechanisms may underlie the restoration or expansion of thymine synthesis in an almost thymineless bacterial strain following infection with phage (Barner and Cohen, 1955).

### 3. *Syntheses Related to Phage Maturation*

If we accept the concept of phage DNA acting as genetic material in integration with the cell genome and controlling heterocatalytically a number of biosynthetic processes, it becomes natural to consider also the proteins of the mature phage particles as special products of the functional activity of the phage genome.

The proteins of a phage particle comprise a variety of antigenically distinct fractions (Lanni and Lanni, 1953), some of which are probably active enzymatically (Brown and Kozloff, 1957). Some are located in the head of the phage, others, in the tail. The tail proteins include the organ of phage attachment to the cell. When a bacterium is infected with two related phages, whose tail proteins differ in antigenic specificity or in requirements for adsorption cofactors (Anderson, 1945), the progeny particles exhibit "phenotypic mixing" (Novick and Szilard, 1951). That is, the specificity of the tail proteins may correspond, not to the genetic characteristics of the phage particle that carries them, but to the characteristics of the other phage type that was growing in the same cell, or to a mixture of the two. The association between genetic and phenotypic properties is almost random (Streisinger, 1956; Brenner, 1957). This indicates that the two kinds of tail protein are synthesized "at large" in the infected cells and are then utilized, as available, in assembling the coats of the maturing phage, in the same way as gene products controlled by different allelic genes may be utilized side by side in a heterozygous cell, or as gene products controlled by genetically different nuclei in a heterocaryotic cell or mycelium.

Other specific proteins, besides those destined to become part of the mature particles, are produced in the process of phage maturation. These include a number of agents that act enzymatically to dissolve the surface layers of bacteria, some digesting capsular polysaccharides (Adams and Park, 1957), others attacking the bacterial cell wall (Huppert and Panijel, 1957; Murphy, 1958; Jacob and Fuerst, 1958). Such enzymes play a role in bacterial lysis, in the release of the newly formed phage, and in

the removal of external cell constituents that might interfere with attack on new host cells.

### *E. Phage Maturation and Infective Heredity*

The interpretation of phage maturation as the terminal assembly of a specific core of viral DNA with specific proteins synthesized by the virus-infected cell under viral control leads to several predictions as to possible events that may take place at maturation or affect the occurrence of maturation.

#### 1. *Transduction*

In the course of vegetative replication and maturation, a phage particle may occasionally come to include in its protein shell some fragment of the bacterial genome. This gives rise to "transduction," as observed in *Salmonella* (Zinder, 1953), in the coli-dysentery group (Lennox, 1955), and probably also in the genus *Bacillus* (Brown *et al.*, 1955). In this type of transduction, the phage can transfer from one cell to another any group of closely linked host genes. If the cell survives infection, it may show one or more of the transduced characters.

In at least one other instance, with phage  $\lambda$ , the only host genes that can be transferred are some that were chromosomal neighbors of the prophage in a lysogenic cell, including a group of factors controlling utilization of galactose (Morse *et al.*, 1956). Here the transducing particles appear to have incorporated the fragment of host genome in the place of a portion of the phage genome itself (Arber *et al.*, 1957). This transducing phage thereby becomes incomplete and ineffective in initiating its own reproduction, although it can still produce cell lysis. This "defective" phage has become a specific transducer of the galactose determinants, which behave here as infective genetic factors. There is now evidence (Luria *et al.*, 1958) that other instances of transduction may also reflect associations of bacterial genes with defective phage.

#### 2. *Defective Prophages*

If maturation is the culmination of a process of specific phage-controlled biosynthesis, we may expect that both environmental agents and genetic changes will affect the very occurrence of maturation. An example of an environmental effect is the specific prevention of successful phage assembly and maturation by inhibitors such as the acridine dye, proflavine (DeMars, 1956). The defective prophages, on the other hand, provide examples of genetic effects on maturation (Appleyard, 1954; Jacob and Wollman, 1956a).

Here, lysogenic bacteria lose by mutation the ability to produce normal mature phage, without losing some of the prophage-controlled properties, such as immunity to lysis by superinfection or production of phage-controlled antigens. The mutations to defectiveness occur in the prophage itself, and the nondefective prophage form may be restored by back mutation. With some phages the defect leads to incomplete maturation. Lysis will then result either in production of no recognizable phage elements, or of fragments of phage coats, or of some particles that carry the genetic defect (Appleyard, 1956).

The notable fact is that the defective prophages, being genetically competent in other respects, but incompetent to determine production of infectious virus, have lost their "viral" aspect. They have become operationally indistinguishable from any other fragments of genetic material of the cell. Yet, we know the exogenous origin and the potential transmissibility of these genetic determinants, revealed in some cases by their back mutations to non-defectiveness. Since we have independent evidence, from transduction, that most or all elements of the bacterial genome are transferable from cell to cell if a suitable viral vehicle is available, we are led to ask how many of these genetic elements either possess or can acquire by mutation the potentiality to determine their own specific incorporation into a viral vehicle formed under their own control. That is, we ask whether all portions of a cell genome might become viruses and whether in so doing they would manifest an ever present potentiality, or acquire a novel cytomorphogenetic function, or recover a function that had been lost by mutation.

There are in bacterial genetics a number of situations that can be interpreted in terms of special genetic elements or "episomes" (Jacob and Wollman, 1958), with the ability to behave at times as chromosomal elements, at other times as units multiplying vegetatively in the bacterial cell. Prophages may be considered as a category of such episomes capable of assuming an effectively transferable form. Other episomes might conceivably acquire this capability by mutation.

As we interpret phage infection as genetic parasitism, we identify phages more and more closely with wandering portions of the cell genome. More generally, we must ask what role infective heredity has played and may still be playing in the evolution of genetic systems (J. Lederberg, 1952; Luria, 1953).

### III. MULTIPLICATION OF TOBACCO MOSAIC VIRUS

#### *A. RNA as the Initiator of Infection*

Little is known about the multiplication of tobacco mosaic virus (TMV) at the cellular level, but several lines of evidence are relevant to our discussion.

As far as the initiating material in infection is concerned, there is clear evidence that this is the RNA portion, which in the mature virus particles is contained within a spirally assembled shell of protein units (R. E. Franklin *et al.*, 1957). The purified RNA fraction extracted from the virus particles can initiate infection by itself, although less efficiently (per unit weight of RNA) than the complete nucleoprotein particles (Gierer and Schramm, 1956; Fraenkel-Conrat, 1956).

The early development of the infection, as revealed by changes in the radiation sensitivity of the virus-producing capacity of the infected cells, shows significant differences between infection with complete virus and infection with the RNA fraction alone (Siegel *et al.*, 1957). In infection with complete virus, there is an early phase during which the sensitivity to ultraviolet light is very high. In infection with the viral RNA alone, this early phase is missing; the whole situation evolves as though the process started directly at a later stage. These observations suggest that in infection with complete virus particles a first necessary step is the release of RNA from its protein shell, so that it can act as the *primum movens* in the process of virus multiplication.

It seems probable that with TMV infection, as with phage infection, the multiplying form of the virus consists of nucleic acid not associated with the protein found in the mature product. Here again, the viral protein may be a specific product of the virus-infected cell, utilized for coating the essential nucleic acid and providing it with a protective apparatus that enhances its chances of successful transmission to other plants. It is conceivable that transmission of virus from cell to cell within an infected plant may occur by the transfer of RNA elements, rather than of complete nucleoprotein particles.

### *B. TMV Protein and Virus Maturation*

What is known of the properties and biosynthesis of TMV protein fits the hypothesis that we have outlined. In infected cells, TMV protein is found, not only in the virus particles, but also as a noninfectious material, presumably not associated with viral RNA or at least readily separated from it by extraction (Jeener, 1956). Isotopic experiments show that at least some of this noninfectious viral protein behaves as a true precursor of the virus particles, into which it becomes incorporated (Van Rysselberge and Jeener, 1957).

The viral protein is made up of small subunits, about 17,000 in molecular weight. These appear to be uniform in structure and composition, at least within the limits of present analytical methods (Knight, 1957). The protein extracted from infected cells or from virus particles has a remarkable tendency to aggregate, under suitable conditions, either alone or around a core

of nucleic acid, to give the typical helical arrangement of the protein in the virus particle (Schramm, 1947).

Complete virus particles can be reconstituted by recombining RNA and protein separately extracted from virus. The reconstituted particles have some infectivity (Fraenkel-Conrat and Williams, 1955). If RNA from one virus strain and protein from a different strain are combined, the progeny to which they give rise has the genetic characteristics contributed by the RNA.

Thus, the TMV protein appears to be a specific material, without intrinsic genetic function, produced under the genetic control of the viral RNA, and utilized in the morphogenetic process of virus maturation.

Here again, this time with a typical RNA virus, the nucleic acid must be considered as the primary genetic material of the virus, and the mature particle as one product of the genetic activity of the virus. The occurrence of other virus-related proteins, which are probably not precursor proteins, indicates that the mature virus is not the only specific product of virus-infected cells. Which other cell functions this virus may control is not known.

The amount of RNA in a TMV particle can probably carry more genetic information than is needed to determine the specificity of the viral protein. The additional information, if any, may control other functions of the virus in the cell. We may also find, in such an RNA virus, some "transduced" elements of host cell RNA.

### *C. Other RNA Viruses*

A few scattered observations on other RNA-containing viruses support the conclusions reached for TMV virus. With poliovirus, Mengo, and West Nile encephalitis viruses, successful transmission of infection has been reported by means of an RNA fraction extracted from infected cells (Colter *et al.*, 1957a,b). Conversely, with turnip yellow mosaic virus, there is found in infected cells a fraction of particles, similar to the infectious virus particles in size, structure, and protein composition, but without RNA and completely noninfectious (Markham and Smith, 1949). These particles are probably a product of faulty maturation, the essential RNA failing to be enclosed into the protein shell. In the complete virus particles, as well as in the noninfectious ones, the protein actually appears to constitute a shell composed of repeated subunits (Klug *et al.*, 1957). It seems a useful hypothesis to assume that with these viruses, and probably also with others like poliovirus, whose particles contain only RNA and protein, the synthesis of virus protein is always a terminal event, leading to the maturation of the virus and to the cessation of the reproduction of its essential genetic material.

## IV. MULTIPLICATION OF ANIMAL VIRUSES

*A. Myxovirus Group*

The myxovirus group includes the viruses of influenza, Newcastle disease, mumps, and fowl plague. The particles of these viruses contain at least three antigenically distinct fractions: an RNA-protein element, called the S (or G) antigen; a hemagglutinin (HA) element; and a lipid-containing fraction. Extraction with ethyl ether destroys the particles and permits separation of the S and HA fractions from other materials (Hoyle, 1952), some of which cross-react serologically with antigens of the host tissue (Knight, 1946).

The changes undergone by the virus particles upon initiation of infection are not yet definitely established (Hoyle, 1957; R. M. Franklin *et al.*, 1957). It is known, however, that the various components appear at different times and in different parts of the cell, the S antigen, first, in or around the nucleus, the HA in the cytoplasm (Liu, 1955; Breitenfeld and Schäfer, 1957). Infectious virus particles appear later than the S and HA elements. Complete particles are never seen within the cells, but only at the cell surface (Morgan *et al.*, 1956). All new infectious virus present at any one time in the infected cells is subject to inactivation by external agents, such as antibody (Rubin *et al.*, 1957), and can be released readily from the cell by treatment with a receptor-destroying enzyme. The unescapable conclusion seems to be that the various virus constituents are formed at different sites within the cell and that their assembly and maturation take place as terminal processes at the cell surface. The assembly process, however specific it may be, permits or even requires the incorporation into the virus particles of certain materials whose antigenic specificity is host-determined. Such a relatively unspecific process of assembly may at least partly be responsible for the genetic complexity of virus particles produced in cells that receive a mixed infection with two related viruses of this group (Burnet, 1955). It also provides opportunities for transduction like phenomena in these viruses.

The RNA-containing S element seems the natural candidate for the primary genetic function in these viruses. Isotopic studies on influenza and other myxoviruses suggest a breakdown of the infecting particles at the surface of the infected cell and an initiation of growth by multiplication of S antigen (Hoyle, 1957). This evidence is somewhat beset by technical difficulties, due to the relative instability of the influenza virus. Whether the RNA component can initiate infection by itself, and whether it becomes separated from viral protein as part of the initiation of infection, remain subjects for future study.

*B. Other Viruses*

The rather fragmentary observations on the multiplication of animal viruses of other groups, although they add little to the picture developed in

the preceding pages, are fully compatible with it. There is, in the first place, a general finding of an eclipse of infectivity following infection. This may be taken as an indication of a drastic change in the structure of the virus in passing to the multiplying state. There is also ample cytochemical and microscopic evidence for a series of stages, different for different groups of viruses, through which the virus materials must go before becoming organized into mature virus particles. Often, the first virus materials to appear in an infected cell are seen in electron micrographs as an undifferentiated matrix, within which the typical virus particles are then formed by a stagewise process of maturation (Gaylord and Melnick, 1953). The frequent intranuclear or perinuclear location of these foci of virus production suggests that some interaction with the host cell DNA may be required to initiate reproduction, even for viruses of the RNA group. It seems possible that production of an RNA-containing virus may require some genetic alteration involving a change in the cellular DNA.

With viruses of the psittacosis group, microscopic observations have suggested that multiplication entails a binary fission of viral elements, which differ morphologically from the mature virus particles (Sigel *et al.*, 1951). Such a finding, if correct, would by no means be incompatible with the hypothesis of a multiplying form of the virus distinct from the infectious mature particle. The elementary act of virus multiplication must always be a reduplication of the genetic elements of the virus. It is not surprising that the reduplication process of vegetative (and possibly noninfectious) virus elements may express itself in morphologically recognizable acts of binary fission. Repeated reduplications of virus elements must underlie the exponential kinetics of virus production observed in some viral infections of individual cells (Dulbecco and Vogt, 1953).

For some insect viruses, a complex reproductive process has been postulated on the basis of morphological studies (Bergold, 1953). Although the basic mechanisms are still doubtful, the existence of separate phases of replication and maturation seems very probable.

## V. VIRUS MULTIPLICATION, CELL FUNCTION, AND CELL ORGANIZATION

### *A. Restatement of the Dual Hypothesis*

The picture of virus multiplication outlined in the preceding sections has a number of unifying features. In all cases, multiplication appears to be initiated by a genetic portion of the virus particles, which contains nucleic acid and which is either noninfectious or, at least, less infectious than the mature virus by the available tests. The production of new virus entails both

the replication of the genetic material, a clearly autocatalytic process, and the maturation of new virus, in which the genetic elements stop replicating and become assembled into virus particles, by joining up with materials synthesized under the heterocatalytic control of the virus. Cell-specific materials, either genetically competent (as in transduction) or presumably with structural functions (as in influenza viruses), may also become included into virus particles. In its functional state within a cell, the genetic material of a virus can control, not only the synthesis and assembly of constituents of the mature virus particles, but also the production of essential enzymatic mechanisms and other biochemical processes, which manifest themselves as altered cell functions. Some of these functions of a virus may be instances of pleiotropic gene action, by which genetic functions essential for virus multiplication accidentally affect other cell functions. It is equally conceivable, however, that a virus, as a transmissible fragment of cell heredity, may contain the genetic determinants of functions unrelated to its own perpetuation as virus.

A number of questions may now be raised: How does virus multiplication lead to the cellular dysfunctions observed in many viral infections? Which cellular properties are determined by genetic elements that can act as viruses? And what relationship exists between these elements and the other genetic elements of the cell?

### *B. Cell Damage and Virus Multiplication*

Cellular dysfunction may result from any of the phases of interaction between viruses and cells. With certain "intemperate" phages, for example, the mere attachment of a virus particle, even unable to multiply, can cause irreparable damage and cell death. Other changes in cell properties, like the antigenic changes in phage-infected bacteria, are observed whenever viral multiplication occurs, either in the vegetative or the prophage state. Still other forms of damage, such as lysis of bacteria by phage or destruction of animal cells by certain viruses, are probably tied up with the process of virus maturation.

Replication of a virus in a noninfectious form, either as vegetative virus or as provirus, is often compatible with continued cell life and cell division, as in lysogenic bacteria. Virus maturation, which involves extensive changes in the pattern of cellular biosynthesis, is probably more directly related to cellular damage leading to cell death. Even in some proliferative virus diseases the mature, infectious virus particles might be produced only in a few cells that are prevented from further growth. At least for Rous sarcoma, however, there is now some evidence of production of mature virus by living, multiplying cells (Rubin and Temin, 1959).

In general, cellular damage appears to be an incidental manifestation of virus infection, even though it is a frequent correlate of virus maturation. Cellular dysfunctions of a variety of types, ranging from simple metabolic alterations in some lysogenic bacteria to unrestricted cell proliferation in virus-induced tumors, must be considered as the expression of the genetic functions of the virus acting in integration with the host cell genome.

These virus-controlled functions are not necessarily different from cellular functions that may arise, be modified, or be suppressed by genetic changes such as mutations. Once we visualize virus infection as a form of infective heredity, the problem of the possible determination of apparently normal functions by virus-like elements reduces itself to the problem of the potential ability of various genetic elements of the cell to behave as viruses: that is, to control their own maturation into readily transferable forms. At present, this problem can be defined precisely only for phages, which, by their reduction to chromosome-linked prophages, their interactions with neighbouring chromosomal elements, and their mutations to defectiveness, demonstrate the possible transitions between virus and chromosomal element. In bacteria, at least, transformation and transduction give proof of the intrinsic transferability of all the genetic material as functional DNA. With other cells, the occurrence of latent infections and the activation of unsuspected viruses upon transfer of tissue extracts into new hosts have repeatedly suggested the possibility of a transformation of cell components into viruses.

Specific cellular components released by growing cells may play a role in growth regulation phenomena (Weiss, 1955), as well as in tissue compatibility (Billingham *et al.*, 1956). It is conceivable that some of these regulatory substances may contain nucleic acids and may be able to reproduce in cells into which they gain access.

### *C. Viruses and Cellular Constituents*

Our discussion has led us to consider the natural relationship of viruses to constituents of normal cells. We are not stretching our imagination too far if we consider the phage DNA as a transmissible fragment of bacterial DNA. Some of the RNA viruses, on the other hand, may ultimately prove related or homologous to cell microsomes, which, as part of the cytoplasmic reticulum, are probably the carriers of the coded determinants for protein synthesis in the cell protoplasm (Simkin and Work, 1957).

A detailed discussion of virus origin would be outside the scope of this chapter. We may point out, however, that even if viruses are genetically related to certain cell constituents, it is unjustified to expect a detailed homology between the genetic structure and physiological functions of a virus and the structure and functions of constituents of the cells in which

we happen to observe it. A great deal of independent, divergent evolution may have taken place both in virus and in cells since a virus first arose from a cell constituent through the acquisition of an apparatus for successful transfer from cell to cell. Conceivably, most cells may have great latitude in the variety of genetic elements, exogenous or endogenous, whose reproduction they are potentially capable of supporting. Only those elements that have evolved both a mechanism for successful transfer and a set of functions observable in other cells may be recognizable as viruses. Transferability of a virus may be extended by mutation to cells that are phylogenetically very distant from one another and from the (hypothetical) cell whence the virus first came. Suffice it to recall the plant pathogenic viruses that can multiply in the cells of plants and of insects (Maramorosch, 1955).

To return to virus multiplication, the hypothesis of a genetic relationship between viruses and cellular organelles does not in itself contribute to our present understanding of virus multiplication. In fact, we know very little as yet about the mechanism of reproduction of these organelles. Rather, we may be certain that the study of virus multiplication will, directly and indirectly, be a major contributor to the elucidation of the mechanisms of replication of the basic units of life.

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