Essays in
Biochemistry

Hans T. Clarke

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SAMUEL GRAFF
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Preface
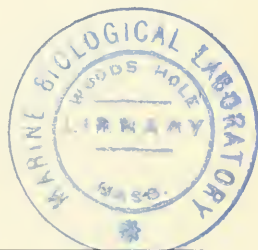
These essays were written in honor of Hans Thacher Clarke, on the occasion of his retirement as Professor and Chairman of the Department of Biochemistry, College of Physicians and Surgeons, Columbia University. Through their contributions to this volume, the authors, all former students or academic associates of Professor Clarke, sought to express their affection and esteem for him, and their gratitude for his generous aid and counsel. If merit be found in these essays, it is a consequence, in large measure, of the high standards of chemical scholarship to which the authors were exposed, early in scientific life, through their association with Professor Clarke. For this opportunity to learn from him not only some of the facts of science but something of its spirit as well they are deeply grateful.

The essayists were accorded free rein in regard to subject and style. The articles, for the most part, are neither reviews nor experimental reports. Some are critical discussions of the status of a biochemical problem *at the time of writing,* whereas others are frankly speculative or deliberately provocative. The absence of editorial directive permits the discerning reader to glimpse the personalities behind the printed page and to contrast diverse approaches toward scientific goals. Nature sometimes yields her secrets upon the skillful use of but a few tools; frequently, the techniques of many scientific disciplines are needed. But always there is the excitement of the search.

EARL A. EVANS, JR.
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Some Metabolic Products
of Basidiomycetes

MARJORIE ANCHEL

Investigation of the metabolic products of fungi has yielded results of considerable chemical and biological interest and practical importance. The chemistry of the actinomycetes, penicillia, and aspergilli has been studied intensively, but the chemistry of the higher fungi, including the basidiomycetes, has received much less attention. The compounds discussed in this paper were all obtained from culture liquids of basidiomycete fungi. Their isolation was followed by microbiological assay, specifically, activity against certain bacteria. Had a chemical assay been used, it would be expected that the compounds isolated would have in common some particular chemical characteristic, although this might be only a small, even an insignificant, part of their general chemical make-up. Since the assay was biological, a common denominator of biological activity must be accepted, namely, in this case, that the compound interferes with the metabolism of the test organisms. Since it is to be expected that the same gross biological effect may be arrived at by different mechanisms, it is not surprising that compounds which have in common the property of antibiotic activity are frequently entirely unrelated chemically. Further, such compounds sometimes represent types which are unusual from the point of view of synthetic organic chemistry as well as little known from a biological standpoint.

The antibiotic compounds isolated from the basidiomycetes investigated at the New York Botanical Garden offer one more illustration of these general conclusions, in that they represent diverse chemical types, some of which have no exact synthetic counterparts, and/or are biologically unfamiliar and, one might almost say, unexpected. On the other hand, certain rough groupings may be made into which fall the greater number of the compounds isolated. A surprisingly large

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group consists of polyacetylenic compounds, with which the major part of this paper will be concerned.

Since the chemistry of most of the compounds discussed has not been completely elucidated and since their biological significance is entirely unknown, much that will be said is purely speculative. It is hoped that this will justify itself in some measure by suggesting further paths for exploration in relation both to chemical and to biological questions. For purposes of discussion, the compounds may be divided into groups comprising quinones, sesquiterpenes, larger polycyclic molecules, halogenated aromatic compounds, and polyacetylenes.

Quinones

This group, of which there are many examples among products of fungal origin, as well as among those produced by higher plants, is represented in our compounds by 5-methoxy-p-toluquinone [*] [1] and possibly by fomecins A and B.[2]

Fomecins A and B are compounds isolated from culture liquids of *Fomes juniperinus*. Both have the molecular formula $C_8H_8O_5$. Fomecin A has one acidic grouping. Its ultraviolet absorption spectrum (in 95% ethanol) shows maxima at 241 mμ ($E = 10{,}760$) and 305 mμ ($E = 14{,}260$), maxima not typical of quinones.[3] When an aqueous suspension of fomecin A was treated with SO_2 the crystals dissolved, as would be expected on formation of a hydroquinone, but the orange-red color was not discharged nor could a hydroquinone be recovered. When the SO_2 was removed with nitrogen and the solution somewhat concentrated, crystals of fomecin A (identified by ultraviolet absorption spectrum and by analysis) were recovered. On the other hand, it reacted with 2,4-dinitrophenylhydrazine to yield a mono-2,4-dinitrophenylhydrazone, $C_{14}H_{12}N_4O_8$, and with amines to yield deeply colored solutions which deposited colored crystalline derivatives. The aniline derivative analyzed for $C_{14}H_{13}NO_4$ ($C_8H_8O_5 + C_6H_5NH_2 - H_2O$).

Fomecin B is isomeric with fomecin A ($C_8H_8O_5$). It has absorption maxima in the ultraviolet at 262 mμ ($E =$ ca. 20,000) and at 337 mμ ($E =$ ca. 7000). Its behavior toward 2,4-dinitrophenylhydrazine and amines is similar to that of fomecin A, but no crystalline derivatives have been isolated. If the fomecins are quinoidal, they apparently are not simple p-quinones.

The compound 5-methoxy-p-toluquinone (I) was isolated from cul-

[*] Unpublished work.

ture liquids of *Coprinus similis* and *Lentinus degener*. It fits into a series of oxygenated quinones, two closely related members of which, fumigatin (II) and spinulosin (III), were isolated from species of *Penicillium* and *Aspergillus*.[4] Another closely related quinone, 2,5-dimethoxyquinone (IV) has been isolated from the basidiomycete, *Polyporus fumosus*.[5]

$$\text{I} \qquad \text{II} \qquad \text{III} \qquad \text{IV}$$

(Structures I–IV: quinone ring diagrams)

I: quinone with CH₃, CH₃O substituents
CH_3O / CH_3 quinone — **I**

II: HO, CH₃, CH₃O quinone — **II**

III: HO, CH₃, CH₃O, OH quinone — **III**

IV: OCH₃, CH₃O quinone — **IV**
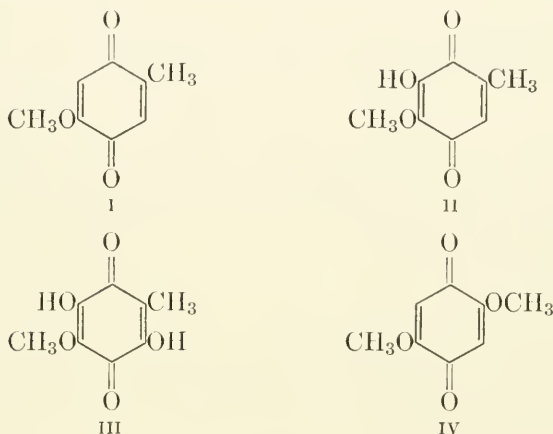
Sesquiterpenes

The compounds included here, solely on the basis of their molecular formulas, are illudin M and illudin S, isolated from culture liquids of *Clitocybe illudens*, and marasmic acid, from culture liquids of *Marasmius conigenus*. Illudin M, $C_{15}H_{20}O_3$, and illudin S, $C_{15}H_{20}O_4$ * (previously reported as $C_{15}H_{22}O_4$),[6] show striking resemblance in their chemical behavior to santonin, $C_{15}H_{18}O_3$, and pseudosantonin, $C_{15}H_{20}O_4$, which have been isolated from higher plants. All four compounds possess carbonyl groups. The santonins, and probably the illudins, possess a lactone ring. Pseudosantonin and illudin S yield acetates. Oxygen is lost on catalytic reduction of the illudins, but in the santonins straightforward saturation of the double bonds and reduction of the carbonyl group occur (also hydrogenolysis of the lactone ring of pseudosantonin).[7] The behavior of the two groups of compounds towards reduction is not understood because the mechanism of oxygen loss in the illudins is unknown. All four compounds undergo acidic rearrangements which yield more than one product, and, in each case, one of the products is phenolic. Marasmic acid,[8] $C_{15}H_{18}O_4$,* like

* Unpublished work.

the illudins, has a free carbonyl group, but unlike them it also has a free carboxyl group which can be esterified to yield a monoester.

Larger Polycyclic Molecules

This group includes ochracic acid and pleuromutilin. Ochracic acid was isolated from culture liquids of *Corticum ochraceum*. It has not yet been crystallized, but a pure freeze-dried preparation analyzed as a dicarboxylic acid of molecular formula $C_{35}H_{44-46}O_7$ and was active against *Staphylococcus aureus* at a concentration of about 0.1 μg. per ml. (about one-tenth the activity of penicillin in our assays).

Pleuromutilin, $C_{22}H_{34}O_5$, was isolated from culture liquids of *Pleurotus mutilus*, *Pleurotus passeckerianus* Pilat,[9] and *Drosophila subatrata*,[10] and its structure was partly elucidated as that of a polycyclic compound containing two hydroxyl groups, a hindered carbonyl group, and, possibly, a lactone ring.[11]

Halogenated Aromatic Compounds

This group is represented only by *p*-methoxytetrachlorophenol (V),[12] a rather effective antifungal agent [13] obtained from culture liquids of *Drosophila subatrata*.[10]
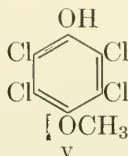
Halogenated aromatic compounds have been reported previously as fungal products, but the mechanism of biological halogenation is still obscure.[14] It is interesting, in this connection, that MacMillan [15] isolated the bromine analog of griseofulvin from culture liquids (of *Penicillium griseofulvium* and *Penicillium nigricans*) in which potassium bromide had been substituted for the chloride.

Polyacetylenes

The term polyacetylenes, as used here, indicates compounds with two or more triple bonds, in conjugation. This class of compounds has proved to be the largest group and comprises about as many compounds as are included in the other four groups combined. It includes nemotin, nemotinic acid,[16] drosophilin C, drosophilin D,[10, 17] agrocybin,[16, 18] biformyne 1, biformyne 2,[19, 20] diatretyne amide,[16, 21]

diatretyne nitrile,[16, 22] and two newly isolated polyacetylenes from *Coprinus variegatus*.[23]

The polyacetylenes, intensively investigated by synthesis, were almost unknown in biological material until quite recently. They have now been isolated from higher plants, from fungi (*Basidiomycetes*),[24] and from *Actinomycetes*.[25] The probable isolation of propiolic acid from a bacterial culture has also been reported.[26] Matricaria ester and its corresponding alcohol, originally isolated from higher plants,[27] have been obtained from cultures of the basidiomycete, *Polyporus anthrocophilus* Cke.[28]

Although polyacetylenes containing as many as eight conjugated triple bonds have been synthesized,* structural variety is better represented in the biologically produced polyacetylenes, some of which contain groupings not yet synthesized. Most polyacetylenes of biological origin contain double bonds as well as conjugated triple bonds; several contain an allene grouping, and some a benzene ring. Carlina oxide, a monoacetylenic compound, contains both a benzene ring and a furan ring.[29] Among the functional groupings which have been found, alone or in combination, are: hydroxyl, carbonyl, carboxyl, ester, amide, lactone, and most recently, nitrile. An unbroken series of polyacetylenes with chain lengths C_8 to C_{13} have been reported [24] in addition to the C_{17} and C_{18} compounds.

Nemotin and nemotin A, the first of the polyacetylenes characterized in our laboratory, were not immediately recognized as such,† although they had been studied spectrophotometrically and the characteristic alkali conversion of nemotin to nemotin A had been examined in some detail.[16] It was only after application of the too-little-known rule of Hausser, Kuhn, and Seitz [30] on spacings of the absorption maxima of polyacetylenes and polyethylenes, about 65*f* for polyacetylenes and 37–47*f* for polyethylenes, that these compounds, as well as the rest of the series, were placed in the polyacetylene group.

The chemistry of nemotin has proved to be of considerable interest. The ultraviolet absorption spectrum of this polyacetylene indicated an endiyne system of unsaturation. On reduction, nemotin yielded undecanoic acid. Since nemotin itself was not acidic, it was postulated

* The synthesis of diphenyloctaacetylene was reported from the laboratory of E. R. H. Jones, *Nature*. *168*, 900 (1951).

† A remark by Sörensen seems appropriate here. In discussing Semmler's preference for an allenic rather than one of the possible acetylenic structures for carlina oxide, he says that Semmler, "led by an irrational aversion against the occurrence of the acetylenic compounds in nature . . . ," chose the wrong formula.

that it might contain an unsaturated lactone ring, which underwent hydrogenolysis on catalytic reduction to yield the reduced deoxy acid. Since it underwent a rearrangement strikingly similar to the myco-mycin to isomycomycin isomerization and since the product, nemotin A, also yielded undecanoic acid on reduction, it was further postulated that nemotin, like mycomycin, contained an allene grouping which was isomerized to an acetylenic linkage on treatment with alkali. These two postulates proved to be partially correct, since a lactone ring and an allenic system have been demonstrated by Bu'Lock, Jones, and Leeming.[31] On the basis of the mycomycin-isomycomycin isomeriza-tion, which involves a shift of bonds as well as an allene-acetylene shift, the conversion might be pictured as follows:

$$HC\equiv C-C\equiv C-C\equiv C=C=C-C\equiv C-C-C=O \qquad VI$$

$$H_3C-C\equiv C-C\equiv C-C\equiv C-C\equiv C-C-COOH \qquad \begin{array}{l} VII\ (A = OH) \\ VIII\ (A = H) \end{array}$$

$$A$$
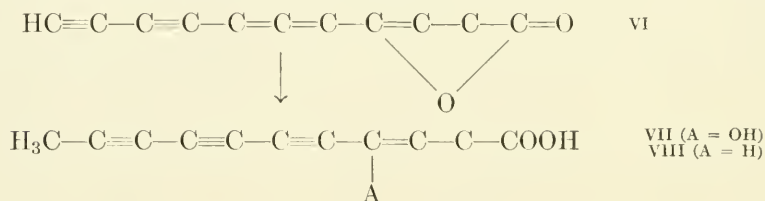
The hydroxyl might be lost on reduction of nemotin A [32, 33] or during the conversion of VI to VIII. The acid VIII was synthesized by F. Bohlmann[34] who very kindly sent us a sample for comparison with nemotin A. This compound proved to have less antibiotic activity than nemotin A against a number of bacteria and fungi and to have an ultraviolet spectrum in which the maxima, as compared to those of nemotin A, were shifted consistently 2 to 3 mμ toward higher wave-lengths (Fig. 1). The explanation for both of these differences has been provided by the findings of Bu'Lock, Jones, and Leeming, who have demonstrated the conversion to be:

$$HC\equiv C-C\equiv C-C=C=C-C-C-C-C=O \qquad IX\ \ Nemotin$$

$$HC\equiv C-C\equiv C-C\equiv C-C=C-C-C-COOH \qquad X\ \ Nemotin\ A$$
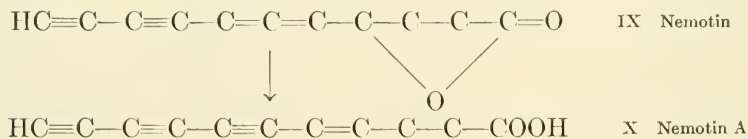
That is, nemotin contains a saturated lactone ring, which opens on alkali treatment, with loss of water, providing a new double bond. No shift of acetylenic bonds occurs, only an allene to acetylene shift. The fact that nemotin A (X), as opposed to Bohlmann's acid (VIII), contains a terminal acetylenic carbon atom presumably accounts for

the difference in its ultraviolet absorption spectrum and also for its higher antibiotic activity.

Drosophilins C and D, isolated from culture liquids of *Drosophila subatrata*, undergo the same characteristic allene to acetylene shift

$E_{1\ cm}^{1\%}$
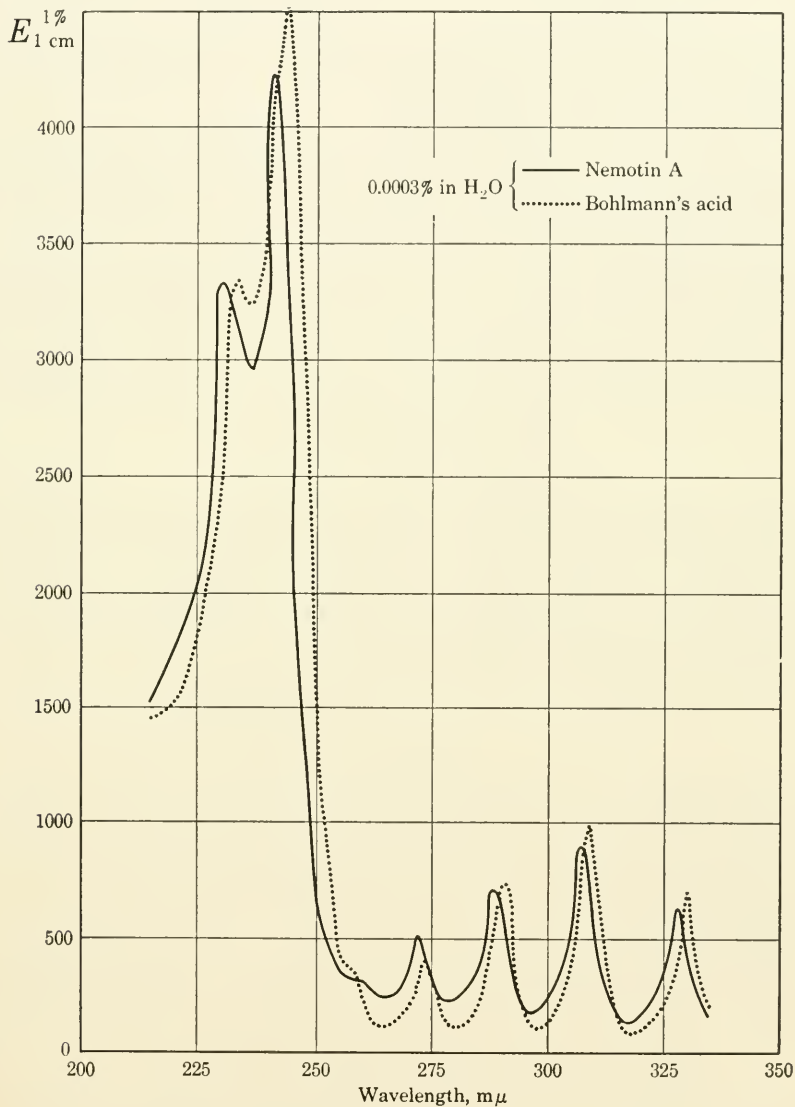
Fig. 1. Ultraviolet absorption spectra of nemotin A and Bohlmann's acid.

as judged by spectrophotometric evidence, but the chemistry of these compounds has not yet been investigated.

Agrocybin, isolated from culture liquids of *Agrocybe dura*, was characterized as XI.[33]

$$HOH_2C—C\equiv C—C\equiv C—C\equiv C—CONH_2 \quad \text{xi}$$
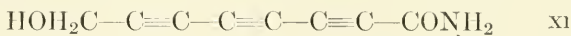
This compound bears interesting relationships both to biformyne 2 and to the diatretynes, discussed below.

Biformyne 1 is a polyacetylene which on catalytic reduction yields a 9-carbon diol.[20] The positions of the ultraviolet absorption maxima of the compound suggest a diendiyne system of unsaturation. The low extinction coefficients of the maxima, however, would speak against such a system, but, as was kindly pointed out to us by F. Bohlmann, they are in better agreement with those of symmetrical triyne diols.[35] Oxidation of the reduction product with chromium trioxide in acetic acid yields octanoic acid,* suggesting adjacent hydroxyl groups, one of which is terminal, as in formula XII for the reduction product.

$$H_3C(CH_2)_6CHOHCH_2OH$$
$$XII$$

The presence of a 1,2-diol grouping is supported by the results of periodic acid oxidation of the reduction product, which yields formaldehyde. Formula XIII is suggested for biformyne 1 by formula XII, its reduction product; by its absorption spectrum; and by the yield of an immediate precipitate with silver nitrate.

$$HC\equiv C—C\equiv C—C\equiv C—CH_2CHOHCH_2OH$$
$$XIII$$

Biformyne 2, like agrocybin, apparently occurs in a bound form in the culture liquid and is freed on boiling. The nature of the complex is unknown in both cases. The fact that both agrocybin and biformyne 2 possess hydroxyl groups suggests that this group may be involved in the attachment.

The two polyacetylenes, diatretyne amide (XIV) and diatretyne nitrile (XV), along with agrocybin (XI) form a rather interesting series.

$$HOOC—C\equiv C—C\equiv C—C\equiv C—CONH_2 \quad \text{xiv}$$

$$HOOC—C\equiv C—C\equiv C—C\equiv C—C\equiv N \quad \text{xv}$$
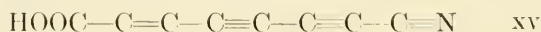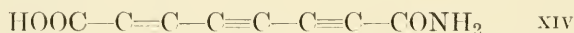
* Unpublished work.

Diatretyne amide differs from agrocybin only in that the former possesses a double bond in place of one of the triple bonds of agrocybin and a carboxyl group instead of the terminal hydroxyl group. It differs from diatretyne nitrile in that it possesses an amide group in place of the nitrile group. The rest of the molecule is, apparently, identical, but the possibility that XIV and XV differ also in their configuration (*cis-trans*) at the double bond has not been excluded. The effect of this difference, or these differences, on the antibiotic activity is rather striking. Diatretyne nitrile is active against *Staphylococcus aureus* at a concentration of about 0.1 μg. per ml., whereas diatretyne amide is inactive at a concentration 8000 times as great.

The antibiotic compounds isolated from basidiomycetes suggest problems which are of interest both in their chemical and biological aspects.

If any one tendency, from a chemical standpoint, appears to be common to this group of compounds, it is that they seem to possess, in general, an enhanced reactivity compared with other compounds of related structure. Thus, the illudins, which are notably similar to the santonins in their behavior, undergo the characteristic change with acid, described above, under considerably milder conditions. A large proportion of the basidiomycete polyacetylenes, in contrast to the synthetic ones and to those isolated from higher plants, are too unstable to be obtained in crystalline form but can be handled only in solution. Further, the allene to acetylene conversion of nemotin to nemotin A takes place under milder conditions than those reported for other similar rearrangements.[25, 36] The behavior of other antibiotic compounds isolated, on the other hand, does not support the idea of enhanced reactivity. Pleuromutilin, ochracic acid, and diatretyne nitrile are relatively stable chemically, although all three of these compounds have a high degree of antibiotic activity.

The basidiomycete compounds present other interesting chemical problems and shed further light on little-explored questions. The illudins, fomecins, and pleuromutilin exhibit what appears to be somewhat unorthodox or at least unusual chemical behavior. The structures of the biformynes and of the precursors of biformyne 2 and agrocybin need clarification. The availability of compounds such as the nemotins and the drosophilins (along with mycomycin) provides models (not readily available synthetically) for study of the allene to acetylene shift as influenced by other structural features of the molecule. The behavior of agrocybin on catalytic reduction, using platinic oxide catalyst (loss of hydroxyl oxygen), serves as a reminder, particularly important in working with those polyacetylenes which are too unstable

to be analyzed, that caution is necessary in deducing the formula of a polyacetylene from that of its reduction products.

Data useful for spectrophotometric correlations have been obtained in some instances. For example, the absorption maxima of diatretyne amide, in which the endiyne system is conjugated at either end (to a carboxyl carbonyl at one end and an amide carbonyl at the other end), are essentially the same as would be expected for an endiyne system conjugated only to a carboxyl group, and the spectrum of diatretyne nitrile resembles closely that of an entriyne system. Thus, the nitrile grouping as it occurs in this compound acts chromophorically like another acetylenic bond, whereas the conjugated carboxyl grouping again has no pronounced effect on the spectrum.

The existence of polyacetylenic compounds of biological origin * poses many questions of general biological interest, and their isolation and structural elucidation pave the way for attacking some of the problems raised.

From a metabolic standpoint, the existence of polyacetylenes of biological origin presents several problems. The first question which arises, naturally, is one concerning the origin of these compounds. By what series of reactions do these highly unsaturated compounds, of chain lengths as short as C_8 and as long as C_{18}, arise? What are their immediate precursors? Are they formed by combinations of shorter-chain unsaturated compounds or by dehydrogenation of compounds of similar chain length? Growth of polyacetylene-producing organisms using an isotopic carbon source may help to provide some of the answers. The fate of these compounds in the organism is also an unexplored problem. It seems reasonable to suppose that highly reactive compounds like the polyacetylenes may be not merely end products of metabolism but, rather, intermediates. By using labeled polyacetylenes produced either by the fungus or synthetically, it should be possible also to attack this problem.

The general question of relation of structure to antibiotic activity arises also in connection with polyacetylenes. Neither the synthetic polyacetylenes nor those isolated from higher plants have been tested systematically for antibiotic activity. However, there are several facts

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* The term "naturally occurring" as applied to polyacetylenes isolated from fungal culture liquids was used in contradistinction to "synthetic" but is, perhaps, somewhat ambiguous in its implications. Nothing is known about the production of these compounds by fungi in nature since they have been isolated only under laboratory conditions. A term such as "biologically produced," suggested by Dr. Selman Waksman, or "of biological origin" might, therefore, be preferable.

which indicate that such activity is not a general property of conjugated acetylenic linkages but depends, at least in part, on other groupings present in the molecule. Thus, among the basidiomycete polyacetylenes, compounds with similar unsaturated systems may show pronounced specificity in their bacterial and fungal spectra. A more striking demonstration of this fact is afforded by comparison of the compounds diatretyne nitrile and diatretyne amide discussed below. As mentioned previously, the latter is at least 8000 times as active against *Staphylococcus aureus* as the former.

More specific questions are raised by the frequent occurrence of pairs of closely related compounds in a culture liquid. Diatretyne amide (XIII) and diatretyne nitrile (XIV) offer one of the most interesting examples of this. Are these compounds synthesized independently by the organism, or do they arise from a common, closely related precursor, or is one the precursor of the other? The likelihood of the last possibility is enhanced by the close structural similarity of the compounds: the difference between them consists only in possession of a nitrile grouping by one and an amide grouping by the other. If this last mechanism does represent the actual sequence, it would further suggest the possibility of the existence in the organism of an amide dehydrase. This would be a novel sort of enzyme, since reports of nitriles of biological origin are comparatively rare,[37] and nothing is known of their close association with the corresponding amide. Diatretyne amide and diatretyne nitrile appear to be the first such pair reported, and diatretyne nitrile is the first reported polyacetylenic nitrile of biological origin.

The possibility of taxonomic implications of basidiomycete products is interesting to consider. In higher plants, rather extensive investigations have been made by Erdtman's group on the wood of Gymnospermae (softwoods) and by Sörensen's group using plants of the Composite family. Erdtman has reported[38] that pinosylvin (3,5-dihydroxystilbene) or its methyl ether is present in the heartwood of most species of the genus *Pinus,* and that other genera of coniferous trees do not contain them. An instance in which "chemical taxonomy" has lent support to a taxonomic classification which apparently is in some question on morphological grounds is furnished by Sörensen's group. On the basis of their investigations of acetylenes of the Composite family, they state:[39] "The power to synthesize acetylenic compounds thus separates *Tripleurospermum* distinctly from *Matricaria.*" Although it appears, on examination of the basidiomycete compounds

from a taxonomic point of view, that these exhibit no relationships of taxonomic interest, it is, of course, too soon to draw conclusions since the number of basidiomycete compounds characterized is still relatively small. One facet of the group of findings is, perhaps, somewhat more apparent than the others. The isolation of acetylenic compounds from basidiomycetes suggests their rather general biological occurrence since they have been shown to be produced by higher plants, by the basidiomycete group of fungi and by an actinomycete. The isolation of a propiolic acidlike compound from *Escherichia coli*, mentioned above, is of further interest in this connection.

From the findings and the discussion presented in this paper, it is apparent that no conclusions of a general nature can be drawn which would make a cohesive story of the particular corner of "polychemism" [40] observed in the compounds isolated in our laboratory from basidiomycetes. It is hoped, however, that some interesting trends have been pointed out and that possible paths have been suggested toward desirable goals.

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Heterogeneity of Deoxyribonucleic Acid (DNA)

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A great many explorations have led to the view, which few people now question, that the assertion of biological characters and their transmission from one generation of cells or organisms to the next require the intervention of DNA. In fact, there is a growing conviction that the actual genetic determinants of the cell are composed of DNA. This conclusion has received its most impressive support from the knowledge that DNA preparations from many microorganisms can carry out heritable transformations. There are, of course, a great many heritable factors in the total genetic complement of any cell. It would seem necessary, for the gene: DNA relationship to be valid, to postulate that the total DNA of the cell consists of a great many DNA molecules each concerned with one phenotypic expression and differentiated by a particular chemical structure. Or, alternatively, these macromolecules may be few in number but possessing structures that are heterogeneous along the chain. According to this idea, biological characteristics are associated with specific regions on a DNA molecule; hence, a given molecule may be polyfunctional. It is not the purpose here to choose between these alternatives; indeed, both may be involved. Rather, this essay will deal with several lines of evidence which have revealed the heterogeneous nature of DNA.

Perhaps the earliest published account [1] that is suggestive of this idea is the report that the great bulk of the DNA of isolated "chromosomes" is soluble in M NaCl, but a little DNA was still detected in the insoluble "residual chromosomes." This residual DNA was thought [1] to be a contaminant, but, in the light of more recent developments, it would be good to re-examine this finding since it may reflect heterogeneity in distribution of DNA within the cell. The same applies to

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the very brief report [2] of the solubility of only part (over 60%) of the DNA of liver nuclei in strong sucrose solution.

Under defined conditions, the DNA's of calf thymus and of wheat germ yield dialyzable fragments and small non-dialyzable "cores" (about 7% of the total) following prolonged treatment with deoxyribonuclease.[3, 4]   It has not yet been decided whether this result is due to the presence in these DNA's of more than one DNA or whether the nucleic acids are composed of molecules possessing regions differing in susceptibility to the enzyme.   The type of heterogeneity those experiments reveal may be elucidated if the same technique is applied to the various DNA fractions which are now available (vide infra).

Heterogeneity with respect to the sites of binding of positively charged dyes to DNA has been described.[5]   These results may arise from identical molecules whose linear structure is discontinuous or may be due to mixtures of dissimilar molecules, each possessing its own set of binding characteristics.

Evidence of an inhomogeneous distribution of DNA within the nucleus has also come from studies [6] on the susceptibility to deoxyribonuclease.   Depending upon the species examined, only 40 to 89% of the DNA of isolated nuclei can be removed with the enzyme.   The resistance of some of the DNA to the action of the nuclease is not due to the same phenomenon as the resistance of the non-dialyzable "cores" referred to above, since removal of the basic protein with dilute HCl renders the resistant DNA fraction in nuclei susceptible to digestion.[6]

The DNA of *Streptococcus faecalis* is present in that organism in two forms; one is soluble in dilute sodium hydroxide solution, whereas the other is not and is apparently bound to polysaccharide.[7]

These various findings are reminiscent of our early studies [8] on the heterogeneity of DNA in mammalian tissue.   It was found that two gross fractions could be obtained by subjecting the total DNA (extracted with strong salt solution) to high-speed centrifugation in 0.87% NaCl.   In this fashion, an insoluble (DNA_1) and a soluble (DNA_2) fraction were obtained.   With the exception of the normal livers, a number of organs of the adult rat yielded the two fractions in varying ratios [9] depending upon the organ (Table 1).   Normal liver contains DNA_2, but little if any DNA_1.   During regeneration following partial hepatectomy, DNA_1 appears in large amounts but the DNA_2 content remains constant.   It is not known whether the high ratios for small intestine and for regenerating liver are due to the high mitotic activity of these tissues.

Table 1. Base Composition of DNA Fractions from Various Organs of the Adult Rat * †

DNA Fraction	DNA$_1$			DNA$_2$		
Organ	Thymine	Guanine	Cytosine	Thymine	Guanine	Cytosine
Intestine	0.84	0.79	0.72	1.03	0.74	0.83
Kidney	0.79	0.73	0.70	0.97	0.69	0.77
Spleen	0.84	0.74	0.68	0.98	0.78	0.88
Pancreas	0.65	0.80	0.67	0.82	0.74	0.82
Brain				0.76	0.67	0.68
Testis	0.77	0.77	0.68			

* Molar ratios with adenine taken as 1.00.

† J. R. Fresco, P. J. Russell, Jr., and A. Bendich, unpublished results.

This possibility was explored, in part, by examining the livers of rats which were subjected to partial hepatectomy. The DNA$_1$ to DNA$_2$ ratio remained high 3 to 23 days following the operation, but, thereafter, the ratio dropped rapidly. The regenerated livers 2 and 3 months after partial hepatectomy resembled normal liver in that little or no DNA$_1$ could be isolated.* The virtual absence of DNA$_1$ in the normal livers of adult rats has been confirmed. It was found, further, that the DNA content of rat liver increases as a result of treatment of rats with alloxan. This increase was attributed specifically to the formation of DNA$_1$, whereas the DNA$_2$ content was unchanged.[10]

The isolation procedure which permits the isolation of DNA$_1$ and DNA$_2$ was originally based upon an arbitrary concentration of salt, 0.87% NaCl. Yet, unexpectedly, it does not seem to be so arbitrary after all inasmuch as it reflects the formation of DNA$_1$ in the rapidly growing tissue; i.e., the fractionation procedure appears to have a biological basis in fact in this differentiation of DNA.

Analysis of the soluble DNA fractions for base composition reveals (Table 1) them to be different from the insoluble ones, and, further, that there are significant differences among tissues.† These results reveal a heterogeneity in the composition of DNA from various organs of the rat and in this regard are in sharp contrast to findings [11] from only a few organs of other mammalian species. The basis of this apparent conflict is not clear at present but may be related to the fact

* Unpublished results, with J. R. Fresco.

† Unpublished results, with J. R. Fresco and R. J. Russell, Jr.

that differences among DNA fractions may be obscured when the total DNA containing such fractions is analyzed.

To compare the metabolic behavior of these two gross DNA fractions, and the metabolism of the DNA of various organs, isotopic formate was administered to a group of rats. Half the animals were examined 1 day after administration of the formate and the remainder 23 days later. The DNA fractions isolated from a number of organs were broken down to free bases and the percentage change in the isotope contents during the 23-day period determined (Table 2); the

Table 2. Per Cent Apparent Retention of C^{14} during 23-Day Interval Following Administration of Labeled Formate to Adult Rats *

DNA Fraction	Base	Small Intestine	Spleen	Regenerating Liver	Normal Liver	Pancreas	Kidney	Testis
	Thymine	4.1	20	92		52	66	85
DNA_1	Adenine	2.4	11	55		42	39	82
	Guanine	2.1	15	56		40	65	68
	Thymine	4.2	22	85	53	38	93	62
DNA_2	Adenine	2.7	6	57	15	35	92	105
	Guanine	3.6	15	82	33	42	116	89

* Taken from A. Bendich, P. J. Russell, Jr., and G. B. Brown, *J. Biol. Chem.*, *203*, 305 (1953).

change is referred to as "apparent retention." These results [9] show that the two fractions are metabolically dissimilar in any one organ and that the metabolic picture is different from organ to organ. With the possible exception of the DNA_2 fraction of pancreas, the "apparent retention" of isotope differs from base to base for the individual fractions. (An analogous result has been obtained in mouse- and rat-liver DNA in studies with P^{32}.[12]) These studies suggest that the DNA fractions of various organs of the rat have a heterogeneous metabolic origin. Thus, in addition to differences between the two fractions insofar as solubility and chemical composition are concerned, they could be distinguished on the basis of their anabolic behavior.

Further evidence for chemical heterogeneity of DNA has recently been obtained by three independent techniques. One may be termed fractional dissociation of calf-thymus nucleoprotein; the second involves chromatography on columns of histone, now itself found to be heterogeneous; the last to be described employs an anion exchanger that is ostensibly homogeneous.

Calf-thymus nucleoprotein, in the form of a loosely packed gel with chloroform-octanol, yields a few nucleoprotein fractions upon extrac-

tion of the gel with NaCl solutions of increasing concentration.[13] When freed of protein, corresponding DNA fractions are obtained which show decreasing proportions of guanine and cytosine and increasing amounts of adenine and thymine. (Similar fractions may be obtained [14] by successive extractions of the gels with salt solutions of constant concentration.) An analysis of the 5-methylcytosine contents reveals a disproportionate distribution of this pyrimidine among the fractions, and this [13] constitutes newer, but striking, evidence of the heterogeneity of DNA.

Despite the fact that twenty-one preparations of calf-thymus DNA afforded by several procedures showed [13] very similar compositions, this similarity obscured the large differences in the constitution of the components of the mixture which is known as calf-thymus DNA.

Almost simultaneously, work was described [15] in which histone (from calf nucleohistone) was immobilized on columns of kieselguhr. Solutions of DNA from either calf thymus, *Escherichia coli,* or human white blood cells were placed upon such columns and then eluted with NaCl solutions of increasing concentration. Convincing evidence of the heterogeneous character of these DNA preparations was obtained from an examination of the fractions in the eluates. A gradation in the base composition was also observed in several fractions from calf-thymus DNA.

Although it furnished a clue to the heterogeneous nature of DNA, the fractionation procedure which yielded the soluble and insoluble fractions DNA_1 and DNA_2 could, at best, be described as crude. Better methods were therefore sought. If DNA were indeed composed of different individuals, a DNA preparation should consist, in neutral solution, of a number of polyelectrolytes anionic in character due to phosphate dissociation. Accordingly, columns of anion exchangers were employed in attempts * to effect a separation of individual polyanions, or groups thereof. Initial attempts with commercially available strong and weak base anion exchanger resins furnished a few encouraging results. In one experiment with the chloride form of the Amberlite IR-4B, over one hundred chromatographic fractions were obtained with calf-thymus DNA. But the experiments were difficult to repeat, and the various resins showed many undesirable properties.

The success in the fractionation of proteins [16] by means of anion (and cation) exchangers prepared from cellulose prompted an investigation of their suitability for the fractionation of DNA. A cation

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* Unpublished experiments, with J. R. Fresco and H. R. Rosenkranz.

exchanger containing carboxymethyl groups attached to the cellulose was without any affinity for calf-thymus DNA at neutral pH. However, one containing the basic diethylaminoethyl group removed DNA from solution, and fractions could be obtained by salt elution. A new anion exchanger, Ecteola-Cellulose, prepared by the treatment of alkaline cellulose with a mixture of epichlorohydrin and triethanolamine,[17] has given the most provocative results. A diagram of the chromatographic behavior of highly polymerized calf-thymus DNA on a column of this anion exchanger is shown in Fig. 1. Many chromatographic fractions of DNA were obtained by continuous gradient elution with NaCl solution of increasing concentration followed by graded changes in pH. About 65% of the original DNA was recovered at neutrality, some 20% up to pH 9.9, and the remainder required strong alkali for complete removal. Analogous chromatographic patterns result from the application of a discontinuous salt concentration or pH gradient.

Fractions of DNA obtained this way are non-dialyzable and retain their chromatographic properties on rechromatography; the reproducibility of the patterns is indeed gratifying.

With this method at hand, a solution of transforming DNA from pneumococcus * was passed through a column of Ecteola-Cellulose and all of the transforming activity as well as of the DNA was retained by the exchanger. Fractions of DNA exhibiting transforming activity were obtained at several places along the chromatogram following the elution; wherever the activity was detected it always coincided with the presence of DNA. Perhaps this can be taken as additional argument that DNA can carry and transmit genetic information. At any rate, retention of biological (transforming) activity following its removal from solution by the exchanger and its subsequent elution indicates that little tampering with the integrity of the DNA preparation had resulted.

The pneumococcal DNA preparation originally possessed four demonstrable genetic properties; these included transformations to penicillin, streptomycin and sulfonamide resistance, and mannitol utilization.[18] The DNA preparation, isolated from bacteria arising from a single clone, induced these heritable transformations at random essentially as single, independent events. However, a significant number of the mutant cells showed two properties, those of mannitol utilization and of streptomycin resistance which were acquired, not at random, but rather as related or connected events.[18] It would appear, then,

* Unpublished experiments, with S. Beiser, J. R. Fresco, and R. D. Hotchkiss.
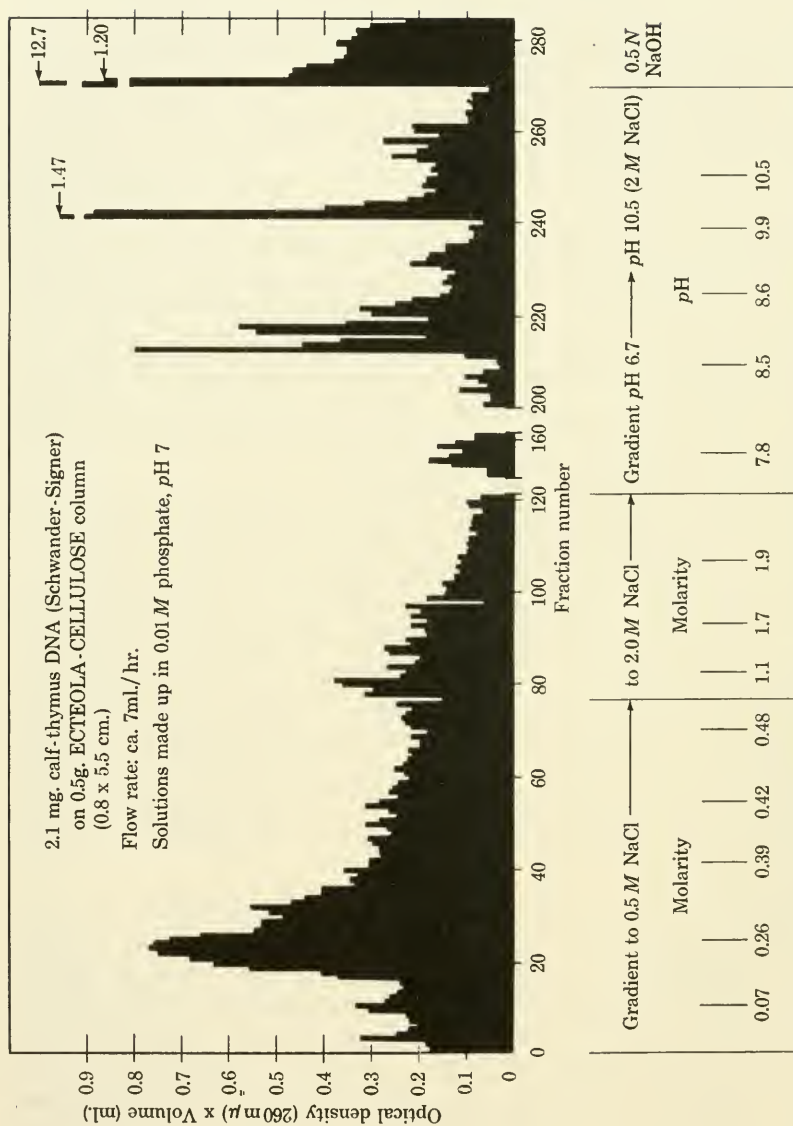
FIG. 1. Chromatographic fractionation of calf-thymus DNA on the anion exchanger Ecteola-Cellulose (A. Bendich, J. R. Fresco, and H. S. Rosankranz, unpublished results).

that the preparation of DNA consisted, at least, of five DNA fractions (or molecules); each of four of these was associated with just one identifiable genetic property. Two of these properties appeared to be linked together in the fifth species of DNA.

The initial attempts to separate this pneumococcal DNA into fractions of discrete activity were somewhat equivocal. The method appears to have a high resolution. In consonance with the evidence cited above, it supports the notion that DNA is highly heterogeneous. It remains to be seen whether this heterogeneity of DNA will help explain some of the pressing problems of biology.

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Biosynthesis of Branched-Chain Compounds

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The branching of carbon chains is a structural feature that is typical of a wide array of naturally occurring compounds. Transformation of a straight carbon skeleton to one which is branched, i.e., substitution of a carbon-bound hydrogen by the bulkier alkyl group, may be expected to cause profound changes of physical properties, but the biochemical significance of such structural modifications is yet to be defined. For the moment, it is the biological origin of branched molecules which is of special interest, not only as a problem of biogenesis but also as a problem of comparative biochemistry. All living forms carry out syntheses leading to branched chains, but there exist notable restrictions in some organisms with regard to the type of branched-chain compounds that can be formed. Thus the animal cell is dependent on an outside supply of the amino acids valine, leucine, and isoleucine, though it can readily form the closely related acids which are precursors of the terpenes and steroids. Similarly one may point to the fact that the triterpenoid hydrocarbon squalene is synthesized by animals, although other isoprene derivatives such as the carotenes are not. An inability to produce branched chains per se is therefore not the cause for the exacting nutritional requirements which the animal organism has developed.

It is the main object of this essay to consider the branching of carbon chains as it relates to the biogenesis of terpenes and steroids. A critical discussion of this area is particularly tempting because the information at hand, though considerable, has not clarified the central issues. It is interesting to note and it may be stated at the outset that the carbon chains of the branched amino acids on the one hand, and of the terpenes and isoprene derivatives on the other, arise by pathways that are entirely distinct. For isoprene derivatives, acetic acid is the sole carbon source, but this precursor does not enter directly

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into the formation of the branched portions of isoleucine, leucine, or valine. For the moment we shall merely take note of the fact that, for the synthesis of branched chains, generally speaking, there exist at least two mechanisms which are entirely separate. Those aspects of the biogenesis of valine and leucine which are pertinent to this discussion will be briefly considered later on.

Active interest in the biosynthesis of branched carbon chains arose from the concern with two seemingly unrelated processes, the formation of steroids, which Rittenberg and Schoenheimer [1] began to study in 1937 in the Columbia Laboratories, and of rubber, which Bonner and his associates [2] have investigated more recently. The first point of contact between the two areas was made by the observations that in both processes acetic acid serves as the carbon source. Later, in the light of the suggestive relationship of squalene and cholesterol, the link between isoprene derivatives and steroids was more firmly established, and even more recently the biogenesis of carotene [3] and of geraniol [4] have provided additional examples in the terpene field of what appears to be the same basic process, namely, the transformation of acetate units to branched 5-carbon chains. If one accepts the thesis that those natural products which obey the isoprene rule, i.e., which can be formally constructed from isoprene units, also belong biogenetically to the isoprene family, then the number and variety of cell constituents which are products of this special type of acetate metabolism becomes impressively large (carotenes, phytol, mono- and sesquiterpenes, tetra- and pentacyclic triterpenes).

The identity of the repeating units in the terpenes and carotenes has inspired numerous hypotheses dealing with the origin of this class of compounds. A recent formulation is that of Bonner, who, on the basis of two sets of observations, (1) the incorporation of acetate carbon into rubber by isolated guayule leaves, and (2) the capacity of acetate, acetone, acetoacetate, and β-dimethylacrylic acid to enhance net rubber production, postulated the following series of reactions leading to the formation of 5-carbon units:

$$2CH_3COOH \rightarrow CH_3COCH_2COOH \rightarrow CH_3COCH_3 + CO_2$$

$$\begin{array}{c} CH_3 \\ \diagdown \\ CO + CH_3COOH \rightarrow \\ \diagup \\ CH_3 \end{array} \quad \begin{array}{c} CH_3 \\ \diagdown \\ C{=}CH{-}COOH \rightarrow \\ \diagup \\ CH_3 \end{array}$$

$$\begin{array}{c} CH_3 \\ | \\ -CH_2{-}C{=}CH{-}CH_2{-} \end{array}$$
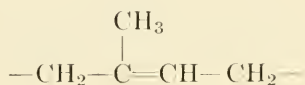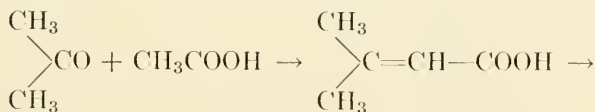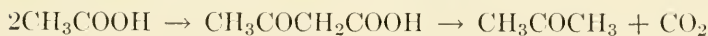
Once the distribution of acetate carbon in the isooctyl side chain of cholesterol had been ascertained, it became immediately clear that the isotopic pattern observed in the sterol could be readily accounted for by the series of reactions shown above. It thus became a logical step to postulate the occurrence of parallel events at some stage of rubber and steroid biogenesis. The details of the above scheme, in particular the suggestion that branching was achieved by a biochemical equivalent of the Reformatskii reaction, have not withstood later scrutiny mainly because acetone fails to show the properties of a specific steroid precursor.[5, 6] In the systems investigated so far, i.e., in the intact animal and in the liver slice, acetone at best equals the efficiency of acetate, a result which reflects the rapid oxidation of this 3-carbon compound to acetate and "formate." Moreover, when Brady and Gurin found that 1-C^{14}-acetoacetate was incorporated into cholesterol apparently without fragmentation of the carbon chain,[7] participation of acetone seemed clearly ruled out. However, it has lately become less certain that the results with labeled acetoacetate were interpreted correctly, and the possibility that acetone participates in a direct way cannot as yet be dismissed entirely.

Searching for an alternative mechanism which would permit the assembly of acetate units to a branched chain intermediate, we have suggested β-hydroxy-β-methylglutaric acid (I) as an intermediate that could be formed from acetoacetate acid and acetate, or acetyl CoA.[8] This condensation would bear some resemblance to the formation of citrate (II) from acetyl CoA and oxaloacetate. The dihydroxy-6-

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\begin{array}{ccc}
\begin{array}{l}
CH_2COOH \\
| \\
CH_3CO \\
\\
+ \\
\\
CH_3COOH \\
\\
\downarrow \\
\\
CH_2COOH \\
| \\
CH_3COH \\
| \\
CH_2COOH \\
\text{I}
\end{array}
&
\begin{array}{l}
CH_2COOH \\
| \\
HOOC-CO \\
\\
+ \\
\\
CH_3COOH \\
\\
\downarrow \\
\\
CH_2COOH \\
| \\
HOOC-COH \\
| \\
CH_2COOH \\
\text{II}
\end{array}
&
\begin{array}{l}
COH-COOH \\
| \\
CH_3CO \\
\\
+ \\
\\
CH_3COOH \\
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COH-COOH \\
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CH_3C-OH \\
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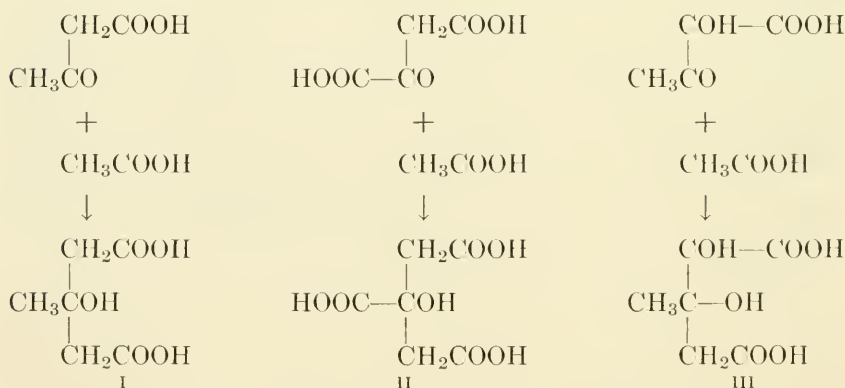
carbon dicarboxylic acid (III) which has been implicated by the work of Tatum and Adelberg as the common precursor of valine and isoleucine in *Neurospora* [9] is most likely formed by an analogous reaction,

a more highly oxidized 4-carbon compound taking the place of aceto-acetate. It is improbable that this hypothetical precursor of branched amino acids in *Neurospora* (III) is a source of isoprenoid intermediates, since precursors other than acetate enter into its formation.

It was fortunate that concurrently with the interest in branched-chain intermediates hydroxymethylglutarate (HMG) was identified as a constituent of various plants.[10, 11] At the same time it was recalled that acids such as β-dimethylacrylic acid (DMA), isovaleric acid (IV), and β-hydroxyisovaleric acid (HIV) are to be found in nature, and hence the moment seemed propitious for an inquiry into their origin and possible relation to isoprene and steroid biosynthesis. With the aid of the now widely used carrier technique the formation of the above-mentioned acids from labeled acetate in animal tissues could be readily demonstrated.[12, 13] Moreover, the number and spacing of the carbon atoms of acetate in the branched molecules conformed, where investigated,[12] with the pattern that had been predicted for the hypothetical steroid precursor. So far, the postulated initial step which leads to a branched compound, i.e., the condensation of acetate and acetoacetate (or their coenzyme derivatives) to a 6-carbon dicarboxylic acid has been elusive, although the reverse reaction has been shown to occur.

Considerable light has been thrown on the interrelationship of the various branched-chain compounds by the discovery of Coon [14, 15] that in the conversion of the 5-carbon acids to acetoacetate a fixation of carbon dioxide is an integral part of the overall process suggesting the sequence of steps shown in Fig. 1. Some of these reactions are closely
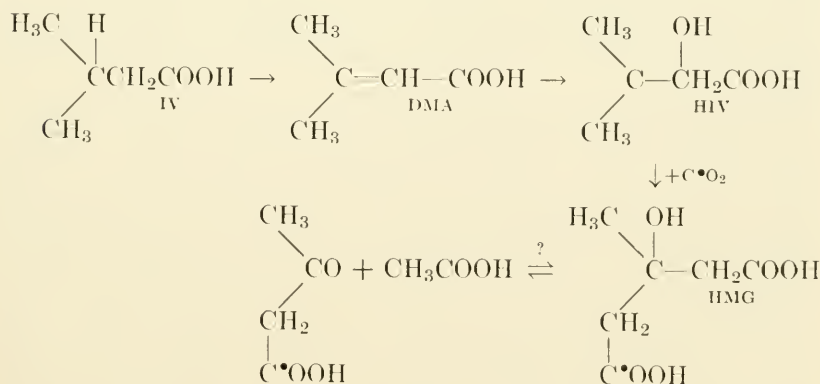
Fig. 1.

analogous to the events which are known to occur with the straight-chain fatty acids, and hence their reversibility may be anticipated. In this event the above scheme would provide for the formation of hydroxymethylglutarate by two pathways: (a) by a $C_2 + C_4$ condensation and (b) by CO_2 fixation of a 5-carbon acid. The second reaction is perhaps not quantitatively important at least in animal tissues, since the supply of isovaleric acid would appear to be limited by the rate of oxidation of leucine which is an essential amino acid. At any rate reactions have now been shown to occur which afford branched 5- or 6-carbon acids, and assuming these acids to be intermediates rather than metabolic end products we may now consider their relation to terpene and steroid biogenesis, the more central issue of our discussion.

When we first took up this problem in 1944 it seemed reasonable to pose the question whether the preformed chains of leucine or valine might serve as carbon sources for some portion of the steroid molecule, and in fact deuterioleucine and deuterioisovaleric acid proved to be efficient precursors of cholesterol. Later, with the finding that only acetate carbons make up the skeleton of ergosterol and cholesterol, it became clear that a carbon source which originated from an indispensable amino acid could not be an obligatory intermediate, at least in the animal body. Nevertheless, it appeared possible that the metabolism of leucine, for example, led to a product that was identical with one of the intermediates in the acetate-sterol conversion, and hence experiments with leucine or isovaleric acid seemed worth pursuing. With the aid of isotopic carbon Zabin [6] showed isovaleric acid to be several times more effective than acetate for cholesterol synthesis. This was true, however, only when the precursor was labeled in the isopropyl portion of the molecule. Carboxyl-labeled isovalerate unexpectedly gave results that were indistinguishable from those obtained with $1\text{-}C^{14}$-acetate.

More recently, we have tested additional branched-chain acids as cholesterol precursors with results that have been both encouraging and puzzling. HMG, HIV, and DMA when labeled at the tertiary carbon atom were incorporated into cholesterol, but only with DMA as the substrate was the transformation extensive enough to indicate specific conversion. On the other hand, partial degradation of the cholesterol samples from the three experiments indicated that in all cases C^{14} was present only at those six positions which one would expect to be labeled if the carbon chains of the acids had remained intact during condensation to the triterpenoid intermediate (Fig. 2).

Metabolic breakdown of the precursors to acetate or acetoacetate would have caused the appearance of labeled carbon in twelve positions of the steroid molecule.   Although the relatively low overall efficiency of HMG and HIV as cholesterol precursors might be ascribed to rate differences in the transformation of the free acids to activated derivatives (admittedly a restatement of the problem rather than an explanation) a more discordant result came to light when 1-C^{14} DMA was tested.   If it is valid to assume that the intact DMA molecule enters into the condensation reaction, then the conversion efficiency
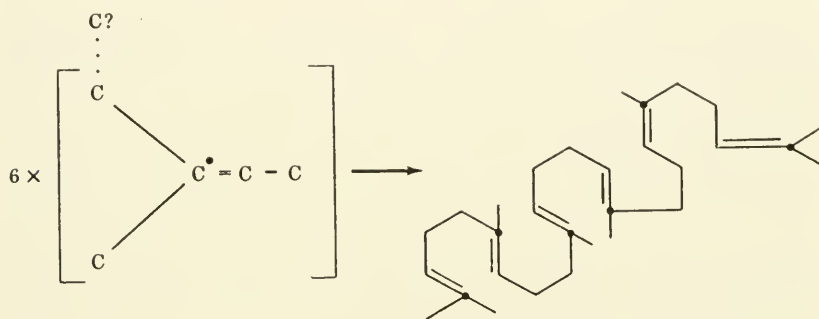
Fig. 2.   Postulated distribution of C^{14} in triterpenoid precursor formed from branched acids labeled at carbon 3.

should be independent of the location of the carbon label in the precursor.   This was not true for 1-C^{14} DMA.   Moreover, C^{14} from 1-C^{14} DMA entered the C-25 position of the cholesterol side chain at a level indicating that extensive breakdown to 1-C^{14} acetate had occurred.   This result in fact confirms our earlier experiences with isovaleric acid and leads to the anomalous situation in which the branched-chain acids behave as if they were direct cholesterol precursors only when they are labeled in the isopropyl group.   The formation of C_2 units from carbon atoms 1 and 2 of isovalerate and of DMA is readily explained by cleavage of the molecule between carbon atoms 2 and 3, perhaps by the reactions suggested by Coon (Fig. 1), i.e., with concomitant CO_2 fixation.   On the other hand, the route taken by the isopropyl portion of isovalerate or dimethylacrylate during conversion to steroids is not immediately apparent.   Acetoacetate formation from the isopropyl portion as depicted in Fig. 1 cannot be the explanation because the observed isotope-distribution pattern in cholesterol [16] differs so markedly from what is found with acetate or acetoacetate [17] as precursors.

There is one further observation which should be mentioned before an interpretation of the relevant data is attempted. Labeled CO_2 when administered to rats is incorporated into cholesterol at a level that is barely detectable. The incorporation values can, however, be substantially increased by the simultaneous feeding of normal isovaleric acid. At the same time the concentration of C^{14} in the acetate pool is raised only slightly as judged by the C^{14} content of the acetyl groups of N-acetylphenylaminobutyric acid. Hence the CO_2-fixing step does not give rise to labeled acetic acid. One is inclined to interpret this result in accordance with Coon's scheme, as reflecting a $C_5 + C_1$ condensation to form a dicarboxylic acid which is subsequently transformed into the steroid precursor. The structure of the substrate that fixes CO_2 is, however, not defined except that it would appear to be a transformation product of isovaleric acid.

Any scheme designed to harmonize the available experimental findings must be capable of accounting for the following observations: (1) the ease of conversion to cholesterol of DMA as compared to other branched acids, at least in the intact animal; (2) the preferential utilization of the isopropyl portions of DMA and isovalerate; (3) the CO_2 fixation into cholesterol, which is promoted by isovaleric acid. Some of the experimental facts enumerated here can be fitted into a scheme that is highly speculative to be sure, but since it is susceptible to experimental test it may prove to be of temporary value as a working hypothesis. It should be understood that at the present time the concept which visualizes terpene and steroid biogenesis as a process involving the multiple condensation of C_5 (or perhaps C_6) units has merely the status of an hypothesis. There are experimental observations which tend to support it, but no proof exists that the basic principle, i.e., polymerization of monomeric units to polyisoprenoids, has biological reality. The speculations which follow are designed to reconcile some of the conflicting experimental data and thereby to strengthen the underlying hypotheses.

As the initial step in the metabolism of DMA, using the 1-C^{11} compound for purposes of illustration, we wish to propose (Fig. 3) a shift of the double bond to the exomethylene position, a reaction which could either proceed directly or by way of β-hydroxyisovaleric acid. Coon [15] regards the hydroxy acid as the substrate for CO_2 fixation because in his enzyme system DMA formed labeled acetoacetate only in the presence of crotonase. It is possible, however, that this finding is the result of a more complex set of events, such as the hydration of DMA to the β-hydroxy acid followed by dehydration to the β-γ

unsaturated acid, which in turn might fix carbon dioxide. This mechanism is preferred because on purely chemical grounds fixation of CO_2 by a hydroxy acid is improbable, whereas both chemical and bio-

$$CH_3\text{-}CH\text{-}CH_2C\bullet OOH \xrightarrow{(1)} CH_3\text{-}C{=}CH\text{-}C\bullet OOH \underset{(2)}{\overset{\pm H_2O}{\rightleftharpoons}} CH_3\text{-}C\text{-}CH_2C\bullet OOH$$

IV   DMA   HIV

$\pm H_2O$ (3)

$$CH_3\text{-}C\text{-}CH_2C\bullet OOH \quad H_2C$$

MVA

(6)

$\pm CO_2$ (4)

$$CH_3\text{-}C\text{-}CH_3 \xleftarrow[(5)]{-C\bullet O_2} CH_3\text{-}C\text{-}CH_2C\bullet OOH$$

CH   DMA   CH   MGA
COOH   COOH

$\pm H_2O$ (7)

$$CH_3\text{-}CO + CH_3C\bullet OOH \underset{(8)}{\leftrightarrow} H_3C\ OH\text{-}C\text{-}CH_2C\bullet OOH$$

CH$_2$ CH$_2$ HMG
COOH   COOH
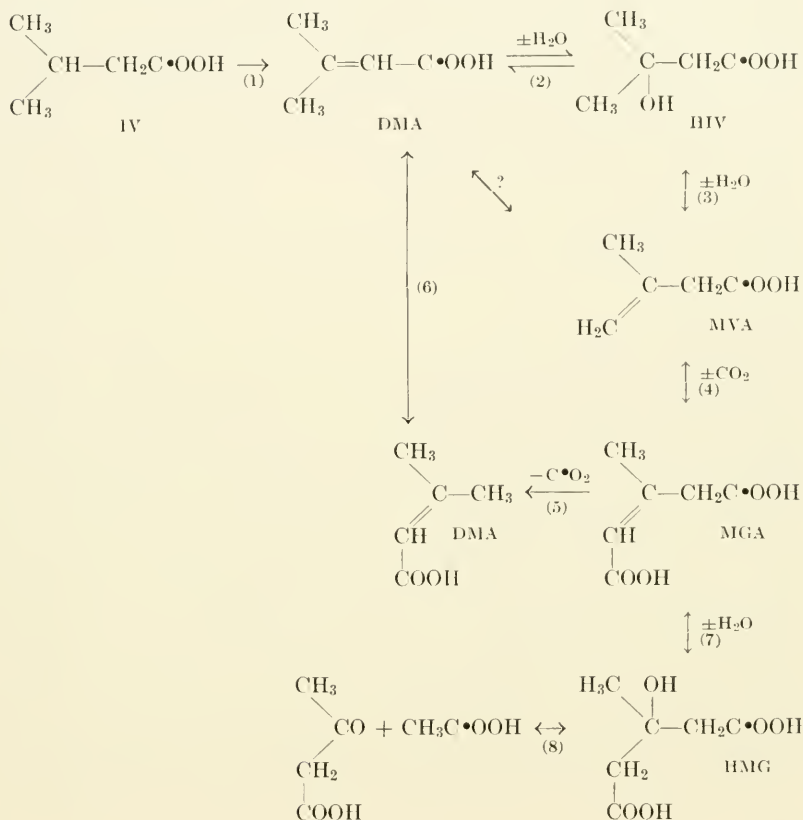
Fig. 3.

IV: isovaleric acid.   MVA: β-methylvinylacetic acid.
DMA: β-dimethylacrylic acid.   MGA: β-methylglutaconic acid.
HIV: β-hydroxyisovaleric acid. HMG: β-hydroxy-β-methylglutaric acid.

chemical analogies exist for the carboxylation of ethylenic compounds. Moreover, it appears that crotonase catalyzes reversible reactions which would allow an equilibrium to be established between the α, β, and β,γ unsaturated acids, and the β-hydroxy acid. If the β,γ acid (methylvinylacetic acid) were indeed the acceptor for carbon dioxide, one of two geometric isomers of methylglutaconic acid would be the product. Their structure makes these acids attractive on several

counts as intermediates in terpene and steroid biogenesis. Decarbox-
ylation, by removal of the carboxyl group which was originally present,
would regenerate DMA with retention of four of the five original
carbon atoms. The molecule becomes reoriented, and the carboxyl
group which was newly introduced will now be linked to the opposite
end of the original molecule. If the cyclic regeneration of DMA
occurred at a sufficiently rapid rate, then not only would the original
carboxyl carbons be lost entirely but at the same time the isopropyl
carbons 4 and 4′ would become equilibrated with carbon atom 2 (see
Fig. 3, reactions 2 to 6). In this event the labeling pattern in the
eventual product of isoprene synthesis, e.g., cholesterol, should not be
specific, i.e., it should be identical with that given by 2-C^{14}-acetate.
This has actually been found to be the case with 4,4′-C^{14}-isovaleric
acid.

It should be emphasized perhaps that in the conversion of acetate
to terpenes and steroids, dimethylacrylic acid need not be a direct
intermediate; possibly it joins the main synthetic path merely by
virtue of its conversion to methylglutaconic acid, and normally this
dicarboxylic acid is formed chiefly from acetate and acetoacetate by
way of hydroxymethylglutarate. The obvious reason why the discus-
sion has nevertheless centered around dimethylacrylate is its superi-
ority over other branched-chain acids as a precursor of cholesterol.

The arguments presented so far imply that DMA (or a coenzyme
derivative) is the monomeric unit which enters into the synthesis of
polyisoprenoid chains. This reaction would be analogous to the β-
ketoacyl condensation which is the well-established mechanism for the
synthesis of the straight-chain aliphatic acids. One may question,
however, the likelihood that isopropylidene groups are sufficiently re-

$$\begin{array}{c}
CH_3 \\
\diagdown \\
C{=}CH{-}COR + H_3C{-}\overset{|}{C}{=}CH{-}COR \rightarrow \\
\diagup \qquad\qquad\qquad\quad | \\
CH_3 \qquad\qquad\qquad\quad CH_3
\end{array}$$

$$\begin{array}{c}
CH_3 \\
\diagdown \\
C{=}CH{-}COCH_2\overset{|}{C}{=}CH{-}COR \\
\diagup \qquad\qquad\qquad | \\
CH_3 \qquad\qquad CH_3
\end{array}$$
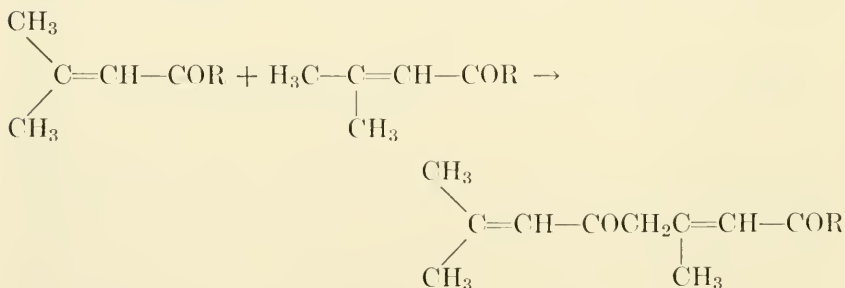
active to enter into a condensation of this type. By contrast the
methylglutaconic group possesses a methylene group which should be

$$\underset{\overset{|}{CH_3C}=CH-COR}{CH_2COOH} + \underset{\overset{|}{CH_3}}{\overset{COOH}{H_2C}-C=CH-COR} \rightarrow$$

$$\underset{\overset{|}{CH_3C}=CH-CO-CH-C-CH-COR}{CH_2COOH \quad COOH} \xrightarrow{-2CO_2}$$
$$\overset{|}{CH_3}$$

$$\underset{\overset{CH_3}{/}}{\overset{CH_3}{\searrow}}C-CH-CO-CH_2C=CH-COR$$
$$\overset{|}{CH_3}$$
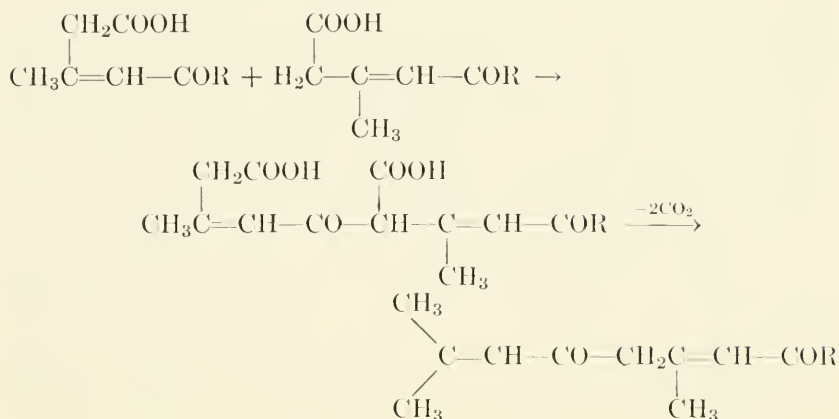
more favorable for reaction in a Claisen-type condensation. In this event the same C_{10} unit will be formed, but decarboxylation would follow or at least not precede the condensation step. It will be noted that the carbon atoms removed by the decarboxylation step will again be the original carboxyl groups of DMA. This second mechanism therefore accounts equally well for the experimental data obtained with 1-C^{14} DMA or isovaleric acid, but cyclic regeneration of a 5-carbon compound would not be obligatory.

The existence of methylglutaconic acid in two stereoisomeric forms is the second structural feature of particular interest for polyisoprenoid synthesis. The natural acyclic terpenes occur both in the *cis* (e.g., nerol and rubber) and *trans* forms (geraniol, gutta-percha, and squalene), and it is pertinent to ask at what stage of the overall synthetic process the geometric configuration of the respective products becomes established. If DMA were the condensing unit, the stereospecific step would follow the condensation reaction. On the other hand, the *cis* and *trans* isomers of methylglutaconic acid would constitute precursors which already possess the configuration of the end product. Present biochemical knowledge is too scarce to predict the nature of the stereospecific steps, although at least one prototype exists. Dehydration of hydroxy acids (malic, citric, and β-hydroxybutyric acids) is a well-established enzymatic reaction which affords geometric isomers, and hence the formation of methylglutaconic acid by elimination of water from hydroxymethylglutarate would not be without precedent. Which one of the two isomers will be formed cannot be predicted, nor can the possibility be ignored that the *cis* and *trans* forms are converted into each other enzymatically by a reaction analogous to the isomerization

of maleylacetoacetate to fumarylacetoacetate which Knox has described.[18]

It is particularly relevant to the present discussion that squalene has been shown to possess the all *trans* configuration.[19] The present hypothesis would therefore anticipate *trans*-methylglutaconate as a precursor of the triterpene and of the steroid derived from it. Two observations have been made which are consistent with the role assigned to the methylglutaconic acids. Rabinowitz and Gurin [20] have shown that hydroxymethylglutarate is dehydrated by liver preparations, and they state that *trans*-methylglutaconic acid is the product. Second, the *cis* form of the same acid has been tested in our laboratory as a cholesterol precursor and found to be inactive. Neither of these two observations are conclusive by themselves, but, taken together with the behavior of DMA as discussed earlier, they strengthen the case for a pivotal role of the *cis* and *trans* isomers of the methylglutaconic acids in terpene and steroid biogenesis.

Though the main object of this essay has been to indicate current lines of thinking and investigation in a selected area of terpene and steroid biogenesis, it has been the intent to look also briefly at the origin of the branched-chain amino acids. Comparison of the two areas of biosynthesis raises the interesting point why higher animals can synthesize one type of carbon chain, but not another closely related one.

Acetyl units are not the precursors of the branched portions of valine and leucine.[21] Instead, it appears that the carbon skeletons of these amino acids are formed from two molecules of pyruvate by a mechanism which may involve an acyloin condensation to acetolactate and a subsequent pinacol rearrangement to an isovaleric acid derivative.[22, 23] There is also evidence that one of the immediate valine precursors, after condensation with an acetyl unit and decarboxylation, furnishes the carbon chain of leucine.[24] It is of particular interest for the pur-

$$
2CH_3CO{-}COOH \rightarrow \underset{\underset{\underset{CH_3}{|}}{\overset{\displaystyle CO}{|}}}{\overset{\overset{\displaystyle OH}{|}}{CH_3C{-}COOH}} \rightarrow \underset{\text{Valine precursor}}{\overset{\overset{\displaystyle OH}{|}}{(CH_3)_2C{-}CO{-}COOH}}
$$

$$
\underset{}{\overset{\overset{\displaystyle OH}{|}}{(CH_3)_2C{-}CO{-}COOH}} + CH_3COOH \xrightarrow{\;-CO_2\;} \text{Leucine precursor}
$$
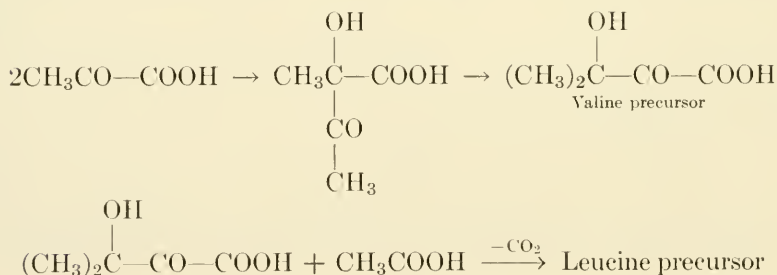
poses of the present discussion that in the synthesis of the carbon
chains of valine and leucine pyruvic acid does not serve as a source
of acetate (or acetyl CoA) but enters as such into the condensation
reactions.

Leaving aside details of the synthetic mechanisms, we may compare
the structures of two of the 5-carbon compounds which have been
implicated in steroid biosynthesis (IV and V), and hence are formed
in the animal body as well as in the plant and microbial cell, with two
precursors of the amino acid valine (VI and VII):

$$
\begin{array}{cc}
\underset{\underset{\displaystyle CH_3}{\diagdown\diagup}}{\overset{\displaystyle CH_3}{}}C{=}CH{-}COOH & \underset{\underset{\displaystyle CH_3}{\diagup}}{\overset{\displaystyle H_3C\ \ OH}{\diagdown\,|}}C{-}CH_2COOH \\
\text{IV} & \text{V} \\[2em]
\underset{\underset{\displaystyle CH_3}{\diagup}}{\overset{\displaystyle H_3C\ \ OH\ \ OH}{\diagdown\,|\quad\ |}}C{-}C{-}COOH & \underset{\underset{\displaystyle CH_3}{\diagup}}{\overset{\displaystyle CH_3}{\diagdown}}CH{-}CO{-}COOH \\
\text{VI} & \text{VII}
\end{array}
$$
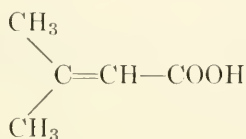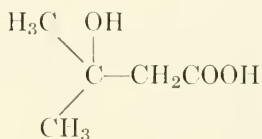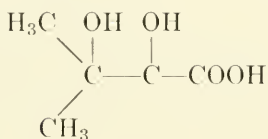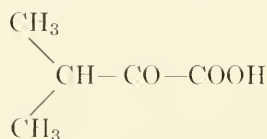
Clearly a transformation of IV or V, to VI or VII does not occur in
the animal body, or else valine would not be an essential amino acid.
The action of crotonase, which catalyzes the addition of water to α,β
unsaturated acids of type IV is evidently restricted to the formation
of β-hydroxy acids, because the α-hydroxy acid, if formed, should be
readily oxidized to α-ketoisovalerate, a compound which is converted
to valine in the animal body. One may argue then that as a result
of the specificity of crotonase the synthetic pathways leading to
isoprenoid intermediates are useless as far as valine and leucine syn-
thesis are concerned, and that as a consequence a synthetic pathway
for these amino acids has evolved that is distinct both with respect
to the mechanism of condensation and with respect to the carbon
sources which it uses.

It appears to be equally true that the valine precursors VI and VII
are not convertible to IV and V since in microorganisms such as *Neuro-
spora* the sole carbon source for ergosterol is acetate, which is used for
the synthesis of IV and V but not of VI and VII. Thus, even in the
absence of the restrictions which exist in the higher animal, the separa-
tion of synthetic pathways for two closely similar structures is rigidly
maintained.

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The Biochemistry
of Lysogeny

ERNEST BOREK

Hope, rather than experience, prompted the title of this essay. "Chemical gropings in lysogeny" would more accurately describe the writer's contribution to this engrossing biological phenomenon. The bold, perhaps brash, title thus merely serves to delineate an ultimate goal: an understanding of the lysogenic mechanism at the molecular level. Such a goal may remain asymptotic to several generations of biochemists; nevertheless, the writer believes that biochemists can already occupy themselves fruitfully with this phenomenon not only with the aim of contributing to an understanding of lysogeny but, equally, with the hope that a study of lysogeny will contribute to our store of knowledge of biochemistry. A paramount problem in biochemistry today is the elucidation of the structures of macromolecules and their correlation with biological function, including the replication of those macromolecules. The writer feels that such problems can be more fruitfully approached at present from a study of the biological functions of the simplest of the self-reproducing systems than from the application of the tools of the organic chemist to isolated fragments of cellular mechanisms. In other words, biological function can reveal chemical structure and mechanism, but the process, at the level of macromolecules, is well-nigh irreversible at present.

If the reader demands evidence of this, let him consider how little biochemistry has contributed to a knowledge of the mechanism of genetics and how much microbial genetics has contributed to our understanding of intermediary metabolism. Indeed, even in classical organic chemistry, function has been the key to structure and not the reverse. Long before the elucidation of organic structures from X-ray analysis was dreamed of, Kekule derived the structure of the benzene ring from its functions, from its behavior during substitution reactions.

Before some of the biochemical objectives in the field of lysogeny can be stated, the biological phenomenon should be described. Fortunately, the reader can be referred for the history of the problem and for a summary of the brilliant recent work, mostly by Lwoff and his school, to a masterly review by Dr. Lwoff himself.[1]

We need, therefore, give only a sketchy outline of the biological phenomenon. A wide variety of microorganisms carry, hereditarily, the seeds of their own and of other bacteria's destruction, either within, or closely associated to their genetic material. Under the influence of random metabolic or physical stimuli the metabolism of such organisms can undergo a profound shift, producing seemingly de novo bacteriophages, which then emerge from the crumbling hulk of the host cell. From this stage on these phages of lysogenic origin apparently differ in no way from other free virulent bacteriophages: their cycle of replication takes place within susceptible host cells which they infect by invasion.

No chemical investigation of the phenomenon was possible as long as we were limited to the spontaneous rate of occurrence of the lysogenic phenomenon. Since the frequency of the occurrence of phage development and lysis in a lysogenic culture is low (1%, or less), any chemical study was precluded by the dilution of the object of the study by its stable, colony-mate cells.

A profound discovery by Lwoff allowed a complete reversal of the above ratio in some lysogenic strains. If cultures of some lysogenic bacteria are exposed to small doses of ultraviolet or X radiation, or to some mutagenic or carcinogenic agent, the fraction of organisms in which the prophage * is induced to proliferate into mature bacteriophage approaches unity. It should be emphasized that not every strain of lysogenic organism yields to the inducers listed above.

The Lwoff effect is an almost startling phenomenon. If a milky culture of *Bacillus megatherium* containing 10^8 viable cells per milliliter is exposed to a small dose of ultraviolet irradiation and then incubated in the dark, the suspension begins to clarify after about 60 minutes and within a few minutes the culture becomes water clear. At the same time, it can be demonstrated, by plating for plaques on a sensitive strain of bacteria, that concomitant to the lysis there is a large increase in the free phage titer.

How can one begin to make a dent in a problem such as this with the tools of the chemist? The great Hopkins [2] gave sound advice on

* Dr. Lwoff's term for the "form in which lysogenic bacteria perpetuate the power to produce phage."

this point. He said that biochemists should strive to be biologists as well as chemists to justify their special designation, for, whereas the chemist is best provided with the machinery for the cultivation of the borderland frontier between chemistry and biology, it is the biologist who best knows the lay of the land.

The next best thing to becoming a biologist is to stick close to one.

Lwoff observed very early that the physiological condition of the lysogenic organism at the time of irradiation determined the extent of the lysogenic response. Organisms which were on a glucose starvation regimen prior to the irradiation seemed to have acquired a resistance to the irradiation. Whereas in logarithmic growth phase only 1% of lysogenic *Escherichia coli* K_{12} will survive a given dose of irradiation as colony formers, about 75% of the same organisms will survive the same dose if the organisms are deprived of glucose for 3 hours prior to the irradiation. The yield of free phage is, of course, proportionally diminished. The organisms have become "inapt." The acquisition of inaptitude by starvation is general for all inducible lysogenic organisms, nor is it restricted to glucose starvation. Nitrogen or specific amino acid starvation in the presence of ample glucose confer inaptitude on lysogenic organisms as well.[3,4] A typical experiment on the development of inaptitude on methionine starvation in a lysogenic, methionine-requiring auxotroph, *E. coli* K_{12} W-6 (isolated by Dr. J. Lederberg), is given in Fig. 1.

Inaptitude, though it is only a peripheral problem related to lysogeny, can be submitted to chemical scrutiny. The known effects of starvation could be probed seriatim as possible sources of inaptitude. In turn, should the search for the mechanism of inaptitude be successful, the findings might not be without bearing on the mechanism of induction, the ultraviolet-irradiation-induced proliferation of the otherwise stable prophage. For often, an understanding of a block to a biochemical mechanism has been revealing of the mechanism itself.

With this, perhaps naive, overall plan in mind, some experiments were devised. In the first place we had to explore, and preferably rule out, the unfruitful possibility that inaptitude is merely the result of the diminished metabolism of starvation. *E. coli* K_{12} in logarithmic growth phase was rapidly chilled to 3°C., kept at that temperature for as long as 20 hours, and then subjected to a normal inducing dose of irradiation at 3°. When they were returned to the warm room such cultures gave the usual lysogenic response. Cultures whose growth was inhibited by an antimetabolite, such as ethionine, also showed but little

change in aptitude. A diminished rate of metabolism at the time of irradiation, whether induced by chilling or by a metabolic inhibitor, was thus ruled out as a possible source of inaptitude.

Next to be explored was the possibility that something accumulates during the aberrant metabolism of starvation which somehow inhibits the lysogenic response. To test this possibility the methionine-requir-
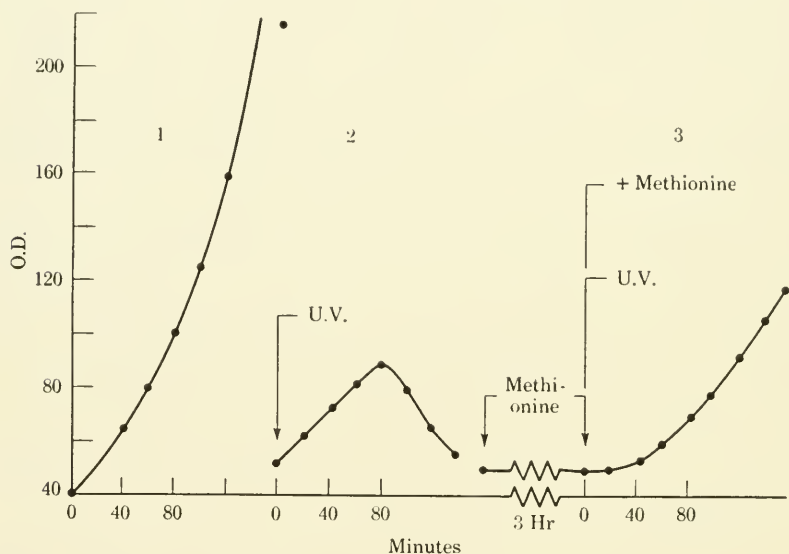
Fig. 1. The effect of starvation prior to irradiation on the lysogenic response. Curve 1 = E. coli K_{12} W-6 in logarithmic growth phase. Curve 2 = same after induction by ultraviolet. Curve 3 = the organisms irradiated after 3 hours of starvation of methionine. The amino acid was, of course, added, after the irradiation.

ing auxotroph E. coli K_{12} W-6 at a concentration of 10^9 cells per milli-liter was subjected to methionine starvation for 3 to 4 hours. After the starvation, the starved organisms were eliminated from the medium by high-speed centrifugation or by sterile filtration. Wild type of E. coli K_{12} in logarithmic growth phase, i.e., with full aptitude, were harvested and suspended to a concentration of 10^8 cells per milliliter in the same sterile filtrate in which the auxotrophs had starved. Such a filtrate should have been an adequate culture medium for the wild type of organism. Indeed, that is precisely the medium on which the wild type of organism is grown. However, even after an immersion of only 5 minutes, the minimum time dictated by the technique, the

aptitude of the organisms was diminished. After prolonged immersion, the inaptitude became more pronounced.[5]

The starvation medium was found to be at pH 5, but the hydrogen-ion concentration could not have contributed to inaptitude for it was found that the pH of cultures in logarithmic growth phase could be lowered to 5 with HCl and, even though the organisms were kept at that pH for 3 hours, their aptitude was not suppressed. A variety of acidic metabolites which *might* have accumulated into the starvation medium were tested for their effect on aptitude. Fumaric acid at a concentration of 0.005 M suppressed aptitude, provided the pH was kept at 5. An assay of the starvation medium for fumaric acid by Racker's method indicated the absence of the acid at anywhere near the effective concentration. Nevertheless, the suppression of the aptitude by fumaric acid was studied in some detail; it did not appear to consist merely of a screening of the irradiation, for it was highly pH dependent and quite specific: the higher homolog, glutaconic acid, was found to be ineffective.[6] Derivatives of fumaric acid which could not dissociate, i.e., the diamide, the diglycyl, and the diglutamyl derivative, were prepared, and it was found that these suppressed aptitude at pH 6.5 as well. Thus the low pH needed for fumaric acid to be effective as a suppressor of aptitude was necessary to repress the ionization of the acid rather than for any effect on the microorganism. It was found that all of the above fumaric acid derivatives are screening agents against irradiation if the irradiation is passed through them, via a quartz dish, when they are out of contact with the microorganism, but they are particularly potent in contact with the organisms. The phenomenon thus seemed to resolve itself into a curiously effective screening by these compounds (by concentration on the bacterial cell?) —and, since it appeared to be not related to aptitude, work on it was shelved.

On return to the study of the starvation medium itself, it was found that it absorbed very strongly at 260 mμ. A cursory examination by elution chromatography revealed the presence of a variety of nucleic acid fragments. The only similar observation we could find in the literature was a brief posthumous note by the late Dr. Marjory Stephenson who found in a study of autolytic ribonuclease in *E. coli* that acid-soluble phosphorus accumulated in the buffer medium in which the organisms had been suspended.[7] We therefore investigated the phenomenon in some detail to determine whether the excretion of nucleic acid fragments is a concomitant of all types of starvation, whether it is limited only to some bacterial species, or whether it is,

indeed, an excretion, or merely the oozings from dying cells and, finally, whether the phenomenon is an attribute of starving cells only.

Since the technique of the experiments bears directly on the answers to the above questions, the salient experimental procedures must be given. For the starvation experiments bacteria were raised on a synthetic medium to logarithmic growth phase from small inocula. They were harvested by centrifugation when they reached a cell count of not higher than 2×10^8 cells per milliliter and were resuspended after sterile washing in a fresh medium lacking the nutrient of which they were to be starved. When cellular concentrations higher than 2×10^8 cells per milliliter were desired, the bacteria were resuspended to an appropriately smaller final volume. This precaution is essential to insure that the experiments are performed with a bacterial population which is preponderantly viable and which approaches physiological homogeneity. For the study of the kinetics of the excretion, aliquots of these aerobically incubating, starving, bacterial suspensions were taken at intervals; the bacteria were eliminated by high-speed centrifugation, and the absorption at 260 mμ of the supernatant fluid was measured. At the same time, aliquots of the bacterial suspension were diluted appropriately for plating for viable cells. No change in the number of viable cells could be detected during the first 6 hours of starvation.

In Fig. 2 a study of the kinetics of the excretion of ultraviolet-absorbing substances by $E.\ coli$ K_{12} W-6 on methionine and on glucose starvation is represented. Analysis of the excreted products indicated the presence of free bases and of nucleotides, but the relative quantities were different on the two types of starvation.

There are several lines of evidence to indicate that the nucleic acid fragments which accumulate in the medium during the first 5 to 6 hours of starvation are excreted products rather than the accumulated debris from dying bacteria. The accumulation is considerably larger on methionine starvation than on glucose starvation, yet, on prolonged incubation, the bacteria remain fully viable for a longer time on methionine starvation. Moreover, comparison of the output of ultra-violet-absorbing material in a culture of 10^9 cells per milliliter with that in a culture of 10^8 cells per milliliter revealed that there was a greater output per cell at the lower concentration, but the measurable death rate, on prolonged incubation, was greater at the higher cell concentration. Finally, the kinetics of the excretion point either to an exhaustion of excretable products or to some equilibrium, for a

plateau is reached, a finding which could not be expected from a steady
death rate of the bacteria.

On the other hand, there is the possibility with respect to the last
point that a number of bacteria, too small to be detected by our
counting technique, die, and their oozed-out cell contents bring the
optical density of the medium to its high level in the first 4 to 5 hours
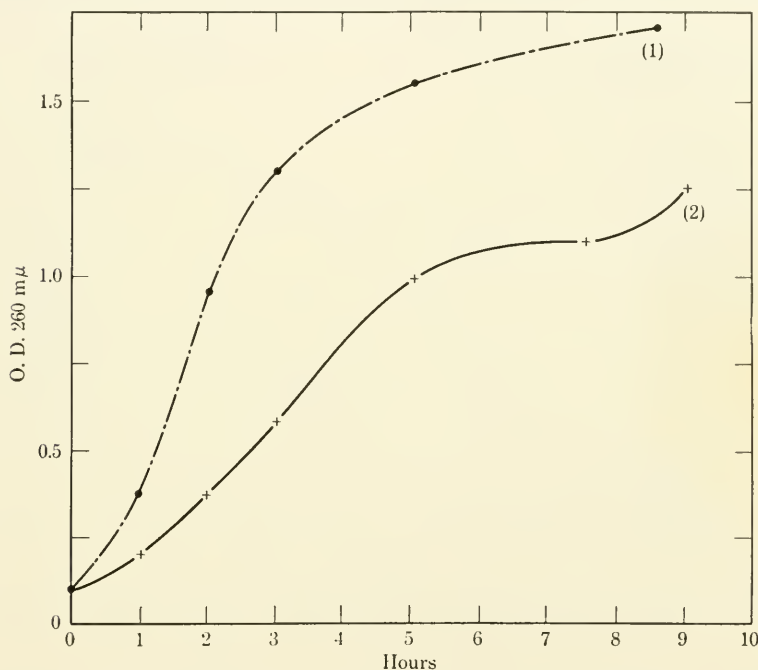
Fig. 2. The kinetics of the excretion of nucleic acid fragments by *E. coli* K_{12}
W-6 on (1) methionine and (2) glucose starvation. Cell count $1:1.2 \times 10^9$ cells
per ml.; $2:1.1 \times 10^9$ cells per ml.

of starvation. The diminished accumulation from this time on could
be visualized as resulting from the adaptation of the remaining viable
bacteria to their debris-laden milieu, and temporary equilibrium might
be reached between the rate of death and the rate of scavenging. The
following findings rule out this possibility. The probable error of
viable-cell counting in our hands is less than 10%. Therefore, a num-
ber of bacteria, 10% or less of the total, would, upon their death, have
to yield an optical density approximating the levels of curve 1 in Fig. 2.
Bacteria in logarithmic growth phase, at a cellular concentration of
1×10^8 per milliliter, were exposed to sonic vibration in a Raytheon

9KC sonic vibrator for 10 minutes. Less than 0.5% of the bacteria remained viable after this treatment. After centrifugation at 5000 r.p.m., the optical density of the fluids at 260 mμ was determined. It was 0.33, or, only about 20% of the optical density of the medium represented by curve 1, Fig. 2.

Of course, this argument excludes consideration of the greater specific absorption of nucleic acid fragments after enzymatic depolymerization.

Low glucose (0.5%)

Fig. 3. The accumulation of nucleic acid fragments in the culture medium of *E. coli* K_{12} in logarithmic growth phase on low glucose.

However, incubation at 38° of such sonically disintegrated cells for 5 to 10 hours produced only a 15 to 20% increase in optical density.

The excretion of nucleic acid fragments during starvation was found to be not restricted to lysogenic organisms, for *E. coli* B and B/r repeated the same pattern.

Since "abnormal" metabolism is often but an exaggeration of "normal" metabolism, we next studied whether bacteria in logarithmic growth phase excrete any nucleic acid fragments into the culture medium.

In Fig. 3 the growth curve of a bacterial culture with a limiting glucose concentration of 0.05%, from start through logarithmic phase to declining growth phase, along with the output of ultraviolet-absorbing substances (its ultraviolet-absorbing shadow, as it were), are given.

As the bacterial population reaches a stationary level upon the exhaustion of the carbon source, the accumulation of ultraviolet-absorbing substances also diminishes. This again is not a characteristic of lysogenic bacteria alone, for *E. coli* B under similar conditions of culture repeats this pattern.

That the excretion of ultraviolet-absorbing material per bacterial cell in a culture in logarithmic growth phase is quite constant is appar-
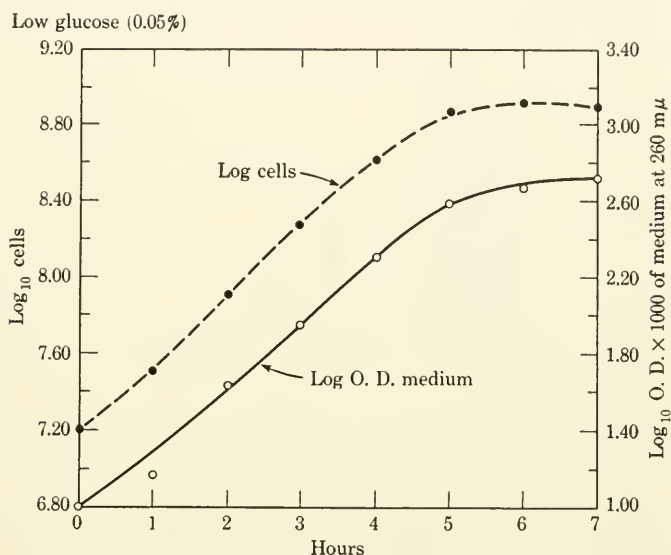
Fig. 4. Same data as Fig. 3 but plotting the log of O.D.

ent from Fig. 4. The plot of the log of the increasing bacterial population and of the log of the optical density of the culture medium are parallel. The constancy of the excretion was also shown by a different method. Cultures of *E. coli* K_{12} were kept in extended logarithmic growth phase at a concentration of 10^8 cells per milliliter by the constant dilution of the culture with fresh medium at 37° at a rate which doubled the volume of the culture per generation time. Cell counts and the optical density of the cell-free supernatant fluid were determined for several hours. In such experiments, the optical density at 260 mμ remained 0.07 with 10^8 cells per milliliter.

The nature of the substances which account for the ultraviolet absorption in the culture media of bacteria in logarithmic growth phase on low glucose was investigated. Paper chromatography of lyophilized concentrates revealed the presence of nucleic acid fragments and of

amino acids. However, the concentration of amino acids was too low to contribute significantly to the absorption at 260 mμ.

The origin of the nucleic acid fragments in the culture media is obscure. The accumulation may be the result of haphazard "leakage" or of the spillage of surplus synthesis. On the other hand, it may represent the microbial counterpart of the excretion of specific endogenous waste products of nitrogen metabolism in metazoa. Such
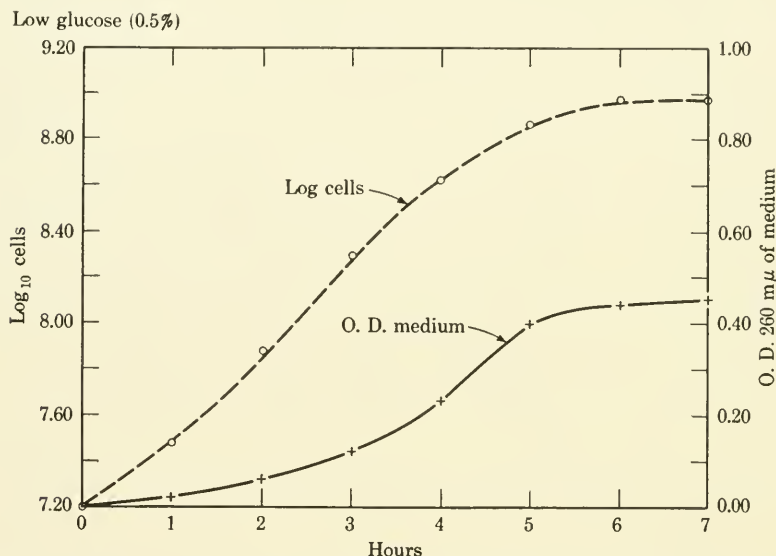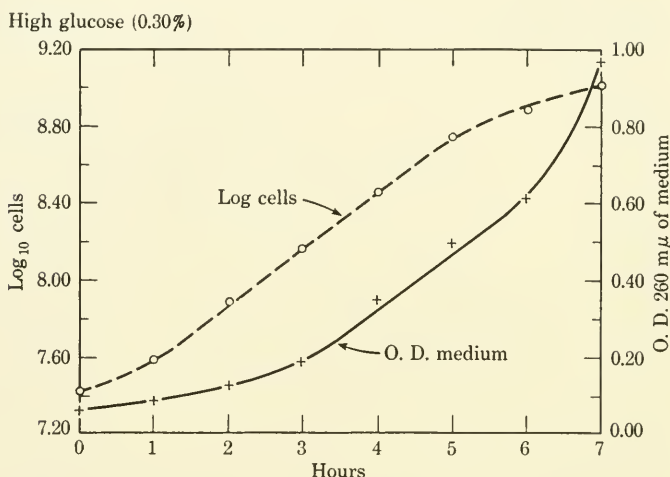

High glucose (0.30%)

FIG. 5. The accumulation of nucleic acid fragments in the culture medium of *E. coli* K_{12} in logarithmic growth phase on high glucose.

an interpretation might be based upon an inversion of Dr. J. Monod's aphorism on comparative biochemistry: "What is true of *coli* is true of elephants—even more so."

The final choice from among the above explanations must await the analysis of the excreted nucleic acid fragments from different microorganisms.

When the bacteria are cultured with the excess glucose concentration, of 0.3%, customarily used by bacteriologists, the pattern of accumulation in the culture medium is completely different (Fig. 5).

As the declining growth phase in the culture is reached, the output of ultraviolet-absorbing materials is substantially increased until it reaches twice the level of the cultures with the low glucose. The different levels of ultraviolet-absorbing substances in the two cases is a reflection of the different conditions which arrest the growth of the bacteria. With the lower glucose concentrations, growth stops upon

the exhaustion of the carbon source. The pH of such cultures even after 24 hours of incubation does not fall below 6.4. However, with the high glucose, the pH of the medium at the end of 7 hours is 5.5. The stationary population under these conditions is the result not only of diminished rate of cell division but of an increased death rate.

The two methods of culture yield similar bacterial densities, but the environment of the bacteria differ. These findings together with those on starving cultures point to the necessity of examining the culture
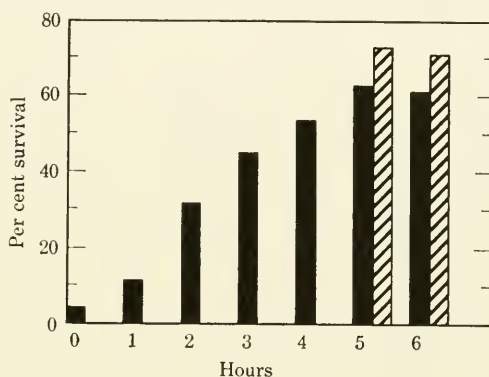
Fig. 6. The kinetics of the development of inaptitude on methionine starvation. Survivors were determined both in the original starvation medium (striped bar) and in fresh starvation medium (solid bar).

fluid in radiobiological studies of microorganisms for, under some conditions of culture, the medium may be laden with nucleic acid fragments and may have variable screening potency. For example, if *E. coli* B in logarithmic growth phase is centrifuged, and the cells are resuspended in a sterile filtrate of optical density 1.5 obtained from the experiment represented by curve 1, Fig. 2, and then exposed to 30 seconds irradiation from a G.E. 15-watt germicidal lamp at 1 meter distance, the survivors will be 70% higher than in a control in which the bacteria are resuspended in fresh culture medium and irradiated.[8]

However, screening by nucleic acid fragments in the culture medium has practically nothing to do with inaptitude in lysogenic organisms as is shown by the data presented in Fig. 6. In this quantitative study of the development of inaptitude on methionine starvation, the survival of the organisms from the same dose of irradiation in their original starvation medium and in fresh deficient medium was compared. Only after 5 hours of starvation were sufficient amounts of nucleic acid fragments accumulated in the medium to screen the radiation meas-

urably; there were 20% more survivors in an aliquot irradiated in its
starvation medium than in one suspended in fresh deficient medium.
Thus, under the conditions of these experiments, the culture of 10^8 cells
per milliliter being washed before the start of the starvation, nucleic
acid fragments in the medium contribute very little to inaptitude. In
the experiments in which we first demonstrated protection by starva-
tion media, 10^9 cells per milliliter were starved for 4 hours and, after
elimination of the organisms by sterile filtration, cells in logarithmic
growth phase were added to the filtrate to yield a final concentration
of 10^8 cells per milliliter. The protection by screening was thus more
pronounced.

However, it should be emphasized that the protection by the starva-
tion medium is not due merely to screening, for if such media are
added to bacteria *after* the irradiation they still suppress the induction:
the number of colony-forming survivors is increased five- to eightfold,
and there is a decrease in the number of infectious centers. The
starvation medium can be added, in a ratio of 1 part to 10, as late
as 20 minutes after the irradiation, and a significant reversal of induc-
tion is still measurable. The starvation medium has no effect on the
non-lysogenic *E. coli* B/r after its irradiation.

As yet we know no more about this induction-reversing agent than
that it is dialyzable and is labile to ultraviolet irradiation.

Since so much of nucleic acid fragments are excreted during starva-
tion the question naturally arises whether inaptitude may not be the
result of *intracellular* shading of radiation-sensitive loci by these frag-
ments as they flow towards the periphery of the bacterial cell. To
explore such a possibility, the nucleic acid content of the methionine
deficient auxotroph was studied after two types of starvation, glucose
and methionine.

For these studies 100-ml. aliquots of either glucose- or methionine-
starved organisms were centrifuged at intervals in a Sorvall angle
centrifuge at 5000 r.p.m. for 20 minutes. A cell count in the superna-
tant fluid revealed the presence of 1%, or less, of the original bacteria.
This finding, together with the finding that there was no change in the
number of viable, colony-forming bacteria during the course of the
starvations, insured the uniformity of sampling at various intervals.
The bacterial pellet containing 2 to 3×10^{10} colony-forming cells was
washed once with 0.9% saline, centrifuged, and subjected to analysis
for RNA, DNA, and dilute-acid-soluble fragments by the method of
Ogur.

In Fig. 7, a typical experiment on the relative changes in RNA and DNA and acid-soluble fragments on glucose starvation of *E. coli* K$_{12}$ W-6 are shown. Since the probable error of cell counting is 10%, the changes cannot be considered as significant except for the decrease in RNA and the increase in dilute-acid-soluble fragments after 4 hours of starvation. The stability of the nucleic acid levels on glucose starvation may be related in this mutant to the presence of excess methionine in the medium or to some metabolic products resulting from its genetic deficiency.
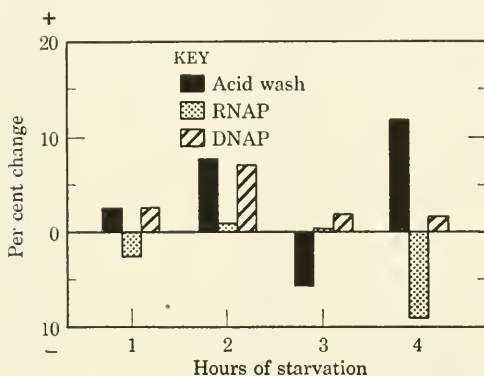
FIG. 7. Relative changes in nucleic acid content on glucose starvation of *E. coli* K$_{12}$ W-6.

In Fig. 8, the results of a typical experiment on methionine starvation of *E. coli* K$_{12}$ W-6 are summarized. There was no detectable change in the number of viable cells throughout the 6 hours of starvation. There was about a 30% increase, however, in the turbidity of the cell suspensions, indicating either an increase in cell size or a change in the light-scattering property of the starved cells. The RNA, DNA, and acid-soluble fragments are represented in the figure as changes from the values found when the organisms were harvested from logarithmic growth phase and resuspended in the medium lacking methionine. No unequivocal interpretation can be offered for the 17% increase in total DNA during the first hour of starvation. It may represent increased DNA per cell, or it may be the result of a correspondingly increased cellular population, from the utilization of residual intracellular methionine, during the first 15 to 20 minutes of incubation in the methionine-deficient medium. The magnitude of the increase in DNA is not quite double the magnitude of the error of cell counting under the best conditions, and, unfortunately, cell counting during the first 30

minutes after the resuspension of a centrifuged bacterial colony yields erratic results due to clumping of cells. However, the cell count remains constant from one-half hour to 6 hours, and longer, on methionine starvation, and, therefore, the increase in acid-soluble material and in RNA which continue significantly after the first hour represents
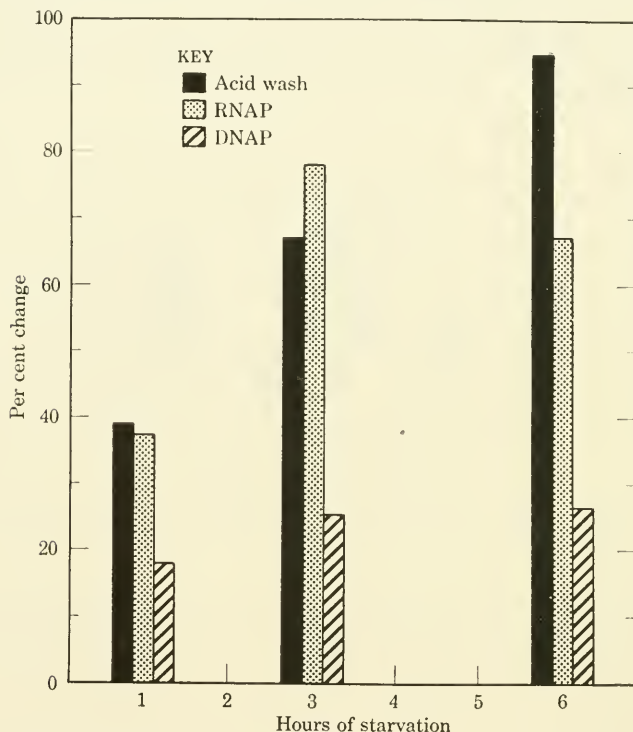
FIG. 8.   Relative changes in nucleic acid content on methionine starvation of
$E.\ coli$ K$_{12}$ W-6.

unequivocally an increase of these components within each starved cell. The large increase in nucleic acid material without increase in the number of viable cells in these starving cultures was confirmed by a simple independent method. A 10-ml. aliquot containing 2.8×10^8 cells per milliliter at the start of the starvation was centrifuged, washed, resuspended in 10 ml. of fresh medium, and the cell suspension was exposed to ultrasonic vibration for 10 minutes. The optical density at 260 mμ of the resulting clear fluid was 0.8. After 4 hours of starvation an identically treated aliquot yielded an optical density of 1.2, or an increase of 50%.[9]

Electron-microscope photographs of this starved mutant revealed enlarged, often misshapen, cells loaded with electron-dense material.

The increase in nucleic acid material on methionine starvation in *E. coli* K_{12} W-6 parallels similar changes in microorganisms when they are exposed to drastic shocks. Mitchell reported an increase in the free nucleotide content of bacteria after the "initial attachment of penicillin to growing cells."[10] Park and Johnson showed that in *Staphylococcus aureus* in presence of 0.1 of a unit of penicillin per milliliter there is about a 40% increase in RNA in 65 minutes with no concurrent change in the cell count.[11] Kelner has noted that there is a marked increase in RNA in *E. coli* B/r following a dose of ultra-violet irradiation which prevents 90% of the organisms from giving rise to visible colonies.[12]

However, the large increase solely in RNA content, upon starvation, seems to be unique to this auxotroph. The appropriate auxotrophs of *E. coli* K_{12} when starved of histidine, tryptophane, and leucine, respectively, yielded values similar to those obtained upon glucose starvation of *E. coli* K_{12} W-6.

We also studied a methionine-requiring mutant of the W strain of *E. coli* which was kindly supplied, as were those listed above, by Dr. Bernard Davis. The W strain is lysogenic but, unlike the K_{12} strain, the frequency of the occurrence of the phenomenon in a given population cannot be increased by radiations or other inducing agents. The methionine-requiring auxotroph of the W strain, W 122–33, appears to have a genetic block analogous to that of *E. coli* K_{12} W-6, as far as this can be determined by the probably not-too-discriminating technique of the determination of accumulated metabolic precursors. This putatively analogous mutant of the W strain did not accumulate nucleic acid material on methionine starvation.

The unique ability of *E. coli* W-6 to synthesize RNA independently of DNA and of protein (for there was no increase in total protein content) may be put to use to study the relations among the syntheses of these three entities, but here we are concerned only in what this mutant may have contributed to an understanding of inaptitude. The two types of starvation, glucose and methionine, have parallel effects on inaptitude, but, by rare chance, they have divergent effects on the nucleic acid content of the starved cell. Intracellular screening by nucleic acids or fragments thus appears to be an unlikely mechanism for inaptitude.

We are thus back where we started. We learned a bit about nucleic acid metabolism, but apart from the essentially negative contribution

that inaptitude is not caused by screening we know nothing more about it. However, the preoccupation with the mechanism of excretion led to the next working hypothesis. We considered the possibility that inaptitude is the result of the loss during starvation, either by excretion or by enzymatic disposal, of some radiation-sensitive locus within the
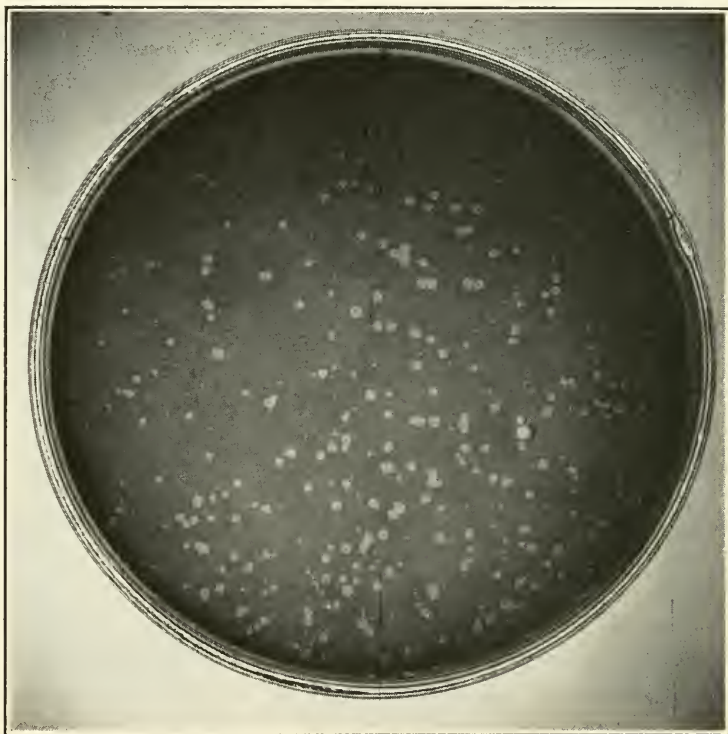
Fig. 9(a). Infectious centers in *E. coli* K_{12} incubated with irradiated leucovorin at a dilution of 2×10^{-4}.

cell. That there is a specific site, an Achilles' heel, as it were, upon which the inducers fall in a lysogenic organism has a compelling plausibility. How else could we account for the homogeneity of the lysogenic response to induction? It must be recalled that close to 100% of an inducible organism will, in logarithmic growth phase, consistently respond to the small inducing irradiation by the proliferation of bacteriophage. The phenomenon is quite different from the mutagenesis induced by irradiation. Irradiation for mutagenesis must be, by comparison to that for induction, prodigious, killing over 99.9% of the

cells, and the surviving "biochemical cripples" run the gamut of known and unknown genetic deficiencies. Of course, the captious may argue that there is an equally homogeneous response in such experiments, too, since 99% of the bacteria die. Most likely, however, the cause of their death is not as homogeneous as in induced lysogenic organisms.

FIG. 9(b). Untreated control at a dilution of 10^{-4}.

Since the lysogenic response to induction is so uniform the development of the phage could be more plausibly visualized as resulting from some metabolic shift in a delicately adjusted equilibrium rather than from random lesions in the genetic material. (The profound change in nucleic acid metabolism induced by invasion of external phage, cessation of RNA and increase of DNA synthesis,[13] does not take place during phage development in induced lysogenic cultures. Neither RNA nor DNA synthesis is interrupted after induction by irradiation.[14])

We first considered the possibility that inaptitude results from the loss during starvation of some radiation-sensitive metabolite which is

needed to mediate the metabolic effect of irradiation. We must admit that we know of no evidence for the existence of a radiation-sensitive cofactor of induction. The hypothesis is frankly rooted in heuristic opportunism: nothing much could be done chemically with an hypothesis postulating some subtle change in a macromolecule during starvation.

We therefore started a search for a metabolite which, upon irradiation, might act as an inducer on unirradiated $E.$ $coli$ K_{12}, either in logarithmic growth phase, or starving. We irradiated strongly a variety of metabolites (purines, pyrimidines, nucleosides, nucleotides, vitamins, and coenzymes) and then incubated $E.$ $coli$ K_{12} in logarithmic growth phase with the irradiation products. We tested the irradiation products for their toxic effects on the organisms by counting surviving cells, and for their inducing effect by assaying on a sensitive strain for an increase in the normal background of infectious centers.

We found that several metabolites upon strong irradiation are converted into products toxic to the bacteria. Some of these had been described in the literature; some had not. But of all the irradiated metabolites only leucovorin, and its derivative anhydroleucovorin, acted as inducers. Such an experiment was performed as follows: Five hundred micrograms of anhydroleucovorin or calcium leucovorin tetrahydrate per milliliter in the usual synthetic medium was irradiated in a 10-ml. lot in a quartz petri dish for 15 hours with a 15-watt G.E. germicidal lamp. $E.$ $coli$ K_{12} in logarithmic growth phase was centrifuged, and the cell clump was resuspended in the irradiated medium to a concentration of about 10^8 cells per milliliter. The culture was then incubated in the dark for 50 minutes, and then appropriate plating was performed for the determination of surviving colony formers and of infectious centers.[15] In Fig. 9 the photographs of the result of such an experiment along with that of an untreated control at twice the concentration are given.

The irradiation-elicited inducing potency resides in the pteridine moiety of leucovorin, since 2-amino-4-hydroxy-5-formyl-6-methyl-5,6,7,8-tetrahydropteridine [16] (by analogy to folic acid a plausible first cleavage product of leucovorin [17]) is converted by 5 hours of irradiation into an inducer, but $para$-aminobenzoylglutamic acid is not.

In Table 1 characteristic data are presented. It should be emphasized that the induction by the irradiated products is only partial. Full induction, by 100 seconds irradiation, would induce, under these conditions, about 60% of the cells. No higher induction could, as yet,

Table 1. The Inducing Effect of Irradiated Leucovorin on _E. coli_ K_{12}

Number	Experiment	Cells per Milliliter at Start	Surviving Cells per Milliliter after 50 Minutes	Induced Cells (Large Plaques) per Milliliter after 50 Minutes
1	_E. coli_ K-12 control	2.0×10^8	3.3×10^8	2×10^5
2	Exposed to 15 seconds direct irradiation	2.0×10^8	2.1×10^8	6×10^6
3	Incubated with 500 μg/ml. of irradiated leucovorin	2.0×10^8	2.4×10^8	5.4×10^6
4	Incubated with 500 μg/ml. of irradiated 2-amino-4-hydroxy-5-formyl-6-methyl-5,6,7,8-tetrahydropteridine	1.9×10^8	2.7×10^8	1.1×10^7

be achieved, because the photolytic products, unlike their unirradiated precursors, inhibit, above a concentration of 500 micrograms per milliliter, the bacterial respiration essential for phage development. Whether induction approximating the direct irradiation of the bacteria can be obtained with the photolytic products will depend upon whether a separation of the inducing and inhibitory potencies can be effected.

The induction by the photolytic products, unlike the mutagenesis by irradiated complex media reported by Wyss [18] and collaborators, is not caused by accumulated hydrogen peroxide. The concentration of hydrogen peroxide after irradiation in the inducing solutions is too low by a factor of 10 to affect _E. coli_ K_{12}. Moreover, induction by hydrogen peroxide is, of course, completely suppressed by catalase, whereas solutions of the photolytic products of leucovorin are completely unaffected by this enzyme.

Since attempts at the identification of the inducer are just starting, one can speculate freely on its nature, untrammeled by the intrusion of obstreperous facts. An organic peroxide is one possibility, since some peroxides are known to act as inducers in lysogenic organisms. The sequence of reactions during the photolysis of folic acid is well known: cleavage occurs at the 6-methylene link, the methylene carbon being oxidized to an aldehyde, yielding 2-amino-4-hydroxy-6-formyl-pteridine. On further irradiation this compound is oxidized to the corresponding 6-carboxylic acid.[17]

Leucovorin differs from folic acid in containing a reduced ring system and a formyl group in the 5 position. By, perhaps oversimplified analogy, one might expect both the 5 and 6 carbons in leucovorin to be involved in oxidation, possibly yielding a peroxide. At any rate, the presence of the formyl group of leucovorin is essential, for the reduced form of folic acid, tetrahydro folic acid, does not yield an inducer on irradiation; nor does 10-formyl folic acid.

However, if the inducer is a peroxide it is an unusually stable one;

it retains most of its activity at 38° for 24 hours, and it is not inactivated by glutathione or ascorbic acid.

Unfortunately, we could not determine unequivocally whether this organic inducer could, in contrast to direct irradiation, induce bacteria which had been rendered inapt by starvation. The photolytic products are effective only if they are in contact with the organism for 40 to 50 minutes. At the same time the lacking nutrient must be provided to enable the induced organism to elaborate phage and the organisms are known to regain aptitude under these conditions.

Although we pay constant heed to the devil's advocate who persistently insinuates that the unique effect of irradiated leucovorin is an artefact, unrelated to true induction, there are circumstantial indications which entice one to pursue the study of the phenomenon: leucovorin is heavily implicated in purine metabolism [19] and the photolytic product of folic acid, 2-amino-4-hydroxy-6-formylpteridine, is known to be an extremely potent inhibitor of xanthine oxidase.[20] At any rate, the phenomenon may not be without value as a possible tool to explore induction: the effect of the photolytic products on isolated enzyme systems can be studied, and its possible concentration in some component of the cell can be explored.

There are, of course, a host of possible mechanisms for the development of inaptitude other than the loss of a radiation-sensitive cofactor of induction. However, we shall refrain from listing any of these. The writer is shackled by his own injunction to overly imaginative students: fruitful biochemical speculation must stand on a bifurcated root, one reaching into the biological phenomenon, the other into the store of immediately applicable methods of chemical exploration; otherwise such speculation is not biochemistry. Unfortunately, we have not been able to nourish the second root too well.

We must finish this essay, as we started, on hope, a hope that the goal may be reached when a detailed series of reactions can be written for the chemical events that are precipitated when an inducible lysogenic organism is exposed to a packet of inducing energy. The writer is conditioned to grope for such a goal, for he belongs to what he likes to call the Hudson River School of biochemistry. Should the name the Hudson River School evoke in the reader's mind an association with the school of painting of the same name, the writer would be neither surprised nor displeased. For it must be recalled that the painters of the Hudson River School painted every tree, every twig, every leaf into a landscape. So, too, the school of biochemistry founded by the man to whom this book is dedicated strives for the

precise determination, atom by atom, of the molecular structure and the molecular function of the components of the living cell. Whereas slavish devotion to minutiae in a painting may be aesthetically questionable, the inspired unraveling of molecular anatomy and molecular physiology by the students from Dr. Clarke's department has proved to be richly rewarding.

The reader must find examples of the shining products of this school in other essays in this book, but it is hoped that this one may not be without some value as an example of how to—or how not to—grope in a brand-new field.

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The Development of a Plasma Volume Expander

MAX BOVARNICK and **MARIANNA R. BOVARNICK**

The innate and apparently incurable predilection of man for the "letting of blood" in one form or another has presented to the medical fraternity one of its oldest therapeutic problems—the problem of finding a substitute for whole blood or its essential components. According to no less an authority on the matter than the biblical record of human history, within the first generation of his appearance on earth man had already succeeded in converting his plowshare into a sword with which he eliminated one-third of the male population of the period. This sort of thing has been going on in one form or another ever since, and constant improvement of human knowledge over the centuries has in no way diminished the magnitude of the problem. On the contrary, modern surgical practice requires the replacement of enormous amounts of blood, and the unbelievable improvement in the science of warfare has threatened to raise the above-mentioned annihilation rate of 33.3% to nearly 100%. It is thought that blood or plasma volume replacement on sufficient scale may possibly help to reduce the latter figure.

Therefore, although it is perfectly obvious that no substitute for blood is likely to be as good as blood itself, the imminent potential magnitude of the replacement problem has stimulated efforts to provide a substitute for plasma in those instances where an insufficient volume of circulating blood threatens to effect vascular collapse and death and where the oxygen-carrying capacity of red blood cells is not needed.

As a result of the extensive experience gained in World War II it has been possible to formulate a set of criteria for any satisfactory plasma substitute. These are as follows: The material must primarily be able on injection to expand plasma volume by remaining in the blood stream a suitable length of time and exerting an oncotic pressure

in the same manner as does the plasma protein. Although opinion may vary as to the optimal length of stay of the substitute in the blood stream, it is generally agreed that a half-life of 12–20 hours is satisfactory; i.e., 50% of the injected plasma substitute solution is to remain after 12–20 hours. A corollary requirement is that the material must not remain in the blood stream indefinitely or be stored in the tissues indefinitely, as it is then liable to give rise to undesirable reactions. Having served its purpose for the time stipulated, it should be excreted or metabolized, preferably the latter if in the process of metabolism it can serve as a source of nutritive energy or as suitable building material for protein lost in the original trauma. The material must not be toxic or produce any undesirable physiologic response such as hypotensive action. It must be non-pyrogenic and non-antigenic. It must not interfere with the clotting and other hemostatic properties of the blood, and it must not be harmful to any of the formed elements of the blood. It should not materially increase the viscosity of the blood.

In addition to these physiological criteria certain physical and chemical requirements are imposed by considerations of practicality. The material must be readily and cheaply available in large quantities. It must be easy to sterilize, stable to conditions of long storage and climatic extremes of heat and cold, must not gel at low temperatures, and if possible should be transportable in minimal bulk volume.

During the course of two world wars many substances have been proposed and tried, which have met the above criteria with varying degrees of satisfaction.

Though the subject is too familiar and elementary to warrant any detailed presentation of these materials, a brief review may serve to provide background for further advances in this field.

Chemically most of the materials proposed and used fall into two general classes: they are either polysaccharides or proteins. Of the latter class human serum albumin or human plasma itself should obviously provide the best substitute therapy for loss of plasma. This, indeed, they do, but there are certain drawbacks connected with their large-scale massive use. The first of these is connected with the amount that might be required in military or mass disaster. It is estimated that an average of some 30–40 units (500 cc. per unit) would be required to treat the average case of severe burn. Requirements for other types of fluid loss or shock vary with the nature of the condition but are generally less than this amount. It is evident then that an enormous amount of human blood would be required to stock-

pile plasma or its derivatives for a major disaster. There is the further difficulty that there is a significant deterioration rate for stored plasma.

A still further major difficulty relates to the problem of processing plasma. This is due to the fact that a certain percentage of the donor population are carriers of the virus responsible for producing hepatitis. It is obvious, then, that any pool of plasma enormously increases the chances of spreading this disease. Thus far no practical effective method has been found of inactivating this virus or of removing it from plasma or its fractions. At best it is possible to minimize the dissemination of this disease by processing and distributing plasma in single units rather than in pools. For these various reasons the problem of finding effective plasma substitutes that could be used as plasma volume expanders has arisen.

Among the various proteins that have been tried are bovine albumin, gelatin, isinglass, globin prepared from erythrocytes of man, and hemoglobin. Attempts to despeciate various non-human proteins, such as bovine albumin and isinglass, have not been successful in that effective despeciation can only be achieved by means which are so drastic as to cause extensive breakdown and loss of necessary molecular size. Human globin, although free of the objectionable antigenicity or other toxic properties, seems to be non-effective in the treatment of shock probably because of rapid excretion. Hemoglobin, similarly, is unsatisfactory.

Gelatin has been accepted but is not entirely satisfactory for wide use because those preparations which are of proper molecular size in relation to retention in the blood stream are apt to gel at low temperatures. Oxypolygelatin, an oxidized polymerized gelatin, has been introduced by Pauling as a material of greater fluidity than gelatin but similar physiological properties. There have been reports stating that gelatin is antigenic in humans, but general experience with it has not demonstrated this to be a serious factor in connection with its use.

Of the various polysaccharides that have been considered, and these have included pectin, methyl cellulose, and dextran, only the last has found wide acceptance. Hazards of antigenicity or deficiencies of staying power in those which have been degraded to remove antigenicity are associated with all the polysaccharides. In addition, there is the difficulty that these materials may not be subject to the metabolic processes of the human body and may therefore remain deposited in organs for undue lengths of time, leading to conditions such as cirrhosis of the liver, which resulted from the use of the gum acacia in the first world war. Although preparations of dextran have been

obtained of such degree of purity and homogeneity that they are acceptable in many respects, prolonged trial has nevertheless demon strated certain unsatisfactory properties such as antigenicity and undesirable prolongation of bleeding time due to an unknown effect on the hemostatic mechanism.

A third class of substances, of which there is one outstanding representative, is that of the synthetic polymers. The polymer of vinyl pyrrolidine known as PVP was used extensively by the Germans in World War II, with considerable success. It has been used to some extent in this country but is felt to be undesirable because the material is taken up by the reticuloendothelial system and remains there indefinitely.

Against this background attention can now be turned to the examination of another substance or group of substances which have been proposed and which offer some uniquely interesting properties with reference to their use as plasma volume expanders. These are the peptides of glutamic acid and polymers of these peptides. These polymers of glutamic acid can be produced both synthetically and biologically, but the biologically produced material will receive major consideration here.

Glutamyl polypeptide was first described and isolated by Ivanovics and Erdos,[1] who observed that it was a component of the capsule of *Bacillus anthracis* and of *Bacillus subtilis*. These investigators, who first isolated the pure polypeptide, demonstrated that it was composed of glutamic acid residues linked in γ linkage.

Our interest in this material first arose in connection with the possibility that this peptide might offer an opportunity of studying the enzymatic synthesis of the peptide linkage. The reason for this was twofold. The peptide was formed as an extracellular product in *B. subtilis* cultures, and it was hoped that a cell-free synthetic enzyme system might be obtained similar to the system successfully studied by Hehre[2] in the dextran-producing organism *Leuconostoc mesenteroides*. Furthermore, it was felt that, since the peptide contained only one amino acid, the enzyme system involved in its biosynthesis might be less complex than those needed for other peptide syntheses, and thus offer a favorable starting point for attack on the general problem of peptide biosynthesis.

It was first necessary to demonstrate that the material obtained from the strains we worked with was truly composed of only glutamic acid and to ascertain the nature of the peptide linkage. Fortunately for the purposes of both these experiments and subsequent develop-

ments in relation to the use of these materials as plasma volume extenders, the *subtilis* organism produced these glutamyl peptides on a simple medium, Santon's medium, the organic constituents of which are glutamic acid, glycerin, and citric acid. The organism grows as a mat on the surface of shallow layers of this medium and produces up to 1 gm. of peptide per liter. By incorporating into this medium heavy nitrogen, either in the form of ammonia or of amino nitrogen in the glutamic acid, it was possible to obtain pure peptide containing 4.00% isotopic nitrogen excess, and this peptide on hydrolysis yielded pure glutamic acid containing 3.99% isotopic nitrogen excess, indicating that this was the only amino acid present. We also confirmed the fact that the glutamic acid residues in the peptide we obtained were γ linked.[3]

At this point in our studies the year of 1941 ground to a close leaving behind it many new and pressing problems, among them the need for a method of preserving blood or developing a substitute for plasma.

It had become apparent to us that here was available an easily and cheaply produced polypeptide composed of a single non-aromatic amino acid linked in a physiologically acceptable peptide linkage and possessing a free carboxyl group for each peptide linkage. There was a reasonable possibility that this material might be non-antigenic, because existing theories, based on considerable evidence, postulated that only large peptides or proteins containing some aromatic amino acids were antigenic. A particularly attractive and provocative feature was that the presence of a large number of carboxyl groups would assure maximal Donnan effects, so that if the molecule were large enough to be non-diffusible from the blood stream it would have an unusually high oncotic efficiency.

Under the circumstances, it was evident that the possibility of the use of this material as a plasma volume extender had to be explored. It was very quickly demonstrated that the material as isolated from *B. subtilis* filtrates by the methods of culture then employed was in many respects an ideal plasma volume extender. In extensive tests in small animals it was proved to be non-toxic and non-pyrogenic. Its osmotic potency in vitro was found to be quite high, and this osmotic potency was found to be additive to that of serum albumin (Table 1[4]). The material was obtainable as a dry white powder which was highly water-soluble as the sodium salt, and solutions of the latter were stable to autoclaving. The material did not deteriorate or develop any unfavorable properties on standing. The ease of preparation from culture filtrate was quite remarkable. After removing

Table 1. Osmotic Efficiency of Glutamic Acid Polypeptide in Serum Albumin Solutions

Albumin Concentration, gm/liter	GAP Concentration, gm/liter	Osmotic Efficiency *
60	0.00	159
50	1.08	158
40	2.16	155
30	3.27	145
20	4.39	144
10	5.53	145
0	6.68	152

* The osmotic efficiency of a non-diffusable substance in a solution is defined as the number of cubic centimeters added to an infinite volume of the solution for each gram of substance when the osmotic pressure, the quantities of all other non-diffusable substances inside the membrane, and the concentration of each diffusable substance outside the membrane are kept constant.

the organisms by filtration or centrifugation, the peptide was precipitated from solution by addition of copper sulfate which formed a green rubbery complex with it. This dissolved readily in citric acid solution, from which copper was removed by precipitation as sulfide or by other methods. On acidification and standing in the cold a white precipitate of peptide separated out. This peptide, after resolution in sodium hydroxide and reprecipitation, is pure enough for physiological work.

Molecular-weight determination by an end-group method showed molecular weight of 12,000 to 15,000. By light diffraction and viscosity measurements it was determined that the average molecular length ranged from 150 A. to 200 A., which is of the order of magnitude of serum albumin, and the molecular diameter was 11 A., approximately one-third that of serum albumin.[4] On physiological testing it was found that this material unfortunately was excreted very rapidly in the urine in normal dogs and humans. In a human the blood stream is cleared of peptide in approximately 5 hours after injection of 20 gm. in a liter of saline. In a dog rendered hypotensive by bleeding, the peptide solution promptly restored the blood pressure to the normal level; whereupon the dog resumed urination, excreted the peptide very rapidly, and urinated himself back into the hypotensive stage.

It was obvious that, although otherwise apparently suitable as an extender, this material was too small and too readily excreted by the kidney to be effective for the desired length of time. Since the molecular length was approximately that of serum albumin it seemed reason-

able that a first attempt to improve the situation should be in the direction of increasing the diameter of the molecule. This obviously could be done by attaching to the free carboxyl groups further amino acids, peptides, sugars, or any biological substance with a replaceable hydrogen. Since the peptide itself had seemed to be pharmacologically acceptable and physiologically promising, side chains of either glutamic acid of glutamyl peptide were the first choice, as it was felt that they would retain all the Donnan effects and contribute nothing new in the way of possible disadvantageous pharmacological effects.

After some exploration it was found that conversion of the peptide to a polyazide could be accomplished, and that this polyazide conjugated readily in pyridine solution with side chains in the form of the pyridine-soluble polymethyl ester of the peptide. After saponification the polysodium salt of the conjugate was obtained. (These steps will be described in more detail later.) The preparation of two such conjugates in small amounts was completed, and these were tested in dogs. Fortunately for this purpose we were able to develop a simple, rapid, and convenient method of microassay. It had been observed that the peptide formed highly insoluble precipitate with some cationic dyes. Dr. Joseph Victor, one of the group * who collaborated in biological experiments with the peptide, had developed this observation into a quantitative method of peptide determination applicable to blood and urine. This has since been elaborated to make it applicable to the assay of peptide content of other tissues.

Of a number of dyes tested, the results obtained with safranine O seemed to be the most reliable. With this material it was found that at pH 5.98 in the presence of excess safranine the peptide was precipitated quantitatively if its molecular weight was 3000 or higher. The presence of excess peptide in the mixtures interestingly enough dissolved the precipitate. This is reminiscent of the behavior of antigen-antibody precipitates with the peptide behaving in a manner similar to a multivalent antigen. In the actual analysis precipitation is complete in 15 or 20 minutes, and the decrease in the concentration of dye remaining in the supernatant is proportional to the amount of peptide present in the solutions being analyzed. Since safranine gives no precipitate with any material in normal plasma or urine which has been diluted 1:1, the analyses can be performed directly on these materials. Where tissue extracts are present, the extraneous safranine-

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precipitable material can be removed by trichloracetic acid precipitation.[5] Peptide and conjugate have been found to be soluble over short periods of time in the trichloracetic acid supernatant. After filtration of the TCA mixture, however, the trichloracetic acid must be extracted immediately with ether, both because it will eventually cause precipitation of the peptide and because it forms an insoluble precipitate with safranine. With these methods available, excretion rates and blood levels were easily followed. As expected the conjugates were retained in the blood stream much longer than the straight-chain peptides.

At this interesting juncture it was decided that a "blood substitute" was no longer urgently needed, as the transportation of chilled whole blood to needed areas was practicable on a desirable scale. Furthermore, glutamyl peptide had suddenly acquired a new importance. It seemed that the available conventional military, industrial, and scientific forces might not suffice to meet the insatiable demands of war. Man, with inhuman ingenuity, successfully met this challenge—somewhat to his later consternation. Among the new offerings at the time was that of biological warfare, and the possibility arose that certain knowledge and skills hitherto devoted to the benevolent purposes of the healing art might be perverted to more destructive ends. It therefore became necessary to prepare defenses not only against the naturally occurring epidemic concomitants of war, but also against the possibility of man-made pestilence. The next phase of our work on the peptide was to be under the auspices of Camp Detrick, Maryland, then the center of biological warfare activity.

The anthrax organism had achieved the high distinction of early consideration as a suitable agent for biological warfare. Glutamyl peptide, as already mentioned, was known to be a capsular component of the anthrax organism and was thought in this capacity to be partly responsible for its virulence.

On this basis it was reasoned that antibodies to this peptide should confer some degree of protection against infection by the anthrax organism, and the development of a vaccine containing the glutamyl peptide as the specificity-conferring component became desirable. It had long been known that protective antipeptide antibodies were not readily produced by anthrax vaccines prepared in the usual fashions, nor, for that matter, by actual anthrax infection and recovery. It was hoped, therefore, that a more potent, protective vaccine for humans might be prepared by attaching the peptide as a haptene group to human protein. In man this antigen should presumably produce antibodies specific for this peptide.

Antigens were accordingly prepared in which the peptide was linked to human globulin both via the azide method listed above and via a diazo coupling reaction achieved by making a p-nitrobenzoyl derivative of the peptide by reacting it with p-nitrobenzoyl chloride, reduction of this to the p-amino derivative, and conversion of this to the diazonium salt. Peptide was readily separated from protein peptide conjugates by alcohol fractionation. The conjugates were tested for antigenicity in mice, guinea pigs, and rabbits, using both in vitro and in vivo methods for the detection of antibodies.

The results of the work along this line can be briefly summarized as essentially negative in that extensive courses of immunization with these antigens, as well as with living and dead bacterial vaccines yielded at best questionable evidence of antibodies in rabbits and guinea pigs, and very dubious protection in mice. The rabbit serum thus produced gave a dubiously positive precipitin test with the peptide used, and the guinea pigs did not become sensitive to the peptide as evidenced by lack of any symptoms of an anaphylactic shock on injection of the peptide. The important point in relation to the present subject of discussion is, however, the fact that under no circumstances were the peptide itself, polymers of the peptide with itself, or vaccines of the peptide-producing $B.$ $subtilis$ ever found to be able to stimulate the production of antipeptide antibodies in animals, and the same seems to be true in humans at the time of the present writing, although human trials are not yet complete.

In the meantime the turbulent pattern of war had created new and more urgent problems, and the subsequent opportunities of peacetime allowed the pursuit of more interesting ones so that shortly after completion of this immunological study our attention was diverted from glutamyl peptide to other fields until 1950. The initiation of work on glutamyl peptide was, however, destined to yield a rich reward indeed. Following the war, interest in the peptide had persisted at Camp Detrick, and investigations relating to its mechanism of biosynthesis had been conducted by Drs. Housewright, Thorne, and Williams.[6, 7, 8] In an excellent series of studies they had been able to obtain an enzyme preparation from cultures of $B.$ $subtilis$ which catalyzed a transamidation reaction in which the γ-glutamyl radical of glutamine is transferred to D-glutamic acid and D-glutamyl-D-glutamyl peptides. Tri- and probably higher peptides were formed by the same system. During the course of these fundamental investigations these workers had developed much information on conditions of formation

of peptide and especially on methods of peptide production in deep culture.

When the possibility arose in 1950 that replacement of blood or plasma might again become an urgent problem, and this time on a scale far exceeding the practical supply of whole blood or its components, it seemed that once again expediency must prevail over interest, and our work on glutamyl peptide as a plasma volume expander was resumed.

At the same time we proposed the use of the straight-chain peptide as a sedimenting agent to be used in the low gravity centrifugal separation of red cells from plasma in the project on blood fractionation and preservation that was underway at Harvard. In connection with this activity testing of the straight-chain peptide in humans was begun. (The material had been thoroughly tested in animals and found satisfactory, in 1941.) No unacceptable properties manifested themselves in these tests, and these findings provided further encouragement to proceed with preparation and testing of conjugates as plasma volume expanders. With increasing promise of eventual usefulness of the conjugate as an extender, the problem of peptide production in deep culture had become more and more important as the available shallow-culture method of production was economically unsatisfactory. In preliminary experiments we had recently determined that peptide could be produced in deep culture by aeration with CO_2-air mixtures, but results in different batches were irregular and generally not too satisfactory. With the aid of the group at Detrick peptide production by the deep-culture methods soon reached a highly satisfactory status. One of the first findings with this deep-culture peptide was that its molecular weight was much higher than that produced in shallow culture. The highest number average molecular weight we ever observed in the latter was approximately 28,000, whereas the former frequently ran as high as 100,000 or over. The availability of this material greatly increased our range and flexibility with respect to size and shape of conjugates, and the ease of production * of peptide in large quantity greatly increased the impetus of the project.

With the availability of straight-chain peptide of molecular weight 120,000, which was presumably about 10 times as long as that previously used, the question immediately arose and was put to the test as to whether this material would show increased retention in the blood stream. The results are of considerable interest in that they demon-

strate that mere increase in molecular length of peptide is not in itself sufficient to effect significant changes in length of retention in the blood stream of humans. Thus, increasing the size of the straight glutamyl peptide chain from 10,000 to 120,000, a twelvefold increase in length, gives no real increase in the blood-stream half-life, which is approximately 1 hour for both, even though the large molecules are now many times the length of the serum albumin molecules. As can be seen in Fig. 1 the large peptide is excreted with almost the same rapidity
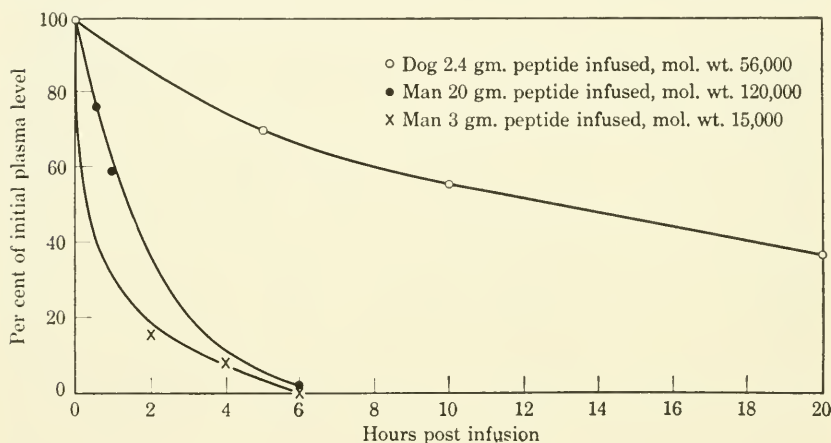
FIG. 1. Plasma levels of straight chain peptide in man and dog.

as is the small. A conjugate with molecular weight of the order of 120,000, having side chains with molecular weights of approximately 8000, shows a half-life at least 10 times that of straight-chain peptide of the same molecular weight.

Strangely enough, the situation is different in dogs. In this animal straight-chain peptides of molecular weights of 60,000 and over have a half-life in the blood stream of 12 hours or longer and bring about the desired hemodilution while in the blood stream. This difference between the two species is of considerable practical interest as the dog is the most commonly used test animal for plasma volume extender tests. In this case, at least, the results with the dog were entirely misleading with respect to behavior of the straight-chain peptide in man. Whether or not this difference between humans and dogs relates to differences in kidney physiology or in the manner in which the peptide is metabolized in the two species has not yet been ascertained.

It was quite evident in view of the above results that the investigations of conjugates and polymers of glutamyl peptide must be resumed.

Our two, and only, previous trials with conjugate had been conducted with dogs as test animals. Although the material injected had been an unfractionated solution of sodium salt of conjugate containing some unreacted peptide, two conclusions were drawn from the experiments: (a) the conjugate remained longer in the blood stream than did the straight peptide and (b) the conjugate we had made was not large enough to give the desired duration of blood-stream retention. The number average molecular weight of backbone in these conjugates had been 12,000–13,000 and that of the side chain had been smaller. The side chains had been obtained by methylation of the peptide by suspending the pure dry peptide of molecular weight 12,000 in dry methanolic HCl for 24 hours, removing methanol and HCl in vacuo, and precipitating the ester from methanol solution with ether. During this procedure the peptide was degraded, the extent of degradation varying with the precise conditions of reaction.

Methods were therefore also developed for preparing side chains from the peptide with no degradation in chain length. By working under controlled conditions of time, temperature, and moisture, it was found possible to prepare a suitable polymethyl ester of peptide by adding ethereal diazomethane to an ethereal suspension of peptide. If methylation is allowed to proceed to completion, there is generally methylation of practically all of the terminal amino groups and loss of their availability for conjugation. Too little methylation gives a pyridine-insoluble product. The best compromise seemed to be a product methylated to the extent of 50–60%. This is usually 60–70% soluble in anhydrous pyridine and retains 80–90% of the end groups as unmethylated amino groups. There is no degradation of the peptide in this process.[9]

The backbone polyhydrazide was prepared as previously by full methylation of another sample of peptide. This is easily achieved by methylation with diazomethane as above, except that a small amount of methanol is added to the reaction, and the methylation is allowed to go to completion, as evidenced by absence of free carboxyl on titration of a small filtered aliquot, with dilute alkali in the presence of phenolphthalein indicator.

Excess diazomethane is discharged by addition of ethereal formic acid, and the ester is filtered off and dried. On dissolving in 50% methanolic hydrazine the ester is converted to polyhydrazide which is precipitated out by addition of methanol. This in turn is converted into polyazide by conventional methods at $-10°C$. Polyazide, which is a white water-insoluble powder at this temperature, is soluble and

fairly stable in cold pyridine. This polyazide in pyridine solution is a backbone ready for conjugation with side chains.

Conjugation is effected by addition of pyridine solution of side chain to pyridine solution of backbone azide in the presence of triethylamine; the reaction mixture is maintained at 0° for 12 hours with stirring and at room temperature for another 24 hours and then precipitated by addition of ether. The solid is dissolved in water and saponified with alkali, and the sodium salt of the conjugate is separated from unreacted peptide by fractional precipitation with alcohol.

Since there is generally a large difference in order of magnitude of molecular weights of the peptide and conjugate, fractionation is not too difficult. The degree of fractionation is easily ascertained by an end-group determination using Sanger's method. The pure conjugate should give no reaction, as it has no terminal amino groups.

It is obvious that molecular dimensions of the conjugate should be subject to variation by alteration of the size of backbone and of side chains, and of the ratio of side chain to backbone. Methods of varying the size of side chain have been described above. The size of the backbone can be controlled by alcohol fractionation of the sodium salt of peptide to be used as backbone. The number of side chains on a given backbone can be controlled by varying the relative concentrations of side chain and polyazide groups in the conjugation mixture. In this connection it was thought desirable to have available a method for quantitative determination of azide concentration in the reaction mixture. A rapid simple method was obtained by using the iron hydroxamic method of Lipmann and Tuttle.[10] In this determination it is important that the aliquot of pyridine solution of azide used should be no larger than 0.1 ml., as the presence of too much pyridine interferes with color formation, and that two drops of gum acacia solution be added before addition of the ferric chloride solution. This latter helps stabilize the solution.

A considerable number of these conjugates have been prepared, and various aspects of their chemistry and biology have been investigated. These include studies of their effects on formed elements of the blood in connection with their use in the blood preservation, of their effects on hemostasis, of their possible pathological effects on prolonged and repeated injection in animals, etc. The results of these studies have to date been negative in that no biologically unacceptable properties of the peptide or conjugate have been observed. Physicochemical studies have been carried out for purposes of characterization of the various polymers produced.

Of considerable importance in connection with the use of these materials is the question of their metabolic fate in man.

It has been ascertained that both the peptide and the glutamyl peptide conjugates are broken down by aqueous tissue extracts of practically all human tissues including red cells, kidney, liver, spleen, and brain. The major exception seems to be muscle. It is significant also that plasma does not break down either peptide or conjugate. The precise extent of breakdown that occurred in these tissues in vitro is not known. It has been observed that if peptide or conjugate solutions are exposed to homogenates of the organs, or hemolyzates, then 70 or 80% of the safranine-precipitable conjugate or peptide disappears within 12 hours at room temperature. Since it is known that, under the conditions of assay used, safranine precipitates quantitatively peptides and conjugates of molecular weights of 3000 and higher, it is obvious that the material that has disappeared in this time must have been degraded to molecules of less than 3000 molecular weight. By microbiological assay and chromatographic analysis it was determined that after precipitation with trichloracetic acid there were present in the digestion mixture low molecular weight glutamyl peptides and some free glutamic acid. The free glutamic acid found was never more than 10% of the conjugate or peptide originally present, but it is possible that some free glutamic acid produced may have been metabolized. This point of completeness of metabolism or excretion of the material is important and will have to be conclusively settled by isotopic analysis. It is interesting that, under the same conditions used in obtaining the above data, synthetic α-linked glutamyl peptide is degraded extremely slowly, if at all, by extracts of human kidney, liver, and red cells.

The potentialities for control of molecular size of conjugate may be exemplified by the following. In three separate preparations where the size of the side chain and the ratio of side chain to azide were kept constant, but where the backbone varied in length in the approximate ratio of 1:2:3, the half-lives of the material in the human blood stream were respectively 12, 18, and 24 hours. In another set of preparations when backbone size was kept constant it was found that enlargement by side chains consisting of glutamic acid (or various other amino acids) caused no significant increase in the half-life in the blood stream over that of the original backbone peptide, whereas side chains of molecular weight of a few thousand increased the half-life by many hours even though the number of amino acid side chains per backbone was higher than that of peptide side chains. In a third instance where

both backbone and side chain were kept constant with respect to size
but varied in ratio, it was found that doubling the ratio of side chain
per azide increased the sedimentation rate of the conjugate from 1.2
to 1.4 S units. The sedimentation rate of the backbone peptide used
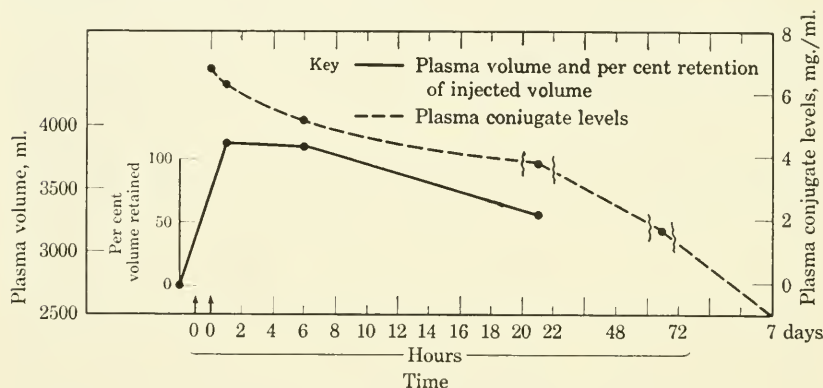in this experiment was 1.0 S units.

FIG. 2.   1 liter of 2.6% conjugate infused in 1 hr.

The experience gained in the work described above has led to the
production of many preparations which, when clinically tested, have
given satisfactory results with respect to blood-stream retention,
plasma volume extension, and absence of unfavorable physiologic or
hematologic effects. A typical example is shown in Fig. 2 and Table 2.

Table 2.   Clinical Test: Hematological Findings

(1 liter of 2.6% conjugate infused in 1 hour)

| | Baseline Preinfusion | Post Infusion | | |
Test		1 hr.	6 hr.	24 hr.
RBC	5,040,000	4,460,000	4,310,000	5,110,000
WBC	5,500	9,000	8,000	5,050
Differential:				
Band forms	1	2	1	5
Segmental forms	43	56	47	55
Lymphocytes	50	36	45	37
Eosinophils	2	3	4	
Monocytes	3	2	2	3
Basophils	1	1	1	
Platelets	297,360	250,200	258,600	332,150
Clotting time (Lee White)	8' 30"	14' 55"	9' 5"	15' 55"
Hematocrit	46%	36.5%	40.5%	44%

It is hoped that further trial will confirm the expectation that these glutamyl peptide conjugates will prove to be useful, preferably under circumstances other than those which prompted their development.

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The Very Big and the Very Small

REMARKS ON CONJUGATED PROTEINS

ERWIN CHARGAFF

It is an old experience in the natural sciences that what is poison for one generation often is honey for the succeeding one. Whether this indicates, in a given case, the dawn of a better era or an inurement by frequent exposure to sublethal doses of truth often cannot be decided without the perspective of centuries. There are important exceptions, but in general a scientific truth fades every 30 years, to be replaced by another equally evanescent. A well-designed and well-constructed chair lasts longer.

It is not very long ago that the extreme contempt for the amorphous and intractable, felt by generations of organic chemists (or at least by the second-rate specimens) has made room for the realization that there is little sense in treating living and growing tissue merely as the starting material for the isolation of well-behaved crystalline substances. In recent times, the respect for nature and its multiform manifestations (as such a very healthy sign) has, in fact, sometimes assumed exaggerated proportions; and it is occasionally necessary to point out that the living cell is not simply a macromolecule with a skin, or the bacteriophage a nucleoprotein with a tail. So-called model experiments often are carried to incredible lengths, prompting one to say that confusion superimposed on complexity may produce papers, but not results, and that a skunk dipped into chlorophyll is not yet an apple tree. The secret of the organization of the cell will not be found by a clever sleight of hand.

It is, however, becoming clear that organization, as observed on the macroscopic and microscopic levels, must be matched, on the submicroscopic and molecular levels, by the existence of patterns in which the varied arrangement of a limited number of constituents serves to

impress individuality and specificity on cells or cell communities. One could venture the opinion that biochemical evolution is accompanied, or indeed caused, by the formation of macromolecules of ever-increasing complexity composed of an ever-diminishing number of constituents. One mechanism by which this may be accomplished is that of conjugation. Many different conjugated proteins are being recognized, such as the nucleoproteins, the lipoproteins, the mucoproteins, or the chromoproteins; many enzymes that carry cofactors, distributed in specific positions on the protein molecule, belong to one or the other of these groups. But we encounter also lipopeptides, mucolipides, etc.; and many more unrecognized compounds of this type must daily be going down the drains of our laboratories than repose in the graveyards of our scientific journals. When I am sometimes told that biochemistry has "run out of good problems," I shudder and reply: "Biochemistry has not even begun!"

The general aspects of the problem of conjugation have rarely been formulated clearly. This is perhaps not surprising, for this class of substances has long found itself between two chairs, as it were: too big to be handled conveniently by the chemist; too small to be seen consistently by the morphologist. The chemist strove for the isolation of the smallest unit endowed with homogeneity; the biologist attempted the recognition of the simplest structure endowed with function. The prize went to the loudest prophet with monomaniac intent. Since scientists in our time have, on the whole, lost the ability to say "We don't know," it is usually the man with the premature explanations that brings home the bacon; and by the time he has been found out he will have devoured it.

Another obstacle to the recognition of the biological importance of the conjugated proteins may be seen in their being usually considered apart from each other under the headings of their respective prosthetic groups. I am aware of only one instance, namely, in a symposium held some time ago (1953) at Rutgers University, in which an attempt was made to consider the conjugated proteins as a family of substances having more in common than the small print that they occupy in the current textbooks of biochemistry. But there can be little doubt that many, if not all, life processes take place on what may be considered the surfaces of conjugated proteins. It is, perhaps, not uninstructive very briefly to compare two groups of conjugated proteins on which my laboratory has spent some effort, viz., the nucleoproteins and the lipoproteins.

As concerns the nucleoproteins, a strict distinction must be made between the complexes containing deoxypentose nucleic acids and those in which a pentose nucleic acid acts as the prosthetic group. When the conjugated proteins associated with the same type of nucleic acid are compared, it will be noticed that their properties are governed by the type of protein they contain rather than by the composition of their nucleic acid moiety. This is undoubtedly due to the ability of proteins to differ much more from each other in their chemical and physical properties than do the nucleic acids. The deoxynucleoproteins are, in many cases, complexes whose protein moiety is represented by a protein of markedly basic properties, such as a histone or a protamine. Although it is inviting to consider such compounds as salts between the cationic protein and the anionic nucleic acid, this would be wrong. It is, for the moment at any rate, much safer to regard the nucleoprotamines and the nucleohistones as specifically conjoined complexes of a complicated and, thus far, little-understood geometry to which both electrostatic and secondary valence bonds contribute. What all these compounds, however, appear to have in common is that they are readily dissociated by high electrolyte concentrations and that under conditions permitting the removal of the protein moiety, by precipitation or denaturation, the nucleic acids are liberated.

Occasionally deoxynucleoproteins are encountered in microorganisms that are exceptional in resembling the ordinary pentose nucleoproteins rather than the nucleohistones. On the other hand, certain plant viruses represent exceptions to the behavior of the most commonly found pentose nucleoproteins. Of the latter, as they occur in the microsomes and the nucleoli, it may be said that they exhibit less of an electrostatic character than the deoxynucleoproteins. The bonds holding the ribonucleic acid to the protein are broken with much less ease; and one gains the impression that in this case the structure of the prosthetic group is inextricably associated with the structure of the entire conjugated protein. Whereas the dissociation of a nucleohistone may be compared to the removal of branches from the trunk of a tree, the separation of the ribonucleic acid and protein moieties of a ribonucleoprotein resembles much more the disentanglement of the warp and the woof of a fabric.

All nucleoproteins share, however, one important feature: they are combinations of two types of giant ampholytes, each of which can, and undoubtedly does, exhibit innumerable specificities as regards shape and constituent sequence. Their combination probably does add

a new dimension; but each partner is, in itself, fully competent to maintain a specific pattern and to convey intricate information.

Of the lipoproteins, on the other hand, it could be said that it is only through the attachment of the monomeric lipides to a protein that a specific pattern of lipide arrangement becomes possible. It is not improbable that the future will show that certain lipides can exist in the cell in a polymerized form capable of exhibiting sequential specificity. But up to the present the lipides seem to be the only bulk components of tissues that must be assumed to exist principally in a monomeric form. If the establishment of specific arrangements is considered as an attribute of cellular organization, the formation of specific lipoproteins is one of the ways in which the lipides can take part in such specific patterns. Certain lipides probably are attached to the proteins by a combination of electrostatic and hydrogen bonds; others may occur as solutions in the lipide moieties of lipoproteins. There is little evidence of the existence of covalent links between lipide and protein.

The two types of conjugated protein considered here very briefly are, to a certain extent, representative of conjugated proteins in general. The prosthetic group may be soluble or insoluble in water; it may be a monomer of comparatively simple structure, a mixture of monomers, or a macromolecule having itself a complicated structure and being capable of an intricate sequential specificity. As may have been gathered from what I said before, I consider the principle of conjugation as the main process through which nature makes big things bigger and small things big. Size, in compounds participating in the life of the cell, is probably not an accident. Moreover, such processes of predetermined aggregation may be one of the ways in which what sometimes is stupidly referred to as the "assembly line" is realized in the living cell. The models of which we can conceive are probably no more than an absurd caricature of the synthetic mechanisms, a multiplicity of templates in space and templates in time, through which the organism maintains patterns of this high degree of complexity, unless we assume (and there is no reason for that) that what is duplicated is not really a duplicate.

Romantic deduction has done much harm in the sciences. But the use, the almost unpredictable use, of imagination is an essential element in the operations of the human mind. The injunction not to be astonished—nil admirari—is one of the most stupid legacies of antiquity. When we consider this ever-repeated giant throw of dice, this internally

regulated cataract of reactions, sequences, and products, our first re-
sponse must be a deep astonishment at a chaotic regularity which has
thus far defied our understanding. Eternal surprise is the engine that
drives the searching intellect. Let us hope that the coming generations
will not have lost the ability to wonder about the many meanings of
these palimpsests of nature.

Unbalanced Growth
and Death

A STUDY IN THYMINE METABOLISM

SEYMOUR S. COHEN

In a volume presented to Sir Frederick Gowland Hopkins in 1937, N. W. Pirie offered an essay entitled "The Meaninglessness of the Terms Life and Living." This effort proved to be quite convincing and had the general effect of eliminating those terms from the working vocabularies of a whole generation of biochemists and biologists. As a biochemist working on viruses, Pirie had found it necessary to be more precise in the description of these organisms than the usual definition of "life" and "living" made possible.

In more recent years, Pirie has discussed various aspects of the problem of biopoesis, or the origin of life, having presumably been led to this subject by a consideration of the question of the evolution of viruses and of their cellular hosts. The problem of the origin of living systems is now moving to the stage of experimental study, and it is evident that the goal of such an experimental program must be defined if a successful conclusion is to be recognized. It is no more than fair, therefore, even if amusing, that, as the need arose, Pirie should have undertaken to restore "life" to our working vocabulary. In the absence of a calculus of biochemistry, which could facilitate a quantitative description of the coming into being of living matter, it was reasoned that the least which must be done was to define the minimal major characteristics of living matter. Living substance has, therefore, been defined by Pirie as a system, containing liquid and catalytically active matter, which is capable of growth and reproduction.[1]

In the last few years we have observed some phenomena for which a satisfactory terminology is rather difficult to find. One might adopt the approach of Humpty Dumpty, "When I use a word, it means just what I choose it to mean—neither more nor less." Nevertheless this has seemed rather dangerous in this instance, and I have seized upon

Pirie's recent contribution and definition with gratitude. The phenomena which I shall discuss seem best described by the term "death." This is to be understood as signifying the situation which occurs when cells have lost one or more of the attributes included in the minimal definition of life or living presented above. More particularly, I shall then refer to cells as "dead" when they have lost the power to multiply.

Of course according to this definition the brain of a man is dead from the moment of birth. I would refer to Pirie all quibblers who have this type of example and argument to offer. On the other hand, it will be recognized that in the field of microbiology this definition is actually in common use. For example, one distinguishes between the bacteriostatic action of the sulfonamides and the bactericidal action of penicillin on the basis of what happens to the ability of the treated cells to form colonies. One speaks of the killing and lethal action of ultraviolet irradiation despite the now-well-known gamut of restoring treatments which bring the dead cells to life, i.e., restore the ability to multiply. I shall use the words "death" and "dead" in this way, even though such a use leads to some curious formulations.

Several years ago it was discovered by Wyatt and myself that a group of bacterial viruses, the T2, T4, and T6 bacteriophages, contained a new pyrimidine, 5-hydroxymethylcytosine.[2] This substance was not present in detectable amounts in the host cell, *Escherichia coli,* and it therefore appeared that we were dealing with the first instance of a building block unique unto a virus. A study of the metabolic relations of the pyrimidine was then begun.[3] Among other questions, we wished to see if 5-hydroxymethyl derivatives of cytosine and uracil could be converted to the 5-methyluracil, thymine. A thymine-requiring strain of *E. coli,* called 15_T-, was obtained and was tested for the ability to use the hydroxymethylpyrimidines. It was found that the mutant organism was incapable of this conversion.

Ordinarily the thymineless mutant might have been dropped at this point. However, we routinely infect our bacteria under various conditions, and we attempted to do this with strain 15_T-, using the bacterial virus, T2. To the surprise of my collaborator, Miss Hazel Barner, and myself, infection of 15_T- in the absence of exogenous thymine led to the synthesis of virus and virus deoxyribonucleic acid (DNA). Indeed, it was found that, in the absence of thymine in the medium, infected cells synthesized and accumulated only thymine and hydroxymethylcytosine, and were in the interesting position of making only those pyrimidines which the bacterium appeared unable to make before infection.[4] This result has led us to a closer scrutiny of the properties

of 15_T- in the uninfected state.[5] The properties of the uninfected cell have proven to be so unusual that we have been unable until just recently to return to the original problem of the behavior of infected cells.

Attempts to obtain an experimentally useful set of relations among turbidity, cell number, and the thymine content of the medium led at first to confusing results. The turbidity of a culture increased significantly in the absence of thymine, in the presence of a carbon source such as glucose, plus the usual salts, including NH_4^+. In cultures from which exogenous thymine had been exhausted, viable cell number varied widely. It was then observed that cells which utilized glucose, nitrogen, and phosphorus in the absence of thymine soon lost the power to multiply, i.e., they died, at the rate of 90% per division time. In fact all of these constituents were essential for death to occur. Mere metabolism, in the sense of some partial metabolic event such as glycolysis, was not the key to the killing process. The bacteria had to grow to die. Such death was irreversible since the power to multiply was tested by plating the cells on a medium containing thymine.

The increase in turbidity of 15_T- in the absence of exogenous thymine was due to growth of the cells. There was an increase of the mass of protein and nucleic acid in the cells and not merely a swelling due to water uptake. The cells increased considerably in length and breadth; the protein and ribose nucleic acid of the cells doubled at least, as did the rate of respiration on glucose; the latter phenomenon suggests an increase in enzyme content as well. However, the DNA content of the bacteria barely increased, according to the usual colorimetric procedures. It appears, then, that the cytoplasmic constituents of the cells increase, but nuclear synthesis is prevented by the lack of a substance found uniquely in a nuclear constituent, namely, the thymine present in DNA. It is suggested that death is caused by this unbalanced growth, that the cytoplasmic growth has created a structural framework within the cell in which nuclear division has become impossible. We have noted that addition of thymine to the dead cells permits DNA synthesis; however, viable count remains unchanged, signifying that matters have proceeded past the point at which the formation of DNA can effect division.

The net synthesis of nucleic acid constituents in 15_T- in the absence of exogenous thymine has been studied by means of uniformly labeled C^{14}-glucose.[5] Thus in a two-hour period RNA synthesis in the absence of thymine equals the amount of RNA originally present in the bacteria, as determined by the radioactivity found in the specific RNA

constituent, uracil. In this interval, a mixture of radioactive ultra-violet-absorbing substances were excreted into the medium. Three of these have been isolated and were shown to be uracil, orotic acid, and hypoxanthine. Their radioactivities per atom of C were identical with the glucose used in the experiment, demonstrating that these compounds were actually synthesized in toto while the cell was growing and dying. Uracil was the major constituent excreted, and it may be suggested that in this system this base or a derivative is the precursor which is methylated to form thymine.

It was observed that a thymine analog, 5-bromouracil, permitted a five- to sixfold increase of turbidity as well as considerable synthesis of both DNA and RNA in the absence of thymine. Indeed cell number doubled, and then, despite a continuing DNA synthesis, death occurred. On examination the cells were seen to be very long and we concluded that the cells had synthesized an inadequate DNA, which, containing bromouracil instead of thymine, was unable to support normal division.[5]

A cell in which cytoplasmic synthesis proceeds in the absence of nuclear synthesis is a useful tool. It has been possible to demonstrate not merely enzyme synthesis in such a system but the synthesis of a new enzyme in response to the presence of an inducing substrate, i.e., enzymatic adaptation. The enzyme tested was xylose isomerase, which catalyzes the equilibrium:

$$\text{D-xylose} \rightleftharpoons \text{D-xylulose}$$
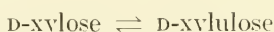
Xylose isomerase is undetectable in cells grown on glucose. When 15_T- grown on glucose was exposed to D-xylose in the absence of exogenous thymine, the bacteria synthesized this enzyme in response to the presence of the inducing substrate. The initial appearance and rate of synthesis of the enzyme were similar to those observed in the presence of thymine. Thus nuclear synthesis does not appear essential to the phases of induction and synthesis. We have concluded that the cytoplasm is the site of such activities.[5] Of course, having made xylose isomerase in the absence of thymine, xylose was now used by 15_T- for unbalanced growth, and the cells died, that is, the cells committed suicide as a result of adaptation.

Death of 15_T- in the absence of thymine does not occur until cytoplasmic growth has proceeded past some critical point. If, just before this point is attained, thymine is furnished to the cells it is found that the entire cell population divides synchronously, and a considerable degree of synchrony is maintained for at least four cycles. When

thymine is first supplied, DNA synthesis begins and rapidly doubles. DNA synthesis then stops and division begins. This process is also completed rapidly, and DNA synthesis begins again, starting a new cycle. It appears that continuing growth in the absence of DNA synthesis has brought all of the cells to the same point in the division process, wherein the supply of the appropriate nuclear constituents triggers the actual division. The relations of DNA and division in this system are entirely analogous to the phenomena observed among higher organisms. In cytochemical studies on many types of higher cells it has been found that DNA synthesis and doubling occur during the interphase and very early prophase.

Since this phenomenon described above provides a considerable degree of synchrony to large populations of cells, it should be possible to investigate many phenomena from the point of view of their occurrence in particular phases of the life cycle of a cell. If the effects of thymine depletion and the inhibition of DNA synthesis are observed to be widespread phenomena, application of appropriate agents should induce synchrony in many kinds of cell populations, tissue cultures, and perhaps even intact tissues.

Let us return to the phenomenon of death caused by thymine deficiency and unbalanced growth. How widespread is it? It is possible to induce thymine deficiency in other bacteria by growth of the organisms in the presence of sulfonamides. The division rate is considerably slower than the rate in the absence of sulfanilamide but it can be increased by the supply of compounds containing the one-carbon fragments dependent on the coenzyme containing folic acid. These compounds include a purine, e.g., xanthine, methionine, serine, histidine pantothenate, and thymine. If thymine is omitted from this fortified medium containing sulfanilamide, the cells die.[5] If any other metabolite is omitted from the medium while thymine is present, death does not occur.

Sulfanilamide is generally considered to be bacteriostatic. It is so because it prevents the synthesis of cytoplasm and nucleus alike. It may be converted to a bactericidal substance by providing compounds essential specifically for cytoplasmic synthesis and not for nuclear division. It has been observed that the folic acid antagonist, Amethopterin, can specifically inhibit the synthesis of thymine and DNA. Is it possible that this compound can be made more useful in killing leukemic cells by the concurrent supply of metabolites important for cytoplasmic synthesis?

In point of fact, there is reason to beieve that many agents exert

their killing action by provoking unbalanced growth. For example, the nitrogen mustards will kill *E. coli* and in so doing produce filamentous cells in which DNA synthesis, but not RNA synthesis, is inhibited. Penicillin kills only when cells are actually dividing. The treated cells become filamentous and accumulate uracil-containing compounds.

E. coli exposed to low levels of ultraviolet irradiation become filamentous as a consequence of cytoplasmic synthesis and inhibited DNA synthesis. Kanazir and Errera have recently shown that such cells accumulate thymidylic acid.[6] We have compared the killing action of ultraviolet irradiation with thymineless death in 15_T-.[5] The two processes appear to be entirely analogous, although some complicated phenomena have been found following irradiation. If irradiated cells are incubated in a liquid medium for 20 minutes, a majority of the cells are restored to life, as determined by plating on a thymine-containing nutrient agar before and after incubation. This restoration has the characteristics of the decay curve for a toxic product. At maximal restoration in liquid medium it has been observed that the restored cells die again whether thymine is present in the medium or not. This may be observed in Fig. 1. Restoration has left a residual lesion, which is expressed in the time necessary for one division, since by this time all restored cells left in the liquid medium have completed their second death. Plating on the solid medium appears to interrupt the second death.

As can be seen in Fig. 1, the second death occurs at the same rate as thymineless death. We have also found that this may be prevented by eliminating utilizable carbohydrate or nitrogen from the medium and from the cells. In a complete medium, the presence of 5-methyl-tryptophan, at a concentration which inhibits growth, also prevents death.[5] It is a matter of some interest that restoration is not significantly impeded by any of these treatments, and it becomes possible to restore and conserve almost all of the cells of an irradiated population, which was originally killed to the extent of 90%. It would appear that in ultraviolet irradiation it is the second death, dependent on unbalanced growth, which is really dangerous. In extending these studies we have found identical phenomena in organisms without exogenous thymine requirements.

It is my feeling at this moment that induction of unbalanced growth describes the common mechanism of action of most of the major anti-tumor agents in use at the present time. The proof of this hypothesis calls for a great deal of work, but the case is so strong with bacteria as experimental materials that it should be possible to use the hypoth-

esis as a working basis in attempting the improvement of antitumor agents. One type of such improvement has been suggested in connection with the use of the antileukemia agent Amethopterin. Perhaps thymidine analogs will also fulfill an important role in tumor therapy, since the nucleoside rather than the free pyrimidine appears to be utilized in higher organisms.
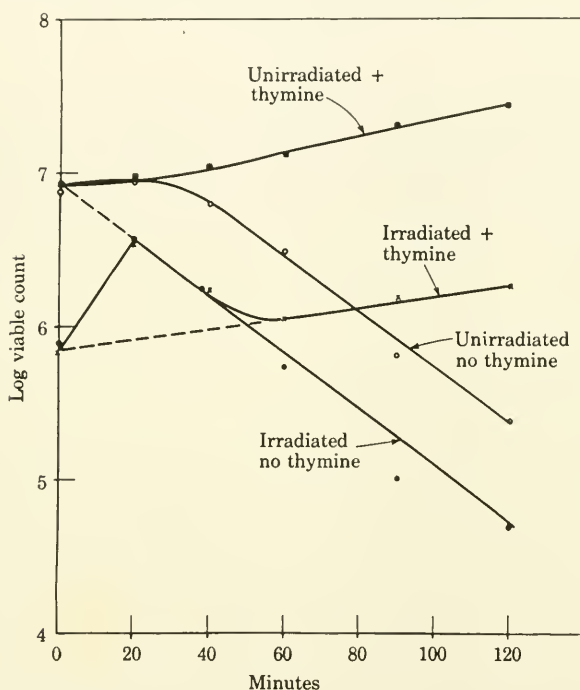

FIG. 1. The numbers of viable cells in cultures of *E. coli* 15$_T$-. Control of irradiated cultures were incubated at 37° in a glucose-NH_4^+ medium in the presence or absence of thymine.

I have described only that type of unbalanced growth which is characterized by inadequate nuclear synthesis. What would happen to a cell in which cytoplasmic synthesis was inhibited while nuclear or chromosome multiplication continued unabated? Such a situation might occur if the synthesis of cytosine riboside were specifically inhibited. This could result in the accumulation of DNA-containing bodies, each of which might also contain a structure preventing the synthesis or utilization of cytosine riboside. Such a DNA body might be liberated when multiplication led an increase of osmotic pressure

and lysis of the cell. If the body were incorporated into another cell and again interrupted cytidine synthesis as it continued its own multiplication, it would evidently be a virus. Perhaps this type of unbalanced growth accounts for the origin of some of these parasites, whose properties more nearly resemble parts of cells than intact cells. Perhaps this hypothesis can also be tested in the near future.

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Some Thoughts on the Biochemistry
of the Steroid Hormones

LEWIS L. ENGEL

The opportunity to write on the biochemistry of the steroid hormones presents an occasion to examine past progress, which has been
most dramatic; to consider current interests; and to speculate upon
future directions for this intriguing field. There are relatively few
fields of contemporary biochemical interest in which the interaction
of the work of organic chemists, biochemists, and clinical scientists
has proved so fruitful. This continual interplay of interests and of
attacks has made possible impressive progress in the last two and one-
half decades. The three major problems in steroid-hormone biochemistry which are of principal interest to the biochemist are biosynthesis,
catabolism, and mechanism of action. The general, overall pathways
of biosynthesis of the steroid hormones are now reasonably well understood, although, of course, many details and individual reactions require further elucidation. It now seems highly probable that the
formation of the steroid hormones in the animal body proceeds from
acetate via cholesterol, or some substance closely related to cholesterol,
to the individual hormones.

Thus, reductive condensation of acetate moieties to cholesterol is
followed by oxidative removal of six or all eight carbons of the side
chain and introduction of oxygen functions at key positions in the
nucleus. In the case of the estrogens, ring A is aromatized with elimination of carbon 19. Although a biosynthetic pathway independent
of cholesterol has been postulated, no unequivocal evidence for such
a pathway has yet been adduced.

The catabolism of the steroid hormones, insofar as it leads to recognizable metabolites, i.e., those which still retain an intact steroid
nucleus, may be a reductive or an oxidative process, or both. Double
bonds at ring junctions and carbonyl groups may be reduced to the

two stereoisomeric forms. Elimination of the 2-carbon side chain occurs with certain pregnane derivatives, and oxidation of hydroxyl groups to ketones sometimes takes place. The reductive processes usually proceed with a high degree of stereochemical specificity, and although both possible isomers may be formed they are usually found in unequal amounts. The structure of the compounds exerts a decisive influence on the relative amounts of epimers formed, and in vivo the metabolic status of the subject may have a lesser effect.

The examination of steroid-hormone metabolites in the urine of normal and diseased persons has yielded a rich harvest of compounds which delineate the general pattern of degradation of the secreted hormones. Over and above the purely scientific interest which attaches to tracing the metabolism of these biologically important substances, there is intense interest on the part of clinicians in the role of the steroid hormones in various disease processes and in the utilization of urinary steroid analyses as diagnostic and prognostic tools. An enormous literature has grown up in this field, and there can be little doubt that these procedures are firmly established in good clinical practice.

However, there are certain points of interpretation of the data which merit closer inquiry. Urinary steroid analyses find their greatest use in the detection of hypo- and hyperfunction of the adrenal cortex and, to a lesser extent, the gonads. These diseases may be tentatively considered as primary diseases of steroid-hormone biosynthesis; normally occurring enzyme systems may be either increased or decreased in amount, but it is improbable that new pathways are introduced. Thus the clinical manifestations of the disease are the result of hyper- or hypostimulation of the target tissues by normal secretory products of the gonads or adrenal cortex. Alternatively, intermediates may be present in excessive amounts owing to absence or deficiency of enzyme systems required for the elaboration of the normal product. The effects seen are also modified by the inherent responsiveness of the target tissues.

There are certain other diseases in which abnormalities of steroid metabolism have been postulated on the basis of less direct evidence. These include neoplastic disease and the collagen diseases. In the former group the evidence, which is particularly strong in the case of cancer of the breast and prostate gland, revolves about the palliative effects of the removal of the gonads and adrenal cortex and/or the administration of certain steroid hormones or corticotropin. In the collagen diseases, the evidence is likewise based upon the effect of cortical steroids and ACTH in modifying the symptoms of disease.

Especially in the first of these two areas, great effort has been made to determine whether there are present in the urine abnormal steroid-hormone metabolites which would help to identify the biochemical nature of the abnormality associated with the disease.

With the increase in precision of methods it has been discovered that steroids which were originally believed to be qualitatively characteristic of neoplastic and connective tissue disease are also present in the urine of control subjects. Thus the differences are more likely to be quantitative and hence less easily definable. Although only fragmentary data are available, it does seem at present that the catabolism of steroids by neoplastic and homologous normal tissues is qualitatively the same.

In considering the urinary excretion of steroids, it has been customary to discuss groups, classified on the basis of the operations employed in their isolation. In a rough way this classification is useful in the detection of gross abnormalities; however, it is generally recognized that individual compounds in these groups are derived from precursors elaborated by either the adrenal cortex or the gonads. Therefore, it would seem profitable to attempt to break down the chemical barriers and to consider the excretion products in terms of the source organ. The index of physiological activity of the adrenal cortex can best be estimated at present from a consideration of excretory products which are distributed in several major groups but can still be related to the primary hormonal secretions with a reasonable degree of certainty. In the case of the gonads there is less scattering, but urinary metabolites of ovarian and testicular hormones are not found uniquely in any one group.

It would be desirable to examine excretion patterns both from the standpoint of estimation of the nature and quantities of gonadal and adrenal hormones secreted under various conditions and from the standpoint of the detailed catabolic fate of the primary secretion products. At present only the latter approach is susceptible to direct attack, but by means of this attack it should be possible ultimately to ascertain whether the abnormalities in disease are associated with biosynthesis, with catabolism, or with both. Even though a significant amount of the hormone secreted may be degraded to non-steroid products, it would seem possible to establish characteristic excretion patterns if representative compounds of each of the chemically definable groups of compounds are subjected to quantitative measurement. This would permit the definition of parameters which could be used to characterize the population of normal individuals and to compare this

population with, for example, patients with malignant disease or with collagen diseases. The chemical and physical techniques for carrying out such analyses on relatively small quantities of urinary extracts are now available and can be applied to answer the important question of whether the abnormality in steroid metabolism in these endocrine-related diseases can, in fact, be defined by study of urinary excretion products.

Now that the major pathways in the degradation of steroid hormones to their urinary metabolites have been established, attention is beginning to turn to the study of the individual biochemical steps in these pathways. The important technical advances of the last decade, and the increasing availability of a wide variety of steroid compounds, have led to significant advances in this area. The diversity of reactions involved in the catabolism of steroids and the high degree of stereochemical specificity in the reactions should make them attractive model systems for enzymologists. Since a wide variety of steroid compounds related to the hormones and differing in structure is available at the present time, opportunity is provided for studying the influence of substituent groups not directly involved in enzymatic transformations and distant from the reacting center of the steroid molecule. Such experiments dealing with substrate specificity should give valuable insight into the number and nature of reactive sites in the enzyme surfaces involved, an insight which could well prove to be applicable to other areas of biochemistry, and which could have important repercussions on our concepts of the mechanisms of enzyme action.

The discussion above has revolved primarily about the catabolic side of steroid biochemistry. It seems appropriate now to turn to the central question facing us as biochemists and physiologists: viz., what is the mechanism of action of these compounds. The steroid hormones occupy a somewhat paradoxical position, since they can be considered both as essential and non-essential for life. Examination of this apparent paradox may lead to some fruitful speculation concerning the mechanism of their action. It is clear in the case of the gonadal hormones that, while they are not required for the survival of an individual animal, they are required and are essential for the perpetuation of the species. In the case of the adrenal cortical hormones, the distinction is not quite so clear. Adrenalectomized animals can be maintained in apparently adequate health provided their diet is supplemented by sodium chloride, and provided further that they are not subjected to gross changes in external or internal environment. Thus

it would seem that the adrenal cortex is not required for life when environmental conditions are held constant. However, alterations in environmental conditions may precipitate changes which can be reversed only by the institution of specific hormonal replacement therapy. If one makes the reasonable assumption that, in the experimental animals cited above, functioning residual gonadal or adrenal tissue is not present, then it would seem safe to conclude that the steroid hormones are not required for the basic, obligatory, biochemical reactions which proceed in the cell. This conclusion would also seem to follow from considerations of the appearance of hormones in the evolutionary sequence above the level of unicellular organisms. It would appear, then, that hormones may be considered as agents elaborated by one cell type which influence the physiologic behavior of other cells. This concept is, in fact, implicit in the definition of a hormone. Thus the hormones may be in a somewhat different category from the vitamins, at least those of the B complex, many of which serve as coenzymes in obligatory metabolic reactions.

In order to examine this question more closely it seems desirable to digress briefly and discuss some general features of steroid structure. The common feature possessed by all these compounds is the tetracyclic nucleus with its complement of one or two angular methyl groups. Side chains of varying length may be attached to carbon 17. Examination of a close-packed model of this nucleus reveals many features not apparent from consideration of the conventional two-dimensional representation. The nucleus occupies space in a characteristic fashion, and the substituents on the nucleus are rather more restricted in their relative positions than is apparent in the planar formulas. When oxygen functions, such as hydroxyl or carbonyl groups, are attached to the nucleus, they confer upon the compounds additional asymmetry which gives to each molecule characteristic aspects of front, back, top, bottom, and two sides. All of the naturally occurring steroid hormones thus far recognized bear an oxygen-containing substituent at carbon 3, as well as other oxygen functions. In addition, unsaturated linkages may be present. The nature and locations and the configurations of the substituents determine both the character and the intensity of the biological activity.

Structural specificity is relatively low in the estrogen series, all three of the principal human estrogens, estrone, estradiol, and estriol possessing a considerable degree of activity. Nevertheless, a change in the configuration of the 17-hydroxyl group converts estradiol-17β, the most potent known natural estrogen, to a relatively inactive compound,

estradiol-17α. It is difficult to set up the requirements for estrogenic activity of organic compounds, since a variety of natural and synthetic substances, some of which are unrelated to the steroid compounds, possess this type of activity.

More rigid restrictions upon structure are present in the androgen series. The minimum seems to be oxygen functions at carbons 3 and 17 and *trans* fusion of rings A and B. Obliteration of the asymmetry at carbon 5 by unsaturation does not interfere with biological activity. As in the case of the estrogens, those 17β-hydroxy compounds which are biologically active are considerably more potent androgens than the corresponding 17α-hydroxy compounds.

Progesterone has the highest activity of any thus far recognized naturally occurring progestational compound. It possesses the unsaturated ketonic linkage in ring A characteristic of most of the naturally occurring neutral steroid hormones, and in addition, a carbonyl group at carbon 20. It should be noted here that, as in the case of cholesterol, the 2-carbon side chain in progesterone possesses the β configuration and that epimerization results in loss of biological activity. Alteration of any of the functional groups on the progesterone molecule results in complete abolition of biological activity. However, a number of compounds closely related to progesterone have been prepared which show activity equal to or even greater than that of the natural hormone.

In the cortical steroid series several qualitatively different types of biological activity are present; among them are the regulation of electrolyte metabolism and of carbohydrate metabolism. Aldosterone is the most potent steroid known insofar as electrolyte regulation is concerned, whereas hydrocortisone exerts its principal effect upon carbohydrate metabolism. However, both of these steroids possess to a limited extent the activity characteristic of the other. In addition, steroids intermediate in both biological activity and in chemical structure have been isolated from adrenal cortical extract and constitute a series in which activity shifts stepwise from predominantly electrolyte to predominantly carbohydrate regulatory as hydroxyl and carbonyl groups are added or removed from specific carbon atoms in the molecule.

This very cursory survey of the relation between structure and biological activity has served primarily to emphasize the point that the biological activity of the steroid hormones seems to be less dependent upon the nature of the functional groups than upon their relative positions on the nucleus and their configurations. Apparently no new kinds of chemical reactivity have been conferred upon the molecules

by the addition of more hydroxyl or carbonyl groups, but, as will become clear below, the rates and directions of certain reactions may be profoundly altered by the presence in the molecule of other groups which may be quite far removed in space.

The concept which occupies a central position is that of the entire molecule as the reactive entity. This concept is supported by a wide variety of examples of marked alterations of reactivity induced in steroid compounds by the presence of distant functional groups. One such example is that of the effect on the rate of development of color in the Zimmerman reaction of etiocholan-3α-ol-17-one by the introduction of a ketone group at carbon 11. As far as is known, the 11-ketone group does not participate in the reaction, and yet in its presence there is a tenfold increase in the rate of color development. Even more striking is the action of acetic anhydride and pyridine upon the epimers, 3α,11β- and 3β,11β-dihydroxyandrostan-17-one. In the former the sole product is the 3-monoacetate, whereas the latter yields predominantly the 3,11-diacetate. Another example is the effect of the presence of an 11-oxygen atom on the in vivo reduction of Δ^4-3-ketones of the androstene series. When testosterone or Δ^4-androstene-3,17-dione is given to a human subject, the principal metabolites found in the urine, androsterone and etiocholan-3α-ol-17-one, appear in roughly equal amounts. However, when andrenosterone (Δ^4-androstene-3,11,17-trione) is administered, the ratio of androstane to etiocholane metabolites is more nearly 4. Many other examples of this type of influence have been observed and serve to strengthen the view that the chemical reactivity of the steroids must be considered in terms of the total molecule rather than isolated functional groups. This concept is neither new nor surprising. What is noteworthy is that in the steroids these effects of distant groups upon chemical reactivity are expressed so clearly and unmistakably. This argument leads to the speculation that one must search for the mechanism of action of the steroid hormones not so much in terms of the reactivity of individual function groups but in terms of the behavior which might be expected of the total molecule. The entire molecule of a steroid hormone may thus be dissected into two portions, the nucleus, a lipide backbone, superimposed upon which are centers of unsaturation and oxygen functions in highly specific positions and with characteristic configurations and conformations. The introduction of the 4–5 double bond which is so characteristic of the biologically active neutral steroids serves in a sense to increase the rigidity of an already highly rigid molecule. Such a molecule would be highly oriented at any lipide-water interface

and might be considered to serve at least some of its functions at such an interface—a cell membrane, for example. In order to explain the high activity of these compounds on a weight basis it must further be assumed that there are highly specific binding sites for steroid hormones. At a cell membrane, a steroid hormone could conceivably exercise control over intracellular biochemical reactions by hindering or facilitating the passage of key metabolites through the cell membrane in either direction. It is equally possible that similar control could be exercised within the cell at some phase boundary.

Is there any evidence for such a view? There are certain indirect suggestions which may be pertinent. One of the most interesting characteristics of the steroid compounds generally is their propensity for complexing. The classical example, of course, is digitonide formation which has now become so commonplace a tool to the steroid chemist that its physical significance has perhaps been overlooked. The ability to form stable, insoluble complexes with digitonin is shared by essentially all steroids possessing β-oriented hydroxyl groups at carbon 3 but is not necessarily limited to such compounds. This is a typical example of a steroid-steroid interaction, of which many exist. If one now considers the interaction of 3β-hydroxy steroids with saturated A and B rings, one can subdivide the interaction into what might be considered as first- and second-order effects. The examination of the solubility products of representative digitonides reveals that, in general, those compounds having the $3\beta,5\beta$ (axial) conformation have higher solubility products than those possessing the $3\beta,5\alpha$ (equatorial) conformation. This would seem to suggest that both the hydroxyl groups and the nature of the ring fusions or a combination of these two factors are concerned in the digitonide formation.

A second type of interaction is that shown by deoxycholic acid and apocholic acid, which form very tight inclusion or clathrate complexes with a wide variety of substances. Although it may be argued that these very stable complexes cannot play any significant biological role because of the extremely low concentration of free bile acids in body fluids, the possibility remains that the bile salts participate in a similar, but looser, form of complexing in the intestinal tract. In any case their surface activity would make them highly oriented at phase boundaries.

A third, biologically important, type of complex involving steroids is the binding to plasma proteins. These complexes, some of which have been subjected to detailed physicochemical study, are undoubt-

edly involved in the transport of steroid hormones from the site of synthesis to the target tissues.

A fourth type of steroid interaction which is now undergoing active exploration involves the steroid hormones directly. It has been found that hormonally active compounds such as testosterone, progesterone, and deoxycorticosterone form complexes with adenine, adenosine, and adenylic acid. Although the complexes have not yet been isolated in the solid state, there can be no question as to their existence. These observations extend the scope of steroid-hormone complexes into the area where they might be considered to have a possible role in the mechanism of action of hormones. Though there is as yet no evidence for the biological significance of such interaction, the possibility exists that by complexing with nucleotides possessing a coenzyme function, rates of enzymatic reactions may be profoundly altered. This sort of phenomenon could be involved in the regulatory function of the steroid hormones.

This discussion has tended to raise questions rather than to answer them. Its purpose will have been accomplished if this discussion leads to experiments designed to answer the central question: What is there about the steroid nucleus which makes it the carrier of so many and such diverse biological activities?

The Biochemistry of the
Bacterial Viruses

E. A. EVANS, Jr.

The contemporary biochemist, being a practical man, is for the most part working in areas that are amenable to his efforts and give him information of a precise, detailed, and orderly nature, such as we have at the moment with respect to the oxidation of carbohydrates in muscle tissue. However, there are certain areas of biological interest which would seem not to lie entirely within our biochemical reach and which are also not clearly beyond it. Although these frequently offer sharp reminders of our limitations, they give also the pleasures of unexpected discovery. The study of the mechanism of viral reproduction is such a field at the moment. The contributions of the chemist to the rapidly developing understanding of this process must necessarily lag behind the more dexterous explorations of the microbiologist and geneticist, but they have the virtue of carrying the description, even though incomplete, to the ultimate molecular level.

However, to discuss the biochemistry of viral reproduction is to speak of special cases. The agents which we define as viruses, purely from an operational point of view, embrace a range of composition and morphology that makes it unwise to expect or predict general characteristics. They may vary in size from the small spherical particles responsible for foot and mouth disease or tobacco necrosis, roughly of the magnitude of certain protein molecules, to agents as large as that of vaccinia, larger than some microorganisms. A similar degree of heterogeneity is shown in what we know of their chemical composition. The smaller particles (the bacterial, plant, and insect viruses) appear to contain only nucleic acid (either DNA or RNA) and protein in varying proportions, but the larger agents contain lipides and a variety of other components. However, the difficulties involved in the

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preparation of the viral agents are very great, and critical estimates of their degree of purity are difficult to make.

A large part of our biochemical information about viral reproduction is derived from a study of the T series of coliphages acting on *Escherichia coli*. Though this has been partly a matter of necessity, since the coliphages can be prepared easily and rapidly in sufficient amounts for chemical work, it has also been hoped that the information thus obtained could be applied, with suitable precautions, to other members of the viral group.

In what follows I have attempted to outline briefly the current status of our biochemical knowledge of the coliphages. Rather than interrupt such a description by continued documentation, I have omitted the names of the individuals responsible for the facts recorded. It is to be understood, of course, that our information is derived from the efforts of a large number of investigators.*

Phages have been classified into two categories, virulent and temperate, on the basis of their behavior towards suitable bacterial hosts. These terms are equated with two types of relationships between virus and host cell, the lytic and the lysogenic respectively. It will be convenient to consider our information, both in regard to the types of viruses and to the kinds of relationships, separately.

Most of our knowledge concerning both chemical composition and the biochemistry of the host-virus relationship is derived from the virulent coliphages. Although there is some variation in the dimensions given by various workers, there is general agreement that these particles are spermlike in shape, with hexagonal heads and tails of varying length. They can be divided into four classes, each serologically unrelated to any other members of the group: the T_2, T_4, and T_6 coliphages, which are particles with fairly long tails ($100-120 \times 20$ mμ); T_3 and T_7, which possess very short stubby tails; T_1, with a long thin tail; and finally T_5, also with a long thin tail.

The virulent coliphages contain about equal amounts of protein and nucleic acid of the DNA type. Complete analyses of the amino acid content of the proteins of all the various coliphages have not yet been made. However, in analogy with what has been observed in the

* The reader interested in the original literature of the subject will find a very complete background in S. E. Luria, *General Virology*, John Wiley & Sons, 1953; E. A. Evans, Jr., Bacterial Virus (with particular reference to synthesis of), *Ann. Rev. Microbiol.*, 8 (1954); and C. A. Knight, The Chemical Constitution of Viruses, in *Advances in Virus Research*, II, edited by K. M. Smith and M. A. Lauffer, pp. 153–182, Academic Press, 1954.

analysis of the protein component of mutant strains of tobacco mosaic virus, one might expect that these will differ qualitatively and quantitatively from phage type to phage type. The amino acid content of T_3 prepared by a variety of procedures has been shown to remain the same and is apparently a specific and unchanging property of the phage particle. There is nothing in the amino acid distribution to suggest any specific characteristics for the coliphage proteins, although the dicarboxylic acids are present in unusually large amounts.

So far as nucleic acid content is concerned, the coliphages contain only DNA. The most recent values for the purine and pyrimidine base content are listed in Table 1. The DNA of T_2, T_4, and T_6 is

Table 1. Composition of Coliphage DNA

Virus		Adenine	Thymine	Guanine	Cytosine	5-Hydroxy-methylcytosine
		moles/100 moles estimated bases				
T_2r^+		32.5	32.6	18.2		16.7
T_2r		32.4	32.4	18.3		17.0
T_6r^+	*	32.5	32.5	18.3		16.7
T_5		30.3	30.8	19.5	19.5	—
T_3 †		23.7	23.5	26.2	27.7	—
λ ‡		21.3	28.6	22.9	27.1	—

* G. R. Wyatt, *Cold Spring Harbor Symposia Quant. Biol.*, *18*, 133 (1953).

† C. A. Knight and D. Fraser, in C. A. Knight, *Advances in Virus Research*, II, 168 (1954).

‡ J. D. Smith and L. Siminovitch, in A. Lwoff, *Bacteriol. Revs.*, *17*, 320 (1953).

entirely unlike the nucleic acid from any other source. In these phages, cytosine is absent and one finds a new pyrimidine base, 5-hydroxy-methylcytosine (5-HMC). In addition, glucose is present, one molecule of the hexose being attached probably to the hydroxyl group of each 5-HMC molecule. However, 5-HMC is not present in the odd-numbered phages (Table 1), and the question of the possible presence of glucose has not been examined.

The manner in which the nucleic acid and protein of the coliphage particle are bound together is not known. It is generally believed, however, that the nucleic acid is completely sheathed by a protective protein layer. If one rapidly dilutes saline suspensions of some of the coliphages, the 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 hexagonal head is empty.

There has been much discussion of the living or non-living nature of the viruses, and there is no need to labor the matter further here. However, such viral particles as the bacteriophages and the tobacco mosaic viruses do not show demonstrable metabolic activity, do not contain energy "reservoirs," nor require any source of energy to maintain their structure. It appears, therefore, that we are dealing with structures which, although enormous in terms of molecular size, are bound together by the usual covalent bonds, together with such other types of intramolecular binding forces as operate, for example, in determining the particular configuration of a protein molecule. An attempt to elucidate the chemical details of such structures is formidable enough but one which can be faced by the chemist with more equanimity, certainly, than the effort to describe in molecular terms such metabolizing systems as a single bacterial cell or an erythrocyte.

The adsorption of virulent viral particles by a sensitive host is followed by a sequence of reactions of which the chemistry is understood only in part. With T_2, T_4, and T_6, the tail constitutes the point of attachment to the sensitive bacterial host. In its initial phase, the adsorption process is reversible and depends on the existence of complementary electrostatic and geometric relationships between the virus and certain portions of the host cell wall. In T_2, the positively charged amino groups of the virus particle bind the negative carboxyl radicals of the bacterial cell wall, whereas in T_1, both types of groups on both surfaces participate in the reaction. The initial reversible binding of the phage is followed by an irreversible phase, which probably involves changes in both the virus particle and the bacterial cell wall.

The difficulty of isolating the earlier phases of the infectious process in the in vivo system has led to a study of the interaction between the coliphages and so-called "bacterial membranes." These are prepared by autolysis of heavy suspensions of *E. coli* in buffer solutions and subsequent treatment with proteolytic enzymes and with lysozyme. Such preparations are homogeneous and specifically adsorb and inactivate coliphages which attack the original bacterial cell. If one follows the interaction between phage and membrane by means of the electron microscope, actual dissolution of the membrane can be shown. The process can also be studied chemically by using bacterial membranes in which the nitrogen has been marked by using N^{15}. When such membranes are treated with coliphage, quantities of soluble nitrogenous compounds are released proportional to the quantity of phage added. The chemical nature of these compounds is presently being

studied in the hope that it will reveal something of the in vivo process of invasion.

Under normal physiological conditions, the irreversible phase of adsorption is followed by a splitting of the coliphage particle, so that the bulk of the nucleic acid of the phage particle passes into the bacterial cell. The protein membrane remains attached to the exterior of the cell and can be removed, without interfering with the further stages of virus reproduction, simply by agitating the infected bacterial cells in a Waring Blendor. All or almost all the phage DNA is transferred into the bacterial cell, and probably all the phage protein is excluded. The further steps in the process of virus synthesis involve the participation of only a portion of the original infecting particle, that is, the whole or the major part of its nucleic acid. This, however, does not deny the physiological importance of the protein portion of the viral particle. The antigenic properties of the virus reside entirely in the protein coat. Further, since protein ghosts, free of nucleic acid, are capable of being adsorbed to and of killing bacterial cells, the protein portion of the phage is capable of initiating some type of intracellular reaction. Finally, no one has yet succeeded in inducing the infectious process by protein-free nucleic acid preparations. It is not possible to conclude that the role of the viral protein is simply that of introducing the DNA into the host cell, but certainly its effect must be exerted in the early stages of invasion.

The nature of the forces responsible for the "injection" of the viral nucleic acid into the bacterial cell is entirely obscure. It has been suggested that the adsorption process involves the splitting off of a portion of the bacterial membrane, that the virus-host cell system can be considered as a single system, and that ordinary osmotic forces would be sufficient to explain the passage of the viral DNA from the head of the viral particle into the bacterial cell proper.

In any event, it is not possible to detect the presence of intact virus during the next phase of viral reproduction. If the cells are disrupted during this period, one finds particles which are protein in nature, related serologically to virus protein, but incapable of inducing the virus infection, and representing probably intermediate stages in virus synthesis. Since the DNA of T_2, T_4, and T_6 contains 5-HMC and is free of cytosine, one can study the synthesis of the nucleic acid portion of the virus by following the changes in phage-specific DNA (i.e., DNA containing 5-HMC) and of host-specific DNA (containing cytosine and no 5-HMC) at intervals after infection has occurred. Phage DNA is found to increase soon after infection, while the bacterial DNA

decreases. At a time approximately half the period between infection and lysis, disruption of the cell shows that mature infectious virus particles are beginning to appear. However, viral DNA continues to increase, and remains in excess of that present as infective particles, until the cell finally lyses.

The mature virus particle may contain material derived from (a) the parent infecting particle, (b) the bacterial host cell, and (c) the nutrient components of the medium. We have already seen that apparently the only contribution of the parental particle is its nucleic acid. By the use of isotopes it is possible to show that, although components of the parental DNA appear in the viral progeny, they are confined to the particles synthesized in the earlier stages of the infectious process, i.e., by the time 25% of the virus yield is completed, most of the parental DNA has been transferred, and the virus particles synthesized subsequently contain little or no parental nucleic acid. Further, the phosphorus transferred is not localized in specific or specially conserved parts of the nucleic acid of the first progeny, but is uniformly distributed in each particle. Experiments with parent virus labeled with both N^{15} and P^{32} confirm the view that extensive rearrangement of the transferred material occurs. Up to now no positive correlation has been obtained between the material transfer of DNA from the infecting phage and the transfer of hereditary units, although parental DNA must be the agent whereby these units are transmitted. If special parts of the virus are conserved and transferred to the progeny as nucleic acids of considerable size and possessing genetic specificity, no experiment has yet revealed their existence. Rather, it appears that, after that portion of the viral DNA which is effective in the synthesis of new virus particles has exerted its effect on the metabolism of the host cell, it is broken down into smaller fragments which are then used for virus synthesis in a non-specific manner.

The most striking feature of the utilization of the components of the host cell for virus synthesis is the use of practically all of the bacterial DNA. This first involves breakdown of the bacterial nucleic acid into smaller fragments which are then used for the synthesis of viral DNA, and especially for the viral particles which are formed in the early stages of the infectious process. To the extent that there is sufficient bacterial DNA to account for the whole of the DNA of the viral progeny, relatively little synthesis of viral DNA from the components of the medium takes place. However, when bacterial DNA is insufficient in amount for the DNA of the viral progeny, or when viral DNA requires the synthesis of a qualitatively new component such as

5-HMC (synthesized in part from the cytosine of the host), then this material is synthesized from the simple components of the medium. There is little or no utilization of bacterial protein or amino acids for virus synthesis so that viral protein is formed largely by de novo synthesis from the components of the medium.

Immediately after infection with virulent coliphages, one observes changes in both the morphology and enzymic spectrum of the infected cells. The cytological effects involve abnormal alterations in the chromatinic or nuclear material of the bacterial cell, as one might suspect on the basis of the chemical changes in nucleic acid metabolism. The oxygen uptake is unchanged, but the metabolism of both the phospholipide and the RNA fractions appears to cease, insofar as these constituents of the host cell show no further incorporation of phosphate after infection. As viral DNA is synthesized, the necessary deoxyribose is derived from an increased rate of formation from triose phosphate and acetaldehyde. Whether this involves an increase in the actual amount of the enzyme systems involved, or whether their activity is enhanced, possibly by the loss of some inhibitory effect, is unknown. In the case of DNAase activity, an apparent increase in enzymic action can be shown to involve the loss of a specific inhibitor which appears to be a ribose nucleic acid. If this should be a general phenomenon, it would be a matter of the greatest interest for our understanding of the metabolic role of RNA. However, we have no knowledge of the cellular role of the DNAase itself, and, in view of our incomplete information of the enzymic changes that take place during infection, it is difficult to estimate the significance of the few which have been described.

The picture of virulent coliphage infection which emerges from all this is one in which the process of virus synthesis is set off by a fragment of the original infecting particle. The machinery that is used for virus synthesis is the normal metabolic equipment of the bacterial cell, and the materials on which it operates are the normal components of the bacterial environment and certain portions of the bacterial intracellular fabric. Many, if not all, of the normal metabolic functions of the bacterial cell cease, and there begins an immediate and rapid synthesis of the specific parts of the virus particle. Viral DNA appears so rapidly after infection has occurred that it is difficult to decide whether new enzymic machinery is synthesized for this purpose, or whether a variation in some normal process occurs. Viral protein can be detected somewhat later, but apparently the two com-

ponents of the mature particle are made independently and are assembled only at a terminal stage of the replication process.

Consideration of the temperate viruses and of the lysogenic relationship between virus and host discloses much less chemical and biochemical data than is available with their virulent analogs. Little is known about the chemistry of the temperate viruses. Large-scale preparations of the temperate virus λ which lysogenizes the *E. coli* strain K_{12} have been made in several laboratories. These preparations appear homogeneous under the electron microscope and consist of tailed particles similar in size and appearance to those of T_1. They contain roughly 50% DNA and are free of 5-HMC (see Table 1 for purine and pyridine composition). Preparations of λ show a tendency towards spontaneous inactivation and are difficult to assay; little has been done with respect to their detailed physicochemical characterization.

When a bacterial cell is exposed to a temperate phage, a variety of interactions may take place. The bacteria may be non-responsive and remain entirely uninfected. Or, infection may occur but the phage disappears in an unknown fashion, i.e., the infection is said to have been aborted, and, although the bacterial cell may die, viral reproduction does not occur. Under other circumstances, phage may be reproduced with lysis of the bacterium, i.e., the phage behaves as a virulent virus, and the situation is comparable to that described earlier. In the case we are most concerned with, the temperate phage is adsorbed and the bacterium survives to give rise to what is known as a lysogenic clone. The infecting virus particle is transformed (reduced) into what is termed a prophage, and the host cell continues to grow and reproduce in a normal fashion. In growing cultures of such cells, one finds that in a very small proportion of the cells the prophage passes spontaneously into what is known as a vegetative phase and mature phage particles are then produced. This results in the lysis of the particular cells concerned, with the liberation into the medium of phage particles identical with the original parent. This occurs spontaneously, to a small extent, and for unknown reasons. However, in the case of certain lysogenic strains, it is possible to cause a practically complete conversion of the prophage by treating the lysogenic cells with one of a variety of so-called inducing agents. These include ultraviolet light, soft X rays, γ rays or radioactive cobalt, thiomalic acid, reduced glutathione, ascorbic acid, organic peroxides, epoxides, ethylene imines, nitrogen mustards, and hydrogen peroxide either added directly or produced by the addition of sulfhydryl compounds in the presence of copper. When such substances are applied

under the proper conditions conversion of the prophage to the vegetative phase occurs, followed by practically complete lysis of the culture and liberation of virus. The phage produced under these circumstances is identical with the original infecting particles producing the prophage. Although the yields obtained here are somewhat smaller than those from the virulent process, it is possible, by use of the induction process, to obtain sufficient quantities of virus for chemical work.

As one might expect, this general pattern can be modified by changes in the variables involved in each phase of the process. Even if the phage is of the necessary genetic strain, it is possible, by changing the temperature at which the cells are maintained or by altering the multiplicity of the infection, to cause the virus to behave in a virulent rather than temperate fashion. Moreover, reduction to the prophage state may be affected by changes in environmental conditions. For example, in $E. coli$ strain K_{12} exposed to the temperate phage λ, reduction requires something less than 1 hour at $37°C$. During this period the intracellular phage is more easily heat-inactivated than free phage, so that it is possible during the reduction period to rid cells of their potential prophage by subjecting them to higher temperatures. On the other hand, once established, the prophage is even more resistant to heat-inactivation at $43°C$. than is free phage. Even when lysogenization has been accomplished, this can in some cases be reversed by changing the medium in which the material is cultured, e.g., if lysogenic $Bacillus megatherium$ is cultured in a synthetic medium containing citrate, lysogenicity is lost after 61 subcultures.

The nature of the conversion of phage to prophage, i.e., the process of reduction, is not known. There is evidence, however, that during the period when reduction is occurring the phage particle behaves as a cytoplasmic unit, whereas after the lysogenic status has been established, the prophage is firmly associated with or bound to a specific chromosomal site.

At present we have no precise information as to the chemical nature of the prophage. Attempts have been made to look for the specific protein of the infecting phage in a lysogenized strain, but without success, and it appears that viral protein is synthesized only after the lysogenic bacteria are induced. In experiments comparing the fate of DNA from a virulent phage with that from a temperate phage, it has been observed that with the virulent phage a considerable portion of the phosphorus of the viral DNA is found in the acid-soluble phosphate fraction of the bacterial cell immediately after infection,

with only a portion of the parent DNA being associated with the DNA of the infected cell. With temperate phage, the bulk of the viral phosphorus remains associated with the DNA of the infected cell, at least during the experimental period. On the basis of the very limited experimental data it seems possible that the prophage is either a nucleic acid or a nucleic acid derivative, a hypothesis that is in harmony with the considerable body of genetic evidence supporting the view that prophage is a genelike unit located at a specific chromosomal site.

With many prophage-containing cells, it is possible, under the correct environmental conditions, to cause a conversion of prophage into the vegetative state. However, not all prophage-containing bacterial strains are capable of induction nor do all inducible strains respond to the same agent. The mode of action of the inducing agents is not known, and it is uncertain whether all of them behave in the same way. It seems probable that their effect is on the metabolism of the lysogenized cell rather than on the prophage itself. After infection with a temperate phage, one observes a delay in the division of the infected cell and transient morphological changes may also occur. Eventually the cells become cytologically normal again and divide regularly.

If induction occurs, bacterial growth proceeds without bacterial division during a latent period corresponding to one or two generations. No phage is released during this period, but if the induced lysogenic bacteria are prematurely disrupted by lysozyme or cyanide, new mature virus particles are found toward the end of the latent period, and increase linearly until a full yield is reached. Many features of phage multiplication in induced lysogenic bacteria coincide almost exactly with those observed after infection of non-lysogenic bacteria with a virulent phage. Such characteristics of phage development as average burst size, distribution of individual burst sizes, etc., are identical in both systems with the exception of the length of the minimum latent period, which is longer in induced than in infected sensitive bacteria.

However, there are marked differences in the chemical and enzymic composition of the induced bacterial cell as compared with the bacterium infected with virulent phage. After a prophage-carrying bacterial cell has been induced, the oxygen uptake of the cell continues to increase and the cell continues to grow although cell division does not occur. In contrast to what is observed in the virulent process, induced cells continue to synthesize RNA and are capable of forming adaptive enzymes almost up to the time mature virus particles can be detected in the cell. Increased synthesis of DNA and protein is a common feature of both types of viral infection, although there is a delay in

the synthesis of DNA during the first phase of the latent period in the induced cells. However, we have no specific marker for viral DNA in the latter case such as 5-HMC in the case of the T-even virulent coliphages; the data quoted relate to total DNA.

It would seem that a comparative study of the induced lysogenic and virulent systems would be a matter of the greatest interest in regard to a further understanding of the reactions involved in viral synthesis. There appear to be greater synthetic limitations in the virulent system, although virus synthesis has a preferential claim on the energy and material sources available to the infected cell in both virulent and temperate infections.

In the case of both virulent and temperate infections, the evidence points to reactions involving the genetic material of the cell. One would suspect, therefore, a considerable degree of analogy between the reactions we have described and those which occur in the course of the normal growth and division of the bacterial cell. If the effective portion of the virulent virus takes over and modifies the synthetic reactions leading to the formation of viral DNA, it seems probable that this material must bear a definite resemblance both in structure and function to normal intermediates in cellular metabolism. When one considers the complexity of the process and the product in which viral DNA is neatly wrapped up into little parcels of protective protein, one might expect again that there should be normal counterparts for such structures. One would like to know something of the structure and cellular location of the normal nucleoproteins of the bacterial cell. At a more detailed level, one might also ask whether the synthesis of the specific viral protein (in which the protein of the infecting particle apparently does not participate) producing a material capable of specific binding to certain portions of the bacterial cell wall does not reflect the normal role of those particular bacterial enzymes used in its manufacture.

As our understanding of coliphage replication increases, the new facts emphasize the intimate relations between this process and the normal life of the bacterial cell itself. Although it is difficult to conceive any physiological advantage in the lytic infection, and one may regard it as a malignant variation of some normal process, there are a number of phenomena associated with lysogeny which hint at some possible biological role, even though it is not possible to grasp just what this might be. In the case of certain strains of diphtheria bacteria which do not produce toxin, lysogenization with an appropriate bacteriophage leads to the prophage-carrying cells producing toxin. Again, bacterio-

phage particles are implicated in the phenomena of transduction in which two different autotropic mutants of *Salmonella typhimurium*, when separated by means of a ground-glass filter, exchange genetic characteristics by way of a filterable agent.   In both cases phage appears to be associated with non-lethal changes in the genetic characteristics of the bacterial cell.   Along with the bacterial viruses, representing a group of biologically active nucleic acid-protein complexes secreted or manufactured by the cell itself, we must recognize also the existence of other biologically active particles, formed by the bacterial cell, which are either DNA or protein.   The transforming factors which are capable of altering the genetic material of the infected bacterial cell appear to be entirely DNA.   On the other hand, the colicines are DNA-free proteins capable of killing bacterial cells but unable to reproduce themselves, although their formation can be induced in bacterial strains by the action of ultraviolet light.   It seems quite certain that the biology of the nucleoproteins involves a whole spectrum of phenomena of which, as yet, we have recognized only a portion.

The Biosynthesis of
Peptide Bonds

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Few current biochemical problems have evoked more imaginative speculation or animated discussion than the elucidation of the metabolic pathways in the biosynthesis of proteins. The challenge of this problem is a consequence not only of the central place of the proteins in the dynamics of living matter but also of the conceptual and experimental difficulties that it has presented. Since there is general agreement that the principal mode of linkage between the individual amino acid units of a protein is the CO-NH bond, the most profitable discussion has centered about the cellular mechanisms for the synthesis of such bonds. However, in limiting ourselves to the consideration of peptide bond synthesis, it is recognized that the characteristic properties of proteins also depend on the integrity of other linkages and that, in protein formation, the synthesis of peptide chains may be closely linked to the creation of these accessory bonds.

It has long been known from physiological studies that the formation of proteins from amino acids requires energy and that this energy is largely derived, in higher organisms, from the oxidative degradation of carbohydrates and fats. However, the means whereby this energy transfer occurs is still unknown, although there is excellent evidence for the view that it involves the coupling of oxidation to the synthesis of the pyrophosphate bonds of adenosine triphosphate (ATP) and the coupling of the cleavage of ATP to peptide synthesis. Before considering possible ways in which ATP may play a role in peptide bond synthesis, it will be useful to discuss some of the available knowledge about the energy changes in the formation and cleavage of CO-NH linkages.

For many years, it was customary to assign the value of about $+3$ kcal. per mole to the standard free-energy change (ΔF°) in the con-

densation of two amino acid units to form a peptide bond. Recent studies have demonstrated clearly that this value applies only to the reactions for which it was determined, the synthesis of a dipolar dipeptide ion from the component amino acid ions (cf. Table 1). Thus, in a condensation reaction leading to the formation of an uncharged peptide, the value for $\Delta F°$ may be as small as $+0.4$ kcal.[1] On the other hand, a condensation reaction in which an acylamino acid is converted to the corresponding amide may be characterized by a $\Delta F°$ value much higher than $+3$ kcal.; this is suggested by the ΔH values cited in Table 1. Although more data are needed for the thermo-

Table 1. Thermodynamic Relations in the Synthesis of Some CO-NH Bonds

Reaction	Temperature	$\Delta F°$, kcal. per mole	ΔH, kcal. per mole	Equilibrium Constant
DL-Leucine$^\pm$ + Glycine$^\pm$ → DL-leucylglycine$^\pm$ + H_2O	37°C.	$+3.3$		0.005
Benzoyl-L-tyrosine$^-$ + Glycinamide$^+$ → Benzoyl-L-tyrosylglycinamide + H_2O	25°C.	$+0.4$	$+1.5$	0.5
Benzoyl-L-tyrosine$^-$ + NH_4^+ → Benzoyl-L-tyrosinamide + H_2O	25°C.		$+5.8$	
Glycyl-L-phenylalanine$^\pm$ + NH_4^+ → Glycyl-L-phenylalaninamide$^+$ + H_2O	25°C.		$+6.2$	

dynamics of peptide formation, the available information is sufficient to indicate the oversimplification inherent in the assignment of an arbitrary value of $+3$ kcal. per mole to the synthesis of the CO-NH bonds of proteins. It may in fact be expected that, if two peptides of moderate length were converted to a single long-chain peptide in a condensation reaction, the energy required would be much less than 3 kcal.

Since thermodynamic data only can tell us what may happen, but give no information about what does happen in a living cell, the only general conclusion that can be drawn from the available $\Delta F°$ values is that, at pH 7, the formation of a peptide bond by a condensation reaction is an endergonic process. These data do not rule out the physiological occurrence of such reactions, especially if the union of two peptides of moderate chain length is considered. In the absence of experimental evidence to the contrary, it would seem premature to discard the possibility of condensation reactions, as has been suggested at various times. Of special relevance is the consideration that the living cell is not a homogeneous system in which the chemical reactants are present in equilibrium concentrations. Hence, it seems reasonable to envisage the enzyme-catalyzed formation of interior peptide bonds

in condensation reactions that are "pulled" by subsequent processes, among which some may be strongly exergonic.[2] This type of energetic coupling has been clearly illustrated in model experiments. For example, in the synthesis of benzoyl-L-tyrosylglycinamide from benzoyl-L-tyrosine (0.025 M) and glycinamide (0.025 M), catalyzed by chymotrypsin, equilibrium is attained when only about 1% of the reactants has undergone condensation. On the other hand, if glycinamide is replaced by glycinanilide, the resulting benzoyl-L-tyrosylglycinanilide crystallizes from the solution in a yield of about 65%. The driving force in the formation of the anilide is its removal from solution, because, unlike the amide, the anilide has a solubility lower than 2.5×10^{-4} M. Thus, the endergonic synthesis of the peptide bond is coupled to the exergonic process of the removal of the peptide derivative from supersaturated solution. Model experiments of this kind, conducted with intracellular proteinases as catalysts, provide the principal support for the view that, in the synthesis of the peptide bonds of proteins, such "pull" mechanisms are operative. It should be emphasized, however, that there is no evidence from biochemical studies with intact cells or organisms that this type of energetic coupling is important in protein synthesis, although it must also be noted that no investigations have yet been conducted that permit an objective decision on this question.

As mentioned before, the studies on the energy changes in the hydrolysis and synthesis of peptide bonds have called attention to the difference in the $\Delta F°$ values for condensation reactions involving peptides, as compared with condensation reactions involving free amino acids. Since the latter reactions are, in general, more endergonic in character, it has seemed plausible to assume that, instead of a "pull" type of coupled reaction, the biosynthesis of a CO-NH bond between two amino acid residues is "pushed" by an exergonic reaction, such as the cleavage of a pyrophosphate bond of ATP. In some of the recent discussion of possible mechanisms of protein synthesis it has been implied that these two types of coupling are mutually exclusive. In the face of the available experimental knowledge, it would appear more profitable to consider the working hypothesis that both types of mechanism are involved in the biosynthesis of proteins from amino acids, but at different stages of the overall process. It is implicit in this hypothesis that peptides are intermediates in protein synthesis, a view that has been challenged recently on the basis of experimental findings to be discussed later in this essay.

Despite the attractiveness of the concept that the cleavage of pyrophosphate bonds of ATP is linked to the biosynthesis of the peptide bonds of proteins, it is not possible at present to specify the chemical nature of the "reactive" form of the amino acids. Efforts to demonstrate a direct enzyme-catalyzed reaction between ATP and the α-carboxyl group or the α-amino group of amino acids have been inconclusive thus far. Although carboxyl phosphates and phosphoamides of amino acids have been synthesized in the chemical laboratory, there is no evidence that they play a role in the biosynthesis of CO-NH bonds. Hence, in a discussion of the synthesis of proteins from amino acids, one cannot write even the first chemical reaction on the way to the completed protein, in the sense that one can specify that, in the biosynthesis of glycogen from glucose, the first step is the formation of glucose-6-phosphate in the glucokinase reaction.

Attempts to identify the "reactive" form of α-amino acids in protein formation have involved the study of the biosynthesis of the CO-NH bonds of compounds such as acetylsulfanilamide. These studies have been of exceptional importance to biochemistry, since they led to the discovery of coenzyme A (CoA) and have shown that, in the presence of suitable enzyme systems, the cleavage of ATP is associated with the formation of acetyl CoA.[3] Like other thiol esters, this acyl mercaptan reacts with amines such as sulfanilamide to form amides. Similarly, in the biosynthesis of hippuric acid, the acylating agent is benzoyl CoA, whose formation from benzoic acid and CoA requires the participation of ATP. However, in the instance of hippuric acid synthesis by animal tissues, the enzyme that catalyzes the reaction between benzoyl CoA and glycine appears to be specific for glycine as the amine. In view of the limited specificity of this enzyme, it cannot be assigned a general role in protein formation, although the synthesis of acylamino acids such as hippuric acid illustrates a biochemical mechanism whereby energy may be "pushed" into the formation of a CO-NH bond. However, there is no evidence as yet for the enzymic conversion of the α-carboxyl groups of free amino acids or peptides to thiol esters similar to acetyl CoA or benzoyl CoA.

In the search for alternative mechanisms that may be operative in peptide bond synthesis, attention has also been given to the formation of pantothenic acid from pantoic acid and β-alanine. Here, CoA does not appear to be involved, and it has been assumed that ATP is cleaved to adenosine monophosphate with the formation of a reactive "enzyme pyrophosphate" compound which reacts with pantoic acid to form a "pantoyl enzyme" compound; this, in turn, is believed to react with

β-alanine to form pantothenic acid. Lipmann [4] has suggested that a mechanism of this type may be involved in the synthesis of the polypeptide chains of proteins, but no experimental evidence for or against this possibility is available at present.

To the two mechanisms of "amino acid activation" proposed on the basis of studies on hippuric acid and pantothenic acid must be added the conclusions of Bloch and his associates, from their important work on the biosynthesis of glutathione.[5] The synthesis of the two CO-NH bonds proceeds in separate steps, in each of which one equivalent of ATP is required; all efforts to demonstrate a role for CoA appear to have been unsuccessful. In contrast to the synthesis of pantothenic acid, the formation of glutathione from the component amino acids is accompanied by the liberation of one equivalent of phosphate, and not pyrophosphate, per CO-NH bond formed, indicating that ATP is cleaved at different pyrophosphate bonds in the two processes. Furthermore, the studies of Speck and of Elliott have shown that, in the biosynthesis of glutamine from glutamic acid and ammonia, which also requires the participation of ATP, inorganic phosphate is formed, as in the synthesis of γ-glutamylcysteine from glutamic acid and cysteine in glutathione formation.

These studies on the biosynthesis of pantothenic acid, glutathione, and glutamine have all led to the working hypothesis that the role of ATP is to make possible the formation of a reactive form of an amino acid, as for example in a "pantoyl enzyme" or a "γ-glutamyl enzyme," where the acyl group is attached to the enzyme protein at a suitable site (e.g., the sulfhydryl group of cysteine, the imidazolyl ring of histidine, etc.). However, no general scheme for the role of ATP in the formation of the acyl enzyme compounds can be offered at present. Nevertheless, the hypothesis that such reactive acyl enzyme compounds are intermediates in the biosynthesis of the peptide chains of proteins is an extremely attractive one and finds support in work to be discussed later in this essay.

In the face of the fragmentary knowledge currently available, it would seem desirable to extend the study of the synthesis of CO-NH bonds in simple compounds to an examination of the metabolism of α-amides of amino acids. Until recently, compounds of this type were not considered to occur in nature, but the demonstration of a terminal glycinamide in oxytocin and vasopressin [6] confers upon such α-amides increased biological interest. Calorimetric studies have shown that the ΔH of hydrolysis for the $CO-NH_2$ bonds of acylamino acid amides is of the same order of magnitude as that found for inorganic pyro-

phosphate.[7] The α-amides of amino acids thus belong to a group of compounds that may be loosely termed "energy-rich" amides, in analogy to the widespread use of the term "energy-rich" phosphate compounds. Systematic studies do not appear to have been made to determine to what extent such α-amides are present in tissues and biological fluids.

Whatever may be the mechanism whereby the exergonic cleavage of ATP is coupled to the endergonic synthesis of proteins from amino acids, the elucidation of this energetic coupling is only a part of the total problem of protein formation. Of equal, if not greater, importance is the task of describing the cellular apparatus responsible for the synthesis of a protein in terms of the chemical specificity that leads to the characteristic arrangement of amino acid residues in the intricate sequence revealed by the systematic degradation of the protein. The complexity of the structure of proteins makes the biosynthesis of these polymeric molecules a unique phenomenon, whose study is made formidable by the difficulties encountered in demonstrating it in cell-free systems, as has been done for the biosynthesis of glycogen from glucose; here, the work of the Coris and their associates has clearly established the nature of the individual catalytic components of the multienzyme system involved in glycogen formation. Although there is general agreement that the biosynthesis of proteins is also an enzyme-catalyzed process, our ignorance of its details have led to widely conflicting speculations about its nature. Specifically, it is frequently argued that protein synthesis does not involve a multienzyme system which converts amino acids, via intermediates, to the peptide chains of a protein. The proponents of this view suggest that the "activated" amino acid units align themselves along a "template" (frequently considered to be a nucleic acid), and the alignment is followed by the simultaneous formation of all the peptide bonds by an undefined catalytic process, with the subsequent release of the completed protein from the "template." It would seem that, if this is indeed the case, and if it should prove impossible to dissect the enzymic apparatus responsible for protein synthesis, the successes achieved in the analysis of other biochemical syntheses may not be attainable in this field. The question may be raised, however, whether this pessimistic view is justified by the experimental evidence now available.

Support for the "template" hypothesis has come largely from studies on the incorporation of labeled amino acids into the proteins of intact animals and on the formation of bacterial enzymes. Work from several laboratories has demonstrated conclusively that, if one or more isotopic

amino acids are administered to an animal, and if discrete proteins are isolated from the tissues or body fluids, every residue of a given labeled amino acid in the peptide chain has the same isotope content. In a striking experiment, Simpson and Velick [8] administered to a rabbit five isotopic amino acids, and 38 hours later isolated from the muscle highly purified samples of two enzymes (aldolase and glyceraldehyde-3-phosphate dehydrogenase). Upon analysis of 11 of the amino acids obtained after hydrolysis, these investigators found that the ratio of specific radioactivity of each of these amino acids in aldolase to the specific activity of the same amino acid in the dehydrogenase was constant. Subsequent work by Simpson, in which the animal was sacrificed 30 minutes after the administration of the labeled amino acids, gave essentially the same result. The logical conclusion drawn from these experiments, and from similar studies performed by others (Neuberger et al., Work et al.), is that the systems involved in the synthesis of the proteins under investigation were drawing on a single "pool" of each of the amino acids. Although these experimental findings are in accord with the "template" hypothesis, it has been pointed out that they may also be interpreted in terms of either the rapid successive formation of peptide bonds without the release from the protein-synthesizing system of intermediates that accumulate, or the existence of very small "pools" of intermediate peptides that equilibrate rapidly with the amino acid "pools."

Experiments on the induced formation of bacterial enzymes, especially those reported by Monod and by Spiegelman, have also been interpreted to indicate that the synthesis of adaptive enzymes proceeds by a "template" mechanism, without the intervention of peptide intermediates. For example, it has been reported [9] that, in the induced formation of β-galactosidase during the growth of *Escherichia coli*, the enzyme protein is derived from free amino acids in the medium rather than from products of the cleavage of pre-existent bacterial proteins. Here again, the results are consistent with the "template" mechanism, but it is difficult to accept them as proof for its existence, so long as alternative interpretations are not excluded. Other studies in bacterial systems, notably those of Gale,[10] have focused attention anew on the role of nucleic acids in protein synthesis and their possible function in serving as "templates." That there is an intimate metabolic relation between cellular nucleic acids and cellular proteins cannot be denied, but it seems premature to interpret the available knowledge as favoring the role of nucleic acids as "templates" in protein synthesis, especially since the "template" hypothesis itself is so insecurely founded.

Of special importance in regard to the question whether peptides are intermediates in protein synthesis are the experiments of Anfinsen and his associates, who have studied the incorporation of labeled amino acids into ovalbumin by minced hen oviduct or into insulin and ribonuclease by beef pancreas slices.[11] In contrast to the results of in vivo studies, the residues of a given amino acid were found to be unequally labeled along the peptide chain of each of these proteins, a finding consistent with the view that peptides are metabolic intermediates between amino acids and proteins. Anfinsen has suggested that the apparent discrepancy between the results of in vivo and in vitro experiments may be ascribed to differences in the rates of metabolic reactions in the two types of systems. It would seem inescapable that the removal of a tissue from the intact animal would lead to a profound alteration in the rates of those enzymic processes that are influenced by such factors as hormonal regulation and normal blood flow. Thus far, no one has reported comparative experiments, at several time intervals, in which a labeled amino acid was incorporated into the same protein in vivo and in vitro and the pattern of labeling along the peptide chain was determined in each case. Such studies are needed to clarify the discrepancy between the results obtained in the living animal and in tissue preparations.

Clearly, the available data on amino acid incorporation into proteins do not permit one to draw any definite conclusions about the chemical pathways of protein synthesis. However, it would seem more likely that useful clues will come from the continued study of those biological systems in which non-uniform labeling of peptide chains is observed and where one may expect the occurrence of well-defined intermediates between amino acids and proteins. It is frequently stated that peptides (other than glutathione, carnosine, etc.) are not present to a measurable extent in mammalian or bacterial cells, but the validity of this assertion may be questioned, since it cannot be reconciled with the numerous reports in the literature on the isolation or identification of peptide material from animals, plants, and microorganisms.[12] Where negative results have been obtained in the search for peptides as cellular constituents, the possibility must be considered that the methods applied were not adequate; for example, because of failure to react with ninhydrin or because of tight binding to the cellular proteins. Also, the question of the variations in the peptide level, which may depend on the physiological state of the cell, may be of importance but cannot be properly assessed at present. A logical difficulty in the

view that peptides are absent from living cells is presented by the
problem of the chemical mechanism of the intracellular degradation
of proteins.   If peptides are not intermediates in this process, is it
suggested that protein degradation, like protein synthesis, occurs on a
"template"?

In the face of the present situation in regard to the question of
specificity in the biosynthesis of proteins, it seems profitable to employ
model systems as objects of study as has been done in attempts to
determine the role of ATP in peptide synthesis.   The model systems
that have proved to be of special interest have involved tissue pro-
teinases as the catalytic agents and, as substrates, peptides or amino
acid derivatives of well-defined structure.   It has long been customary
to speak of proteinases solely as catalysts of hydrolytic reactions, but
the work of the past few years has shown clearly that this is too
restrictive a formulation since proteolytic enzymes are now known to
catalyze replacement (or transfer) reactions of the following type:

$$RCO—NHR' + NH_2X \rightleftharpoons RCO—NHX + NH_2R'$$
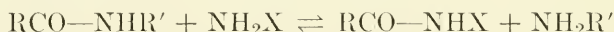
Such reactions have been termed "transamidation" or "transpep-
tidation" reactions.[13]   Extensive studies of the action of purified pro-
teinases (e.g., chymotrypsin, papain, ficin, cathepsin C) in catalyzing
the hydrolysis of peptide derivatives have defined many of the struc-
tural features required in the substrate for enzymic action.   All experi-
ments performed thus far on the catalysis of transamidation by these
proteinases have shown that the specificity with respect to the struc-
ture of the compound containing the sensitive CO-NH bond is the
same for transamidation as for hydrolysis.   However, in the catalysis
of replacement reactions, where water is no longer the common reactant,
the nature of the amine that serves as the replacement agent also is
important in defining the specificity of enzyme action.   For example,
in the reaction catalyzed by the plant proteinase papain, in which
carbobenzoxyglycinamide is the component having the sensitive CO-
NH bond (only the terminal CO-NH$_2$ bond is susceptible to enzyme
action), and glycylglycine, L-leucylglycine, or D-leucylglycine is the
replacement agent, only about 13% of the amide reacts with glycyl-
glycine under conditions (pH 7.5, 37°) where about 65% of the amide
reacts with L-leucylglycine, and about 8% with D-leucylglycine.[14]   The
extent of transamidation observed in each of these three experiments is
a measure of the relative efficiency of the replacement agent in compet-
ing with water for reaction with the amide.   In the case of the papain-
catalyzed interaction of carbobenzoxyglycinamide and L-leucylglycine,

$$\text{Cbzo-NHCH}_2\text{CO—NH}_2 + \overset{\overset{\text{R}}{|}}{\text{NH}_2\text{CHCO}} - \overset{\overset{\text{R}'}{|}}{\text{NHCHCOOH}} \rightleftharpoons$$

$$\text{Cbzo-NHCH}_2\text{CO} - \overset{\overset{\text{R}}{|}}{\text{NHCHCO}} - \overset{\overset{\text{R}'}{|}}{\text{NHCHCOOH}} + \text{NH}_3$$
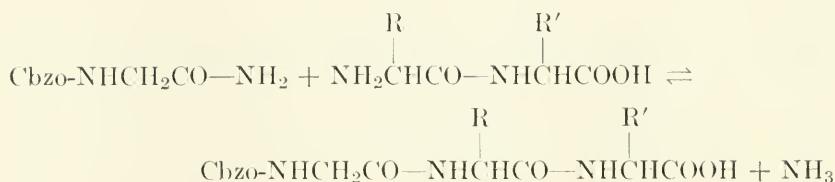
the replacement agent is so much more effective than water at pH 7.5, despite its lower molar concentration, that the predominant reaction is one of replacement rather than hydrolysis. On the other hand, the enantiomer D-leucylglycine is much less effective in this competition, as is glycylglycine. Results of this kind show clearly that, in transamidation reactions, the specificity of enzyme action is more exacting than in hydrolysis.

Studies on the mechanism of transamidation reactions have demonstrated that it is the uncharged amino group of the replacement agent that is reactive; the extent of transamidation at a given pH depends, therefore, on the pK' of the corresponding cation.[15] For this reason, the extent of transamidation observed with the above dipeptides (pK'_2 ca. 8) is much greater at pH 7.5 than at pH 5, the optimum for the hydrolytic action of papain.

The reaction between carbobenzoxyglycinamide and L-leucylglycine illustrates the replacement of a small group ($-\text{NH}_2$) by a dipeptide unit, thus effecting the elongation of the peptide chain from the carboxyl end of the sensitive substrate. In general, transamidation reactions in which an α-amide is converted to a peptide appear to be exergonic reactions, as suggested by the thermodynamic data presented in Table 1. The exergonic nature of enzyme-catalyzed elongation of peptide chains may be further illustrated by results obtained with the intracellular proteinase cathepsin C, purified from beef spleen. This enzyme only acts on derivatives of dipeptides composed of two α-amino acid residues (X may be either NH_2, as in an amide, or OCH_3, as in an ester). If cathepsin C is allowed to act on a dipeptide amide such

$$\overset{\overset{\text{R}}{|}}{\text{NH}_2\text{CHCO}} - \overset{\overset{\text{R}'}{|}}{\text{NHCHCO}} - \overset{\downarrow}{\text{X}}$$

as L-alanyl-L-phenylalaninamide at pH 7.5, the extent of hydrolysis is negligible and there separates from the solution a precipitate which, on chemical analysis, was found to be the amide of the hexapeptide

(L)Ala.(L)Phe.(L)Ala.(L)Phe.(L)Ala.(L)Phe.[16]
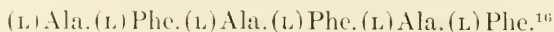
This product was obtained in a yield of 86% of the theory, based on the amount of NH_4^+ liberated. Clearly, what had occurred was the proteinase-catalyzed polymerization of the dipeptide amide by two successive transamidation reactions. It is extremely probable that at each step, an alanylphenylalanyl unit was added to the amino end of the growing peptide chain, in a manner analogous to the action of crystalline muscle phosphorylase, which catalyzes the successive addition of glucosyl units of glucose-1-phosphate to the non-reducing end of a growing amylose chain.

There can be no doubt, therefore, that the known intracellular proteinases can catalyze transamidation reactions in which peptide chains are lengthened and that these enzymes exhibit considerable specificity in these reactions. However, no information is available about the role of the cathepsins in the metabolism of intact animal cells, and nothing can be said at present about the actual occurrence of transamidation reactions in the biosynthesis of proteins. All that can be stated is that the intracellular proteinases are the only well-defined cellular catalysts known to exhibit the specificity to be expected of enzymes that could participate in protein synthesis. One may therefore propose, as a working hypothesis, that a multienzyme system, composed of a number of intracellular proteinases, is responsible for the specific elongation of the peptide chains of proteins. According to this view, the characteristic sequence of amino acid residues in peptide chains is the consequence of the coupled action of a series of proteinases that differ in specificity. An objection that has been raised against this proposal is the difficulty of imagining a sufficiently large number of proteinases that may be expected to be needed to make one type of protein and the much larger number required for the synthesis of all the different proteins. However, there appears to be no valid reason (other than the search for simplicity) against the participation of a series of proteinases in the synthesis of a protein. Also, there does not appear to be any a priori objection to the possibility that a given proteinase could participate in the synthesis of more than one protein. Certainly it would be more in accord with the knowledge gained from the study of other biochemical processes, involving the metabolic synthesis or degradation of molecules much simpler than the proteins, to assume that a multienzyme system is involved. Such an assumption is not incompatible with the experimental results that have led to the formulation of the "template" hypothesis. If the term "template" is considered to represent an organized cellular assembly of enzymic catalysts (plus other constituents such as nucleic acids)

that rapidly catalyzes a series of successive peptide syntheses without releasing into the cellular fluids appreciable amounts of peptide intermediates, and if the nature of this assembly is genetically controlled, then much of the difference in opinion becomes a difference in language.

The significance of the proteinase-catalyzed transamidation reactions that have been studied thus far does not lie in demonstrating processes involving peptide derivatives known to occur in nature, but rather in suggesting new approaches to the study of the energetics and specificity of the enzymic synthesis of peptides. To convert amino acids into a form that is capable of reacting with other amino acid residues, the participation of ATP is required, as has clearly been shown in the studies on the biosynthesis of peptides such as glutathione. On the other hand, once the γ-glutamylcysteine bond of glutathione has been formed, it is capable of reacting with other amino acids or peptides in transpeptidation reactions, as has been shown by Hanes[17] and by Waelsch.[18] Similarly, in the synthesis of glutamine, ATP is required, but, once formed, the $CO-NH_2$ bond can react, in the presence of suitable enzyme preparations, with amines such as hydroxylamine or amino acids (Speck, Elliott, Thorne, and Williams). The most plausible interpretation of these findings has been the suggestion that a "γ-glutamyl enzyme" is an intermediate both in the synthesis of glutamine from glutamic acid and in the transamidation reactions of glutamine.

In the light of these important studies on the intimate relation between the direct synthesis of $CO-NH$ bonds and transamidation reactions in which they may participate, it seems justifiable to extend the hypothesis drawn from the reactions of γ-glutamylcysteine and of glutamine to include the action of intracellular proteinases. This would involve the assumption that, in the catalysis by papain of the reactions of carbobenzoxyglycinamide, there is formed as an "activated" intermediate, a "carbobenzoxyglycylpapain," that can react either with water or with an amine such as L-leucylglycine. Similar "acyl enzyme" compounds could be invoked in the case of other transamidation reactions studied. It may be expected that such "acyl enzyme" compounds would be formed more readily when the reactive carboxyl group is joined in a bond that has a relatively high free energy of hydrolysis. This appears to be the case with the α-amides of acylamino acids and peptides.

As noted earlier in this essay, little is known about the enzymic mechanisms whereby free α-amino acids are "activated" in reactions involving ATP. Until direct evidence for such mechanisms is avail-

able, it seems plausible to assume, as was suggested some years ago,[13, 19] that the coupling between the energy-yielding processes of the cell and the synthesis of the peptide bonds of proteins may be funneled through a relatively small number of amides or peptides such as glutamine or glutathione. At best, this view can still be considered only a working hypothesis. However, it seems a useful concept in attempting to discern new experimental approaches to the difficult task of elucidating the chemical pathways in the biosynthesis of peptide bonds.

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On the Nature
of Cancer

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Cancer is a familiar clinical problem, but its definitive inherent characteristics are still obscure. Many experiments have attested to the similarities between cancerous and normal tissues, but few have revealed unique disparities on which therapy could be based. Under such circumstances imaginative speculation within the framework of evidence is a necessity unless we are to resign ourselves to blind empiricism in the search for the cure for cancer. The biochemist in cancer research soon finds himself in an ever-widening circle of excursions into tangential disciplines in his search for a clue to the intrinsic nature of cancer, for it is only with the aid of observations apparently far removed from formal biochemistry that an hypothesis leading to therapy can be derived.

Cancer is a cellular aberration distinguished by autonomy and anaplasia, that is, by disregard for normal limitations of growth and by loss of normal organization and function. In this essay we will try to clothe these dry definitive bones with experimental conclusions and with plausible speculations, parading our conceptions of the origin of cancer and of possible chemotherapy whenever they contribute to this end. In effect, we set forth a comprehensive hypothesis on *the nature, the cause,* and *the cure* for cancer.

The wide prevalence of cancer among animals permits of controlled experimentation to a degree which is unusual among other degenerative diseases. Carcinomas, sarcomas, lymphomas, leukemias, as solid tumors or as ascitic suspensions, are readily available in variety as regards both host and growth characteristics. Many of these tumors occur spontaneously, and practically all of them can be maintained by serial passage in appropriate hosts. Some workers have the view that it is immaterial which tumor is selected for research, that they

are all fundamentally identical, and that the eventual cure for one is the cure for all. It is the immediate experiment which dictates the choice of tumor or tissue. The many types of cancer enumerated, as well as those occurring in humans where experimental access is not as good, differ markedly in their transplantability, their anaplasticity, and their capacity to grow rapidly. Rapid growth alone, however, is neither a necessary nor a sufficient criterion for cancer; many cancers grow very slowly. Many normal tissues, on the other hand, are relatively hyperplastic or may become so under non-carcinogenic stimuli.*

Embryonic development too consists of rapid cellular multiplication in good part, at least in early phases. It may be instructive, therefore, to compare the growth of a cancer with that of a frog embryo. The latter is comprised of a closed system containing a reservoir of metabolites sufficient for development to proceed to a fairly advanced stage without external nutritional supply. Fertilization stimulates the egg to rapid and orderly cleavage, differentiation, and growth, some phases of which can be studied as discrete processes even though they are not sharply separated in time. Early cleavage is signaled by an increase in oxygen consumption which rises gradually throughout development. The fertilized egg continues to divide without apparent morphological differentiation through the blastula stage, the total mass remaining constant, and segmentation producing progressively smaller cells. Each new cell, however, has a nucleus and nuclear apparatus visibly equivalent to the original single-cell nucleus. It might appear to the microscopist that DNA is being made at a prodigious rate, but, in fact, there is no synthesis of DNA at all in this early phase of development. Nor is synthesis necessary, for it has been shown that the unfertilized egg already contains an enormous amount of DNA, many times, indeed, the amount of DNA which could be accommodated in one nucleus.[1] This is consistent with the fact that the total purines in the embryo are quite constant in amount in the unfertilized egg, the neurula, blastula, and early gastrula stages through which nuclei have been replicated perhaps 40,000-fold.[2] Early cleavage in the frog embryo, then, entails only the reorientation and redistribution of DNA which was preformed in the ovary during maturation. The embryo does not have to bear the burden of synthesis of DNA until comparatively late in development, that is, until the late gastrula stage when differentiation commences. Early cleavage, where no growth or synthesis occurs,

* It is characteristic of carcinogens that the malignant change persists even after the stimulus is removed.

is not expensive in energy. Early respiration only adds to the store of high-energy intermediates which are reserved for the endergonic syntheses of growth and differentiation.

Although the dividing embryo has a high respiration rate, the egg can divide just as well under anaerobic conditions.[3] Some high-energy phosphate bonds are split during anaerobiosis indicating that at least a little energy might be required for cleavage, but glycolysis alone is sufficient to meet this moderate demand. No growth or synthesis takes place during the time when glycolysis alone can supply all of the energy needed.*

The cancer cell, on the other hand, has an inordinate growth obligation which the early dividing embryo does not have. Even so, it lacks not only the reserve of substances which appear to be indispensable for growth and differentiation but also the capacity to make them. The vaunted capacity of cancer cells for anaerobic glycolysis is indeed a drastically insufficient compensation for the inadequacy of their oxidative energy generation mechanisms. So sluggish is the cancer cell aerobically that its only alternative in the face of the excessive demands is to parasitize the host for the necessary energy cycle intermediates, and in this way impair the host further, by reduction of the liver catalase,[4] or by damaging the heart,[5] for example. This inertia of the cancer cell was demonstrated in striking fashion by the experiments of Busch and Baltrush,[6] in which it was found that tumors are incapable of metabolizing either acetate or pyruvate. It has been shown also that the rate of growth of tumor implants is rigorously limited by vascular supply,[7] a fact which also suggests that cancer growth rates depend on parasitic diversion of oxidatively generated factors. It has been observed clinically also that the most rapidly growing tumors are those in blood-rich areas.

In tissue culture too it has been noticed that tumor cells will grow only in the vicinity of the surface, whereas normal cells will often grow throughout the medium, a fact which also can be interpreted to mean that innate glycolysis alone, however large, is insufficient for growth, and that the growing tumor cell must use oxygen. The usual tissue-culture practice involves a discontinuous process which results in alternately feasting and starving the cultures, these rigors being only meagerly compensated for by the frequency of transfer to fresh media. The cells in the center of a hanging-drop culture, for example, sooner or later begin to suffer from oxygen deprivation and intoxication

* High rates of glycolysis are a feature of growing tissues irrespective of malignancy, but no *growing* tissue can survive long under anaerobic conditions.

and become necrotic. This, also, is the way most tumors behave in the intact animal.

Cancer cells, then, appear to be members of a race deficient in oxidative mechanisms.* Their deficiency or immaturity is a permanent inheritable factor. Cytoplasmic inheritance controls differentiation. The mutant we call cancer might well be characteristically deficient in cytoplasmic high energy conversion enzyme systems which are required for differentiation. The different effects of aerobiosis and anaerobiosis could be a reflection of the relative efficiency of the two processes. A 50% difference in oxygen tension such as obtains at an altitude of 20,000 ft. can spell the difference between life and death to an unacclimatized individual.

The Himalayan expeditions have brought into sharp focus the fact, previously established in the laboratory, that human beings as well as animals, adults at least, can be adapted by suitable acclimatization, not only to survive, but also to do hard work in the rarefied atmosphere of altitudes 20,000 ft. or higher. But when acclimatized animals were implanted with tumors and then returned to high altitude the growth of tumors was markedly inhibited.[9] In many instances the tumors regressed completely, and the animals were permanently cured. It seems as though frustration of either division, growth, or differentiation must lead inevitably to cell death. There is no alternative to completion of these developmental compulsions. It appears to be impossible for embryonic cells or tumor cells to revert to resting stages once chromosomal rearrangement for division or cytoplasmic mobilization for growth or differentiation have taken place. A cell is most vulnerable during developmental change, whereas resting adult cells are far more resistant. The widely accepted rule of thumb that cells undergoing active mitosis are more sensitive to lethal agents such as X rays than are resting cells is a restatement of the proposition that a cell dies if its division under urgency is blocked. The energy from glycolysis plus only a modicum of oxidatively generated energy might be sufficient for acclimatized resting host cells but hardly so for voracious growing cells. Cancers or other obligatorily growing cells cannot survive partial anoxia; they cannot be acclimatized as can resting cells.

It does not follow from the above, however, that the cause of cancer is necessarily related to interference with normal oxidative behavior. Mutagenesis is a sine qua non of carcinogenesis. Mutagens are non-

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* This conclusion has been reached before from other considerations. Van R. Potter is well known as an early and active protagonist of the oxidative deficiency hypothesis in cancer.[8]

specific in their effects. Cancer is only one of the many possible muta-
tional responses to injury or other embarrassment of normal functional
processes. Haddow has suggested that malignant transformation is
an artifact of adaptation, that a carcinogen which cannot be lethal
or very virulent provokes an adaptive response in a cell which is
selective within its capacity to retain the carcinogen or in its suscepti-
bility to interference by the carcinogen. The cell can evade for a
long time, but under continued assault or with the passage of time
it succumbs to give rise to a race of mutants which lack the ability
to mature. The price of survival by mutation is high; the price is
the loss of those functional endowments which are presumably pre-
empted by carcinogens. The azo dye carcinogens, for example, have
been described as depriving liver cells of those proteins required for
growth regulation.[10] Thereafter the descendants of the afflicted cells
no longer have the wherewithal to differentiate like normal liver cells.
Many of the chemical carcinogens are known to behave as mutagens
in other biological systems as well.

It appears reasonable to suppose that those mutagens which incite
cancer should exist widely in nature rather than in the laboratory or
in highly industrialized surroundings alone. We shall therefore pay
particular attention to the naturally occurring tumors, rather than
those produced by chemical agents, in tracing the cause of cancer.

Among the animal tumors the mammary carcinoma of the mouse is
of especial interest because it bears more than a superficial resemblance
to mammary carcinoma in the human. This disease occurs at random
among wild mice, but its incidence is truly devastating among certain
highly inbred laboratory strains, occurring in 96% of the Bar Harbor
C3H and the Paris R III strains in adult life. Crossbreeding experi-
ments once made it appear that the disease was an inherited condition,
but on closer scrutiny of the data it became necessary to postulate
an extrachromosomal factor in the transmission of this form of cancer.
From this postulate emerged one of the significant landmarks of cancer
research. By the elegantly simple expedient of foster nursing Bittner
in 1936 demonstrated that this extrachromosomal factor was trans-
mitted with the milk of the nursing mother. When newly born C3H
(high cancer strain) females were foster-nursed on C57 (low cancer
strain) foster mothers the incidence of mammary carcinoma in the
subsequently matured C3H mice was reduced markedly. Conversely,
when the newly born C57-strain females were nursed on C3H foster
mothers cancer of the breast appeared in many of them on maturity.[11]

It is significant that in these foster-nursing experiments the C3H

foster mothers had not developed any visible or palpable sign of cancer at the time they nursed the young C57s. Indeed, the first tumor appeared only after the passage of several months. We can therefore be certain that there had been no transfer of cancer cells to the nurslings, since, if there had been, the transferred cells would have erupted into full-grown tumors in a matter of days rather than of
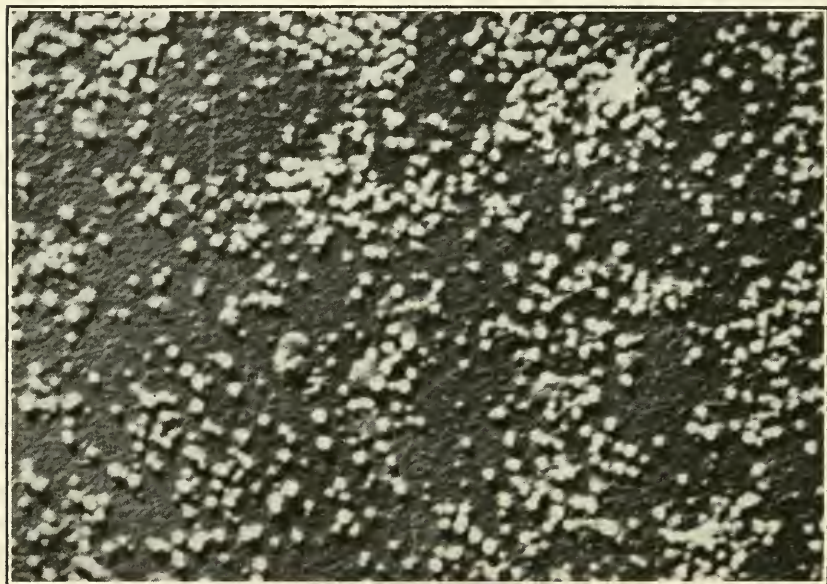
Fig. 1. Electron micrograph of mouse mammary carcinoma virus as purified by convection-current electrophoresis. The drop preparation was shadowed at $\tan^{-1}$ 12° with chromium. Magnification 17,500× (McCarty-Graff).

months. The conclusion is inescapable; mammary carcinoma in mice, a disease of the adult female, is transmitted from mother to offspring by an infectious agent in the milk. Complete confirmation of this conclusion was provided by the isolation and characterization of the virus in the author's laboratory.[12] The viral bodies pictured in Fig. 1 were isolated from high cancer strain milk. The inoculation of these particles is equivalent to the ingestion of "infected" milk.

Now, Rous had already shown, as far back as 1911, that cell-free extracts of chicken sarcoma were able to reproduce the disease on inoculation into new hosts.[13] The comprehensive implications of Rous and Bittner's discoveries are not widely appreciated, but subsequent

developments now compel serious consideration of the proposition that *all forms of cancer are cellular reactions to infectious processes.*

Viruses have already been implicated in a number of other animal cancers, including leukemia, which also has a human parallel. The techniques employed in the animal-tumor experiments, however, are not applicable to human beings since cancer viruses like the cancers they cause are species specific and variable in form and behavior. Perhaps the only applicable property common to all viruses is that they propagate. Fortunately the intact animal host is not essential; suspensions of living cells, tissue cultures, can support the growth of viruses in many instances. It is important to distinguish between the cause of a particular form of cancer, a virus perhaps, and the cellular reaction to that virus infection, the cancer. Often both can be separately cultivated, transplanted, and observed. Tissue culture, now in the full bloom of its renaissance, appears to offer the means for elucidation of the role of viruses in human cancer and also of the metabolic deviations which are the inherent characteristics of cancer. Experiments are in progress wherein human secretions are admixed with tissue cultures on the expectancy that carcinogenic viruses, if present, will reveal themselves either by increase in number of particles or by malignant transformation of host cells.

It is intrinsic to our virus hypothesis that the presence of the virus in the cancer cell is wholly fortuitous, since the mutation produced by virus aggression is self-perpetuating. Although the malignant transformation appears to be an escape mechanism the malignant cell itself is not necessarily unpropitious for viral existence. Porter [14] was able to obtain the beautiful electron micrograph reproduced as Fig. 2 by tissue culture of mouse mammary carcinoma cells in which the virus was still present. This picture demonstrates the exuberant growth of the virus in a cell. Note the resemblance to the isolated particles of Fig. 1.

Now, Bittner's informative foster-nursing experiments established a procedural pattern for other workers who were in possession of inbred strains of animals with high incidence of one form of cancer or another. MacDowell of the Carnegie Institution had been investigating the genetic aspects of leukemia which was endemic in his C58-strain mice when he undertook to test the possibility of milk transmission of the disease by reciprocal foster nursing with Storrs-Little strain which was free of leukemia. His first experiments were inconclusive, and so he stubbornly repeated the experiment, this time with larger numbers. In so doing, however, it was necessary for him to employ all of the

foster mothers he had available, including a number of older mice. The second experiment also failed to throw any light on the *trans- missibility* of the disease, but MacDowell did observe that the inci- dence of leukemia was somewhat diminished in those C58 mice which had been nursed on *old* Storrs-Little mothers. This observation was verified in a third experiment wherein old foster-mother Storrs-Littles
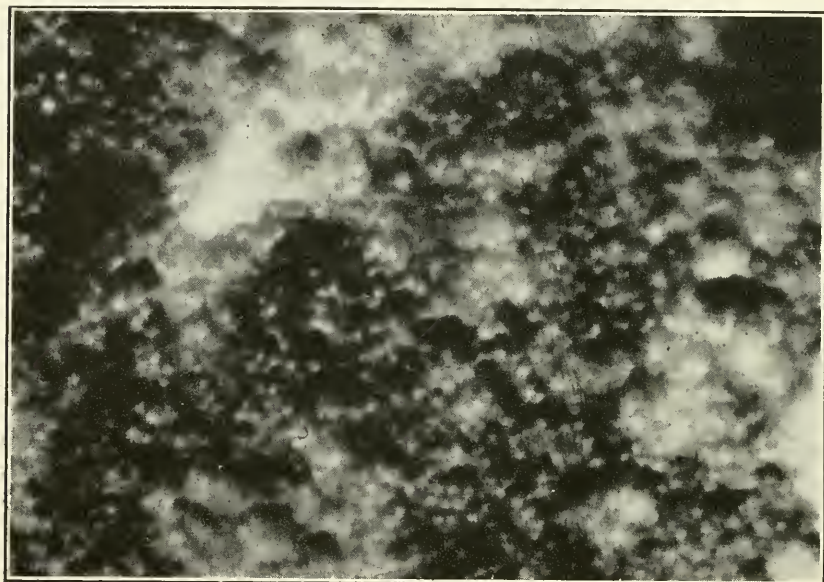
Fig. 2. Electron micrograph of a preparation from a 4-day-old culture of a spon- taneous mammary tumor explant fixed over osmic acid shadowed with gold at $\tan^{-1} 10°$. Magnification $17,500\times$ (Porter and Thompson[14]).

were used exclusively.[15] These experiments established that the milk of old Storrs-Little mice contains a substance which is either prevent- ative or curative of leukemia. Leukemia, like mammary carcinoma, is a disease of the adult mouse. The transmission of the causative agent, whatever it might turn out to be, occurs antenatally or in nursing, probably the former. Leukemogenesis takes place over a period of time. The milk inhibitor was given early in this period, that is, before the leukemic state. It can be inferred then that Mac- Dowell's substance is prophylactic rather than therapeutic. Experi- ments on the isolation and characterization of the substance are still in progress at this writing, but it seems reasonable to suppose that this substance acts by compensating for a deficiency produced by the leukemogenic mutagen.

The deficiencies of cancer, stressed in this article, contrast strongly with the assumed totipotency of cancer so implicit in the concept of autonomy. Chemotherapy would be a hopeless endeavor indeed if the normal cells were forced to compete with complete autotrophes which could avail themselves not only of simple substrates but also of numerous alternate metabolic pathways. The tolerance of adult differentiated host cells to most drugs is limited. Although they do not require as much energy as do growing cells they cannot adapt to radical changes in their chemical functional environment. Indeed, it may be just such adversity which produces cancer! Curiously enough it has been suggested and also actually demonstrated that weak or very poor carcinogens are inhibitors of cancer.

Cancer prevention in man is so far restricted to the elimination of carcinogens from the diet and from the immediate environment. But if it should be found that viruses do in fact play a major role in the dissemination of cancer, then presumably antisera to specific viruses and as yet undeveloped antibiotics would find employment in diagnosis and prophylaxis. It is perhaps fortuitous that some of the experimental drugs against cancer, 8-azaguanine, for example, are also antiviral. The practical aspects of the problem, however, limit therapy to the treatment of cancer after diagnosis has been made, and, indeed, without respect to the precedent chain of events. It must be presumed that diagnosis will not be made until the cancer is well established and even metastatic. Therapy then consists in selective attack on neoplastic tissue.

Most of the effort in design and synthesis of compounds which will either destroy or modify cancer cells *preferentially* is devoted to growth antagonism. Preferential, because normal differentiated cells do not have to divide; cancer cells apparently do. Growth antagonism is often thought of as being synonymous with interference with nucleic acid metabolism. This easy assumption is probably an oversimplification, but it has given rise to many new compounds and a number of interesting biological applications.

Animal cells make their nucleic acids from amino acid; there is no nutritional requirement for either preformed purines or pyrimidines. Adenine is exceptional among the nucleic acid bases, since it is the only one which is accepted and incorporated by the metazoans. Part of this adenine is converted to polynucleotide guanine. Guanine itself, on the other hand, is not incorporated at all. The protozoan *Tetrahymena geleii*, however, has an obligatory requirement for guanine. Kidder, who had interested himself in the dietary requirements of this

organism, also found that 8-azaguanine was a growth inhibitor of *T. geleii* by competition with guanine.[16]   He then discovered that this same compound would inhibit the growth of certain mouse tumors. How or why this drug inhibits tumors is still unknown.*   Tumors inhibited by 8-azaguanine are not arrested metabolically; their nucleic acid turnover is approximately the same as uninhibited tumors. Doses of the drug which might be large enough to effect cure cannot be tolerated by the host. The drug has absolutely no effect on some tumor types such as sarcoma 180. Thus all tumors are not the same, and the selection of tumors for experimentation is not entirely a matter of convenience or indifference.

8-azaguanine, nevertheless a good experimental carcinostatic agent, is useless for human therapy because of its limited solubility and because of its toxicity. These objectionable features led to a number of synthetic maneuvers in an effort to increase the solubility of the active groups, to decrease the toxicity, and above all to increase the activity. Acetylation of the OH group proved to be futile; the ester was inactive biologically, apparently because hydrolysis did not occur with sufficient speed. Neoazaguanine, the sulfoxalate (by analogy with neoarsphenamine) was also inactive. What was most disappointing, however, was the inertness of the two pyrazolopyrimidines, 5-amino-7-hydroxypyrazolo-4,3-*d*-pyrimidine and 6-amino-4-hydroxy-pyrazolo-3,4-*d*-pyrimidine, both isomeric with guanine.

The high expectations for the latter compounds arose from an overly naive reliance on the applicability of competitive inhibition to a completely unknown metabolic system. A competitor is an agent which differs so subtly from a required metabolite that it is accepted without discrimination in the initial incorporative reaction. It is only in the events which follow the diversion of enzymes that the cell discriminates between the true and the false, and biosynthesis grinds to a halt. If the proffered compound differs so subtly from its natural analog, there may be no distinction at all and the cells may utilize the substitute with equal facility. If, on the other hand, the alteration is too gross the compound is completely ignored in the first instance. Even

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* One could now surmise that 8-azaguanine acts by inhibiting an oxidatively generated factor. If this were true, it would follow that the drug would act synergistically with hypoxia, and that its toxicity would be diminished after acclimatization of the host to hypoxia. Furthermore, Drs. Philip Feigelson and J. D. Davidson of this institution have adduced strong evidence that the carcinostatic activity of 8-azaguanine depends on its capacity to inhibit xanthine oxidase which appears to be necessary for the biosynthesis of guanine. (Personal communication.)

when compounds do bring about biological effects they may do so by interference in such involved reactions as to preclude any facile idea of single competitive inhibition. Although 8-azaguanine, its analogs, and the compounds described in the following bear some resemblance on paper to the naturally occurring purines it would be foolhardy indeed at this time to ascribe their activity to structural similarity. Furthermore, there is even no conclusive evidence that simple metabolites like purines or pyrimidines play any role at all in the utilization of foodstuffs for biosynthesis.

Nevertheless our failures with the guanine analogs urged us on to further synthetic efforts. Variations of the bicyclic rings such as benzimidazoles, benzotriazoles, and quinoxalines were prepared as shown in Fig. 3.[17] In addition simpler test systems were sought wherein it was hoped that the finer nuances of biological reactivity could be observed in line with the hypotheses bared above. Microorganisms and *Rana pipiens* embryos were employed to investigate the effects of the compounds.

Several of the compounds did in fact prove to be inhibitors of *Escherichia coli,* but in a special way. They delayed growth, but only by prolonging the lag phase, after which growth was resumed at its normal rate. This behavior could be explained in either of two ways: either the compounds were detoxicated metabolically, or the organisms mutated to resistant types. Both phenomena were found to take place in the various tests. Four of the 35 compounds tested on *E. coli* were mutagenic. It is germane to Haddow's hypothesis alluded to earlier that these four mutagens were all inhibitors and that only toxic compounds were mutagenic. Mutation occurs, however, at concentration levels far below those necessary to produce inhibition, i.e., lengthening of the lag phase.*

It would be an amiable gesture of nature if the bacterial mutagens 4-methoxy-6-nitrobenzotriazole and 6-hydroxy-4-nitrobenzimidazole, for example, should turn out to be carcinogens also. But this is only moderately to be hoped for, since carcinogens are thought to form more or less stable compounds with cellular components. Bonding need not be stable for mutagenic action on bacteria where the cells are bathed in the toxic medium, but combination is probably necessary in more highly differentiated systems where circulatory and detoxicating mechanisms might play a greater part. Neither does it follow

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* Unpublished work of Sheldon Greer and Professor Francis J. Ryan, Department of Zoology, Columbia University.

that agents which affect one species or even one cell type in a species should exhibit parallel or similar effects in another species or another cell type. Thus the application of these substances to *E. coli* must be weighed as an illustration of general principles without expectancy of utility in higher forms.
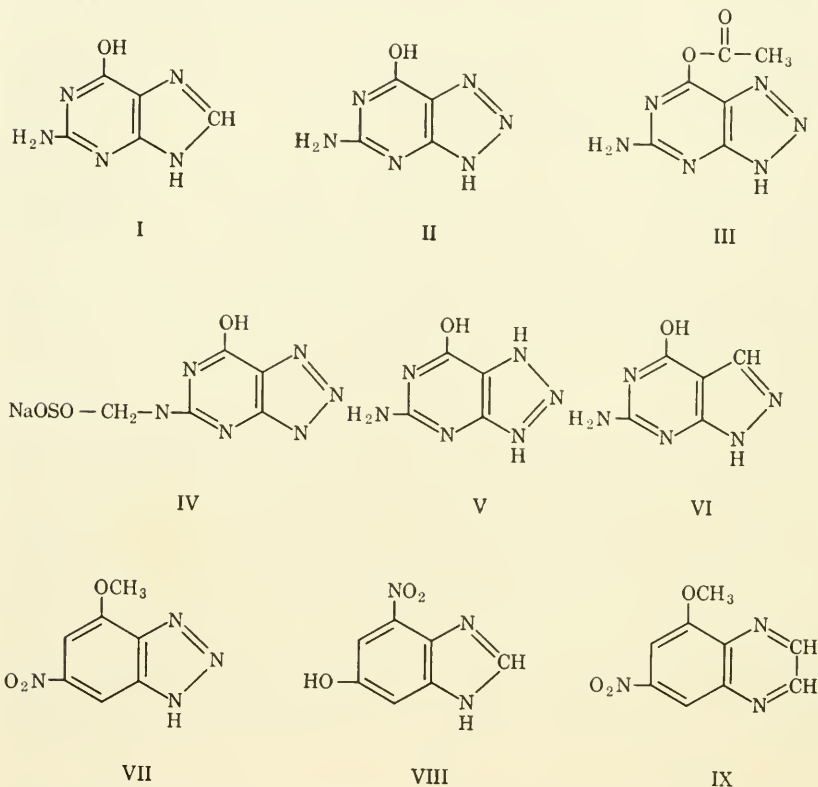
Fig. 3. I. Guanine. II. 8-azaguanine. III. Acetylazaguanine. IV. Azaguanine sulfoxalate. V. 5-amino-7-hydroxypyrazolo-[4,3-d]-pyrimidine. VI. 6-amino-4-hydroxypyrazolo-[3,4-d]-pyrimidine. VII. 4-methoxy-6-nitrobenzotriazole. VIII. 6-hydroxy-4-nitrobenzimidazole. IX. 5-methoxy-7-nitroquinoxaline.

The particular value of experimentation with the frog embryo, however, lies in the possibility of destruction between cleavage and differentiation. The new compounds described above are still to be explored in the light of their effects on energy cycles, but they have already been found to exhibit stage specificity.[18] One group of compounds, mostly quinoxalines, 5-methoxy-7-nitroquinoxaline, for example, selectively inhibits and arrests embryos in early cleavage stages.

Another group represented by 4-methoxy-6-nitrobenzotriazole arrests only late, partially differentiated stages. The specificity in activity is remarkable; even ½ hour of exposure to 0.1 mg./ml. of the quinoxaline brings about immediate arrest of the two-cell stage, whereas exposure of 48 hours or more is required for arrest at the tail-bud stage. Exposing two-cell stage embryos for as long as 30 hours to the same concentration of the benzotriazole still permits development to the blastula or early gastrula stage before death ensues. The selective response to the quinoxaline appears to bear some relation to the mitotic rate and resembles the response to radiation and to radiomimetic drugs. The benzotriazole effects, on the other hand, are characterized by the absence of radiomimicry and by the heightened sensitivity of the embryo with increasing age and with differentiation.

These compounds illustrate the possibility of synthesizing compounds which can selectively attack either growth or differentiation. The finding of such compounds may be a matter of chance and perseverance, since we cannot yet discern any relation between chemical structure and function in this or any other series of compounds so far applied to the problem. We have indicated not only the desirability but also the practicability of such specificity in the chemotherapy of cancer. If the cancer cell is a deficient mutant resulting from a virus infection, then selective chemotherapy is within the grasp of the biochemist who can pin-point the deficiencies and fashion drugs to exploit them. It appears reasonable at this time to suggest that therapy be directed toward energy-cycle mechanisms whether in the tumor or the host.

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Problems in Lipide Metabolism

SAMUEL GURIN

It is now half a century since Knoop [1] fed ω-phenyl-substituted aliphatic acids to dogs and isolated as excretory products either hippuric or phenaceturic acids, depending upon the number of aliphatic carbon atoms in the administered acids. Ever since that time, and with a great crescendo during the past decade, biochemists have sought for a definitive explanation of the way in which fatty acids are oxidized by living tissue. The problem could undoubtedly have been epitomized by a certain well-known writer of detective stories as "The Case of the Mysterious Two-Carbon Fragment." Had an imaginary writer begun his story in 1904, it is only now that he would be able to write his concluding chapter.

Evidence that 2-carbon fragments arise during the biological oxidation of fatty acids has come from so many sources and in such overwhelming volume that there is very little point in belaboring the matter. The isolation of acetyl coenzyme A by Lynen and his group in 1951 [2] brought the field to a new turning point and settled conclusively the nature of the active 2-carbon fragment. It is fitting to pay tribute to the fine work of Lipmann, Nachmansohn, and others who uncovered the role of coenzyme A and ATP in the activation of acetate.

That fatty acids must have their carboxyl groups free prior to their utilization was strongly indicated by Lehninger's work [3] with mitochondria. Lynen's [4] hypothesis, that long-chain acyl CoA derivatives are produced before fatty acids can be oxidized, fitted beautifully with such a notion.

It should of course not be forgotten that the demonstration by Drysdale and Lardy [5] and Mahler [6] that oxidation of fatty acids could be achieved in mitochondrial extracts paved the way for the spectacular advances in this field. With an appropriate electron acceptor it was clearly established that fatty acids could be activated, oxidized, and

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converted by such particle-free extracts to acetoacetate. Furthermore, Drysdale and Lardy were able to demonstrate (1) the formation of acetohydroxamic acid in the presence of hydroxylamine and (2) the quantitative formation of citrate when oxalacetate was supplied. This latter reaction suggested quite strongly the involvement of acetyl CoA, since Stern and Ochoa [7] had isolated the enzyme capable of condensing this active intermediate with oxalacetate to form citrate.

$$\text{Acetyl CoA} + \text{Oxalacetate} \rightleftharpoons \text{Citrate} + \text{CoA}$$
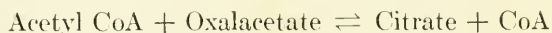
In view of the evidence presented by Stern, Coon, and Del Campillo [8] concerning the formation of acetoacetyl CoA from acetyl CoA:

$$2 \text{ acetyl CoA} \rightleftharpoons \text{Acetoacetyl CoA} + \text{CoA}$$
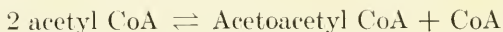
there could no longer be any doubt concerning the nature of the active 2-carbon fragment derived from the oxidation of fatty acids.

At least three separate enzyme systems appear to be involved in the activation of fatty acids: (1) an enzyme system described by Kornberg and Pricer,[9] which is capable of activating acids containing 12 to 20 carbon atoms; (2) enzymes derived from beef liver capable of activating C_4-C_{12} acids (Mahler et al.); and (3) the well-known acetate activating system. All three appear to require ATP and CoA as well as Mg^{++}.

The currently accepted mechanism of oxidation of fatty acids is schematically represented in Fig. 1. Major contributors in this area

Fatty acid oxidation

$$\text{RCH}_2\text{CH}_2\text{CO} - \text{CoA}$$
$$\Updownarrow - 2H$$
$$\text{RCH:CHCO} - \text{CoA}$$
$$\Updownarrow + H_2O$$
$$\text{RCHOHCH}_2\text{CO} - \text{CoA}$$
$$\Updownarrow - 2H$$
$$\text{RCOCH}_2\text{CO} - \text{CoA}$$
$$+ \text{CoA}$$
$$\text{RCO} - \text{CoA} \longleftrightarrow + \text{CH}_3\text{CO} - \text{CoA}$$
$$\text{(recyclized)} \qquad \text{(generated)}$$
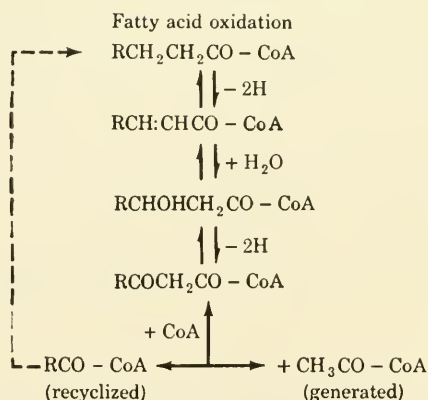
FIG. 1. Generation of acetyl CoA by oxidation of activated fatty acids.

have been Lynen, Ochoa, and Green. The details of this oxidative mechanism have been fully reviewed elsewhere.[10]

It is not surprising that a number of questions are raised by these striking discoveries. One of these concerns itself with the manner in which the fatty acid moieties of phospholipides, triglycerides, and other naturally occurring esters are converted into activated fatty acids. In this connection, a very promising start has been made by Kornberg and Pricer who have demonstrated that acyl CoA derivatives can be enzymatically esterified with L-α-glycerophosphate to yield phosphatidic acids. If this type of reaction should prove to be reversible (and thermodynamically this seems likely), a thiolytic cleavage would be clearly involved as the initiating step rather than a reaction involving the hydrolytic action of lipase. The detailed mechanism of the initial activation clearly needs further investigation.

Although the reversibility of all of the chemical steps of the oxidative cycle seems likely, it has been difficult to establish this experimentally. Stansly and Beinert [11] have been able to demonstrate with purified enzymes the conversion of labeled acetyl CoA to butyryl CoA, provided suitable hydrogen donors were present. Since the thiolytic cleavage of β-ketoacyl CoA proceeds nearly to completion,[8,12] some driving force is necessary to shift the equilibrium in favor of synthesis. Esterification may be an important reaction for this purpose even though this obviously does not provide a complete answer to the problem of lipogenesis.

Before turning to problems of lipogenesis, a brief comment should be made about another aspect of the oxidation problem. The original reports [13] that two types of metabolically different 2-carbon fragments are produced during the oxidation of fatty acids have been amply confirmed. According to this concept, the bulk of the 2-carbon fragments are probably acetyl CoA (originally described as $-CH_2-CO-$ groups). The terminal two carbons of the fatty acid being oxidized give rise to a fragment (originally described as CH_3-CO-) which preferentially contributes to carbons 3 and 4 of acetoacetate. In other words, not all of the 2-carbon fragments derived from fatty acids are symmetrically incorporated into the two halves of acetoacetate. Lynen [14] has invoked the concept of an acetyl-enzyme complex to describe the behavior of the terminal fragment, whereas Beinert and Stansly [15] picture it as an acetyl-CoA-enzyme complex.

$$CH_3CO^*\text{-enzyme } (CH_3CO-CoA\text{-enzyme}) + CH_3CO-CoA \rightleftharpoons$$

$$CH_3CO^*CH_2CO-CoA + Enzyme$$
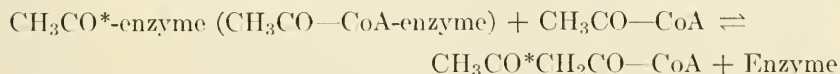
Asymmetrically labeled acetoacetate would in either case be formed. Although these theories are not easy to prove, they are attractive and

explain most of the phenomena that have been observed. Chaikoff and Brown [16] as well as Green [10] have written extensive reviews of this subject.

With the, by now, conclusive evidence that acetyl CoA represents the major if not the sole product of this oxidative cycle, the physiological disposition of this central intermediate becomes of profound significance. It has already been pointed out that it may condense with oxalacetate to form citrate presumably for oxidation to CO_2 by way
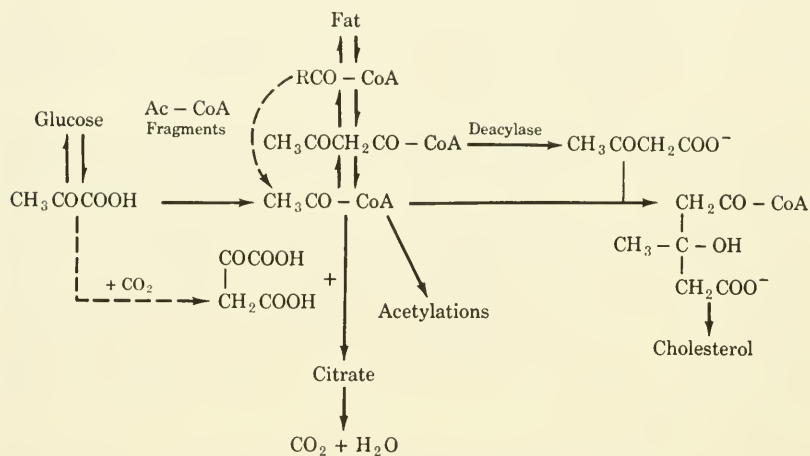
FIG. 2. Metabolic pathways of acetyl CoA.

of the tricarboxylic acid cycle. Its self-condensation to form acetoacetate has also been mentioned. In addition to its function as an acetylating agent, it may also be utilized for the biosynthesis of fatty acids and cholesterol.

It has, of course, been known for a long time that labeled acetate is incorporated into fat by rat-liver slices. Similar experiments have more recently provided evidence that short-chain fatty acids are to a considerable degree cleaved to "active acetate" prior to their incorporation. With homogenates and now aqueous extracts of rat liver [17] it has been possible to demonstrate the incorporation of pyruvate or acetyl CoA into fatty acids. In view of the conclusive evidence that pyruvate is converted in large measure to acetyl CoA by means of pyruvic oxidase, it now becomes clear that glucose must be converted to fat primarily by way of pyruvate and acetyl CoA. The first major metabolic process in the transformation of carbohydrate to fat is therefore glycolysis. This is probably true for most tissues; among

those tested that undoubtedly incorporate acetate into fat have been mammary gland, intestinal mucosa, lung, heart, spleen, testes, ovaries, adrenals, and adipose tissue. It should be pointed out that such positive results simply indicate that the above-mentioned tissues contain enzyme systems capable of transforming precursor carbons into fatty acid carbon atoms. The results do not imply that net synthesis has occurred, nor do they provide much information concerning the driving forces necessary to reverse the oxidative pathway in the direction of lipogenesis.

It is clear that lipogenesis from acetyl CoA is an endergonic and reductive process. Although oxidation of fatty acids may be demonstrated in mitochondria or mitochondrial extracts, it has not yet been possible to obtain incorporation of labeled pyruvate or acetyl CoA in such preparations. The soluble supernatant fluid is required in addition to mitochondrial extracts before this result can be achieved.

A study of the cofactor requirements of the complete water-soluble system [18] indicates that it requires all of the known cofactors of glycolysis, Mg^{++}, ATP, and DPN. In addition, it has been possible to demonstrate a dependency upon CoA. Although there is no doubt that flavoprotein is also required, this has not been demonstrated in such systems. The addition of citrate in relatively high concentrations (0.1–0.2 M) is also necessary for maximal lipogenesis in these systems, whereas equivalent concentrations of glutamate, oxalacetate, and α-ketoglutarate have proved to be not nearly as effective. Although the reason for this stimulating effect of citrate upon lipogenesis is not clear, there is some evidence that the presence or absence of citrate influences profoundly the amount of labeled acetate incorporated into acetoacetate and β-hydroxybutyrate.

Attempts to replace the supernatant fluid with a source of glycolytic enzymes (rabbit-muscle acetone powder) and suitable substrates have been unsuccessful. Although it is obvious that both high-energy compounds and hydrogen donors are essential for lipogenesis, DPNH and ATP have not proved satisfactory nor has it been possible to obtain lipogenesis by enzymatic generation of DPNH and ATP.

Relatively inactive supernatant fluids are obtained following brief dialysis; they are also inactive when prepared from livers of previously fasted or alloxanized rats. Mitochondria or mitochondrial extracts obtained from such animals appear to be essentially normal and can be supplemented with supernatant fluid obtained from livers of normal well-fed rats.

It is of interest to speculate about the defect in such "diabetic" extracts which can readily degrade fatty acids but not accomplish the reverse process. The addition of glycogen, hexose diphosphate, or a number of glycolytic intermediates, produces a marked stimulating effect upon the incorporation of labeled acetate or pyruvate into long-chain fatty acids.[19] In fact, the addition of hexose diphosphate and ADP restores such lipogenic activity nearly to normal. It is significant that glucose is ineffective, whereas fructose has some stimulating effect. Such results are consistent with the data obtained by Chernick et al.[20] who demonstrated that fructose is well utilized by liver slices of diabetic animals. It appears likely therefore that fructokinase and the remaining glycolytic enzymes are relatively intact in the diabetic state. One is therefore tempted to speculate that the diabetic and fasting states may involve a deficiency of glycolytic intermediates. If this is so, then the addition of phosphorylated intermediates of glycolysis to cell-free systems prepared from diabetic animals should be stimulatory. If this concept is correct, then the failure of the diabetic to synthesize fat [21] can in large measure be ascribed to diminished glycolysis resulting from the failure to utilize glucose.

It is attractive to attempt to explain physiological events in terms of chemical reactions, even though the field is still in a primitive state. The fatty livers associated with the diabetic state cannot obviously be ascribed to accelerated lipogenesis, since such livers cannot synthesize fat. Nor can this accumulation of fat be due to a diminished catabolism of fat by the liver since there is abundant evidence to the contrary. It is apparent that this phenomenon must be explained primarily on the basis of accelerated mobilization and transport of fat from the periphery to the liver.

The ketosis of diabetes is understandable if one pictures an over-production of acetyl CoA from fatty acids in the face of a limited supply of oxalacetate. Conversely the antiketogenic effect of carbohydrate can be explained if one assumes that there is a resulting increased production of malate and oxalacetate for condensation with acetyl CoA to form citrate. On this basis, pyruvate which forms acetyl CoA, and should therefore possess some ketogenic activity, fails to do so since it can also provide its own oxalacetate (reactions involving fixation of CO_2).

That acetoacetate is not readily metabolized in liver is consistent with the absence of an effective activating system in liver. The succinyl CoA transferase system occurs elsewhere [8] and is capable of effectively transferring CoA to acetoacetate. Another reaction of acetyl

CoA which occurs readily in liver involves a condensation with aceto-acetate or acetoacetyl CoA yielding eventually β-hydroxy-β-methyl-glutaconic acid.[22] This substance readily yields squalene and cho-lesterol in aqueous liver extracts.[23] Although this particular segment of the field presents many uncertainties which need clarification, it is apparent again that an overproduction of acetyl CoA (and aceto-acetate?) provides a situation which is favorable for increased bio-synthesis of cholesterol. That labeled acetoacetate can be incorporated into cholesterol by liver slices has been well established.[24]

Our rapidly increasing knowledge of the chemical pathways involved in the oxidation and biosynthesis of fat has shown us where the railroad tracks go and something about the location of the switches in this complicated railway system. Turnover studies tell us something about the volume of traffic. We know also that, in certain situations, some switches are open and others are closed. The manner in which hor-monal and physiological regulation influence these biochemical path-ways will challenge the best efforts of biochemists and biologists for many years to come.

It is manifestly impossible to do justice to the many outstanding contributions that have been made in this difficult field. Many have not been touched upon. Such problems as the nature of the linkages in complexes of protein and lipide, the mode of synthesis of the long-chain polyethenoid acids, the biosynthesis of carotenoids and various terpenoid substances will occupy the attention of biochemists for many years. An attempt has been made here only to highlight a few prob-lems that happen to interest the author. Excellent review articles have been published by Lehninger,[10] Green,[10] and Chaikoff.[16]

Current developments in enzymology have depended very heavily upon information obtained with stable and radioactive isotopes. The author was privileged to witness some of the early applications of these research techniques to the study of intermediary metabolism. It is therefore a source of gratification that, from these beginnings, there has developed a renaissance in intermediary metabolism which is now in full tide.

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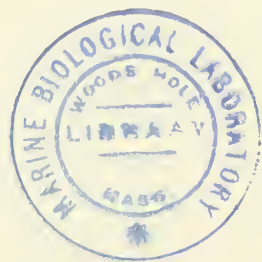
Tetrazoles as Carboxylic Acid Analogs

ROBERT M. HERBST

The acidic character of the tetrazole ring system in which the ring nitrogens are unsubstituted has been recognized since the first preparation of the parent compound by Bladin in 1892. Although Bladin had suggested the name tetrazole for the ring system in 1886, the parent compound was for many years referred to as "tetrazotsäure" because of its acidic character. The acidic nature of several 5-substituted tetrazoles was similarly recognized by nomenclature such as "benzenyl tetrazotsäure" (5-phenyltetrazole) and "amidotetrazotsäure" (5-aminotetrazole). Although it was recognized with the first preparation of these compounds that 5-substituted tetrazoles behaved as acids, it is only recently that a systematic study of the factors influencing the acid strength of these compounds has been undertaken. An attempt will be made to develop the analogy between the factors influencing the acidity of 5-substituted tetrazoles and carboxylic acids in the following.

Until recently, with few exceptions, only the 5-aryltetrazoles have been easily accessible. These were usually prepared by a sequence of reactions due to Pinner that involved the conversion of a nitrile successively into an iminoester, a hydrazidine, an imide azide, and finally a tetrazole. It was also known that certain nitriles such as cyanogen, cyanogen chloride, ethyl cyanoformate, and cyanamide could be converted into the corresponding 5-substituted tetrazoles by interaction with hydrazoic acid (scheme I, R = H, CN, Cl, $COOC_2H_5$,

$$RCN \xrightarrow{HN_3} \begin{array}{c} R-C=NH \\ | \\ N_3 \end{array} \rightarrow \begin{array}{c} HN-\!\!\!-\!\!\!-C-R \\ | \quad\quad || \\ N \quad\quad N \\ \diagdown\diagup \\ N \end{array} \qquad (I)$$

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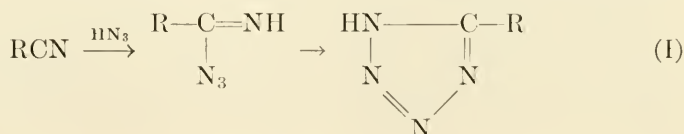
NH_2). The development of this reaction was aborted by von Braun's observation that its application to alkyl and aryl cyanides in the presence of a large excess of concentrated sulfuric acid led to rearranged products such as 1-alkyl- or 1-aryl-5-aminotetrazoles (scheme II). Although the conditions under which von Braun's experiments were done were entirely different from those applied by earlier workers, von Braun did not hesitate to conclude that hydrazoic acid would not add to the cyanide group of alkyl or aryl cyanides.

$$RCN + 2HN_3 \xrightarrow{H_2SO_4} \begin{array}{c} R\!-\!N\!-\!-\!-\!-\!-\!C\!-\!NH_2 \\ | \qquad\quad || \\ N \qquad\quad N \\ \diagdown\quad\diagup \\ N \end{array} + N_2 \qquad (II)$$

R = alkyl or aryl
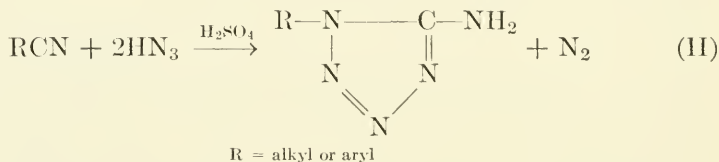
Recent investigations have shown that von Braun's conclusions are not tenable. As a result the addition of hydrazoic acid to nitriles has become the most generally applicable procedure [2-5] for the synthesis of 5-substituted tetrazoles. The symbol R in scheme 1 may be redefined to include in addition to the groups mentioned alkyl and aryl, alkyl- or arylamino, and dialkyl- or diarylamino groups. It must be pointed out, however, that in one instance, when R is a monoalkyl- or monoarylamino group as in the monoalkyl- or monoarylcyanamides, cyclization of the imide azide may take place in either of two ways and the course shown in scheme III is followed exclusively.[4] It is particularly interesting to note that the nature of the group R in the monosubstituted cyanamides appears to have little effect upon the course of the cyclization illustrated in scheme III.

$$RNH\!-\!CN \xrightarrow{HN_3} \begin{array}{c} RNH\!-\!C\!=\!NH \\ | \\ N_3 \end{array} \rightleftharpoons$$

$$\begin{array}{c} RN\!=\!C\!-\!NH_2 \\ | \\ N_3 \end{array} \rightarrow \begin{array}{c} R\!-\!N\!-\!-\!-\!-\!-\!C\!-\!NH_2 \\ | \qquad\quad || \\ N \qquad\quad N \\ \diagdown\quad\diagup \\ N \end{array} \qquad (III)$$
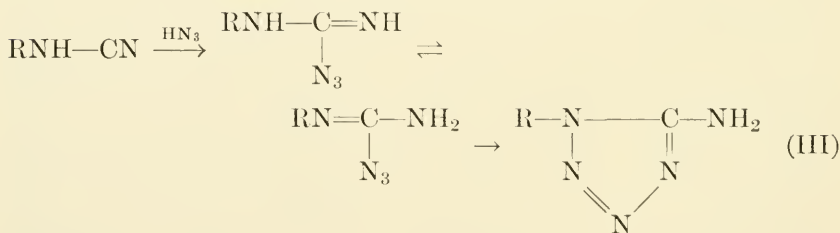
With the accumulation of a group of 5-substituted tetrazoles the determination of their apparent acidic dissociation constants by potentiometric titrations could be undertaken. It soon became apparent that the 5-tetrazolyl group ($-CN_4H$) was quite comparable to the carboxyl group ($-CO_2H$) in bestowing acidic character upon com-

pounds built up around it. In a series of 5-alkyltetrazoles the apparent
acidic dissociation constants were variously from one-tenth to one-half
as large as those of the correspondingly substituted carboxylic acids.
A few examples are cited in Table 1. It will be noted that the dissocia-

Table 1.   Apparent Dissociation Constants of 5-Alkyltetrazoles and Aliphatic Carboxylic Acids

R	R—CN$_4$H * $K \times 10^6$ at 25°	R—CO$_2$H † $K \times 10^6$ at 25°
H	16.2	171.2
CH$_3$	2.74	17.5
	(2.43) ‡	
C$_2$H$_5$	2.56	13.3
C$_3$H$_7$-n	2.47	15
C$_3$H$_7$-iso	2.80	13.8
C$_4$H$_9$-n	(2.38) ‡	13.8
C$_4$H$_9$-iso	(2.45) ‡	16.7
C$_5$H$_{11}$-n	(2.22) ‡	13.2

* Apparent dissociation constants were determined by potentiometric titra-
tion in aqueous solution at 25°C. unless otherwise indicated.[2]

† Constants obtained from conductivity data in aqueous solution.[6]

‡ Apparent dissociation constants determined by potentiometric titration in
25% (by weight) methanol at 25°C.[2]

tion constants are influenced in roughly the same manner by the nature
of the alkyl substituent in both series.

The acidity of tetrazole itself is readily explained on the basis of the
resonance concept, i.e., the resonance stabilization of the tetrazolyl
anion formed upon dissociation of the proton attached to the ring
nitrogens (scheme IV). Similarly, the acidity of the carboxylic acids
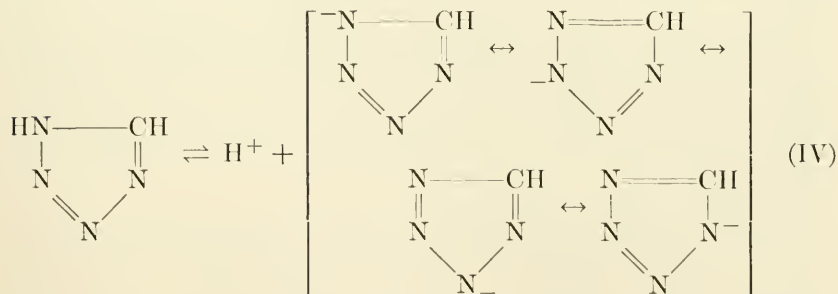
has been attributed to resonance stabilization of the carboxylate ion
(scheme V).

$$\text{HC}\overset{\displaystyle O}{\underset{\displaystyle OH}{\diagup}} \rightleftharpoons H^+ + \left[\text{HC}\overset{\displaystyle O}{\underset{\displaystyle O^-}{\diagup}} \leftrightarrow \text{HC}\overset{\displaystyle O^-}{\underset{\displaystyle O}{\diagup}} \right] \qquad (V)$$
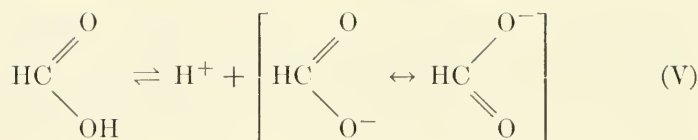
From schemes IV and V it might be anticipated that resonance stabilization would be greater in the tetrazolyl anion than in the carboxylate ion owing to the greater number of forms contributing to the resonance hybrid in the former instance. Although such a situation might suggest that tetrazole and the 5-substituted tetrazoles should be stronger acids than formic acid and the correspondingly substituted carboxylic acids, experimental observations do not support this supposition. It is more likely that other factors, particularly the greater electronegativity of oxygen as compared with nitrogen, are the decisive factors in determining the relative strength of the tetrazoles as compared with the carboxylic acids of the aliphatic series.

In general, factors influencing the electronegativity of the tetrazole system or the carboxyl group should have similar effects upon acid strengths in both series. Thus replacement of the hydrogen attached to the carboxyl carbon of formic acid by an alkyl group is assumed to cause a decrease in the electronegativity (increase in proton affinity) of the carboxyl group through the operation of an inductive effect with a resultant decrease in the apparent acidic dissociation of the carboxyl group. A similar effect is noted in the 5-alkyltetrazoles where replacement of the hydrogen in the 5-position on the tetrazole ring by an alkyl group causes a decrease in apparent acidic dissociation roughly comparable to that noted in the carboxylic acid series.

When aromatic groups are introduced as substituents in the 5-position of the tetrazole ring a different situation develops. Although the apparent dissociation constant of tetrazole is only about one-tenth as large as that of formic acid, the apparent dissociation constant of 5-phenyltetrazole is more than three times as large as that of benzoic acid. A similar relationship exists between a group of substituted 5-phenyltetrazoles and the correspondingly substituted benzoic acids (Table 2).

Since the intrinsic inductive effect of the aryl group upon the 5-tetrazolyl and the carboxyl groups may be assumed to be of about the same order of magnitude, some other factor must be operating to cause the tetrazolyl group to exhibit a greater electronegativity than the carboxyl group in these instances. It will be noted that the phenyl group may participate in the resonance of 5-phenyltetrazole thus in-

Table 2. Apparent Dissociation Constants of 5-Aryltetrazoles and Substituted Benzoic Acids *

R	R—CN₄H $K \times 10^6$	R—CO₂H $K \times 10^6$
C_6H_5	29 (13) †	8.0
$o\text{-}CH_3C_6H_4$	15.2	9.3
$m\text{-}CH_3C_6H_4$	20	4.3
$p\text{-}CH_3C_6H_4$	15.2	3.5
$o\text{-}ClC_6H_4$	57 (25) †	70.8 ‡
$m\text{-}ClC_6H_4$	87	14.5 ‡
$p\text{-}ClC_6H_4$	(32) †	10.0 ‡
$o\text{-}BrC_6H_4$	60	70.8 ‡
$m\text{-}BrC_6H_4$	92 (42) †	13.5 ‡
$p\text{-}BrC_6H_4$	(30) †	9.3 ‡
$o\text{-}CH_3OC_6H_4$	1.2	6.5
$p\text{-}CH_3OC_6H_4$	14	2.8

* Apparent dissociation constants determined by potentiometric titration at 25°C. in 50% (by volume) methanol unless otherwise indicated.[5]

† Apparent dissociation constants determined by potentiometric titration in 75% (by volume) methanol at 25°C.[5]

‡ Dissociation constants determined conductimetrically in 50% methanol (by volume) at 18–20°C.[7]

creasing the number of forms contributing to the hybrid beyond those due only to the resonance of the tetrazolyl portion of the anion (scheme VI). It should also be emphasized that, in addition to forms involving

(VI)
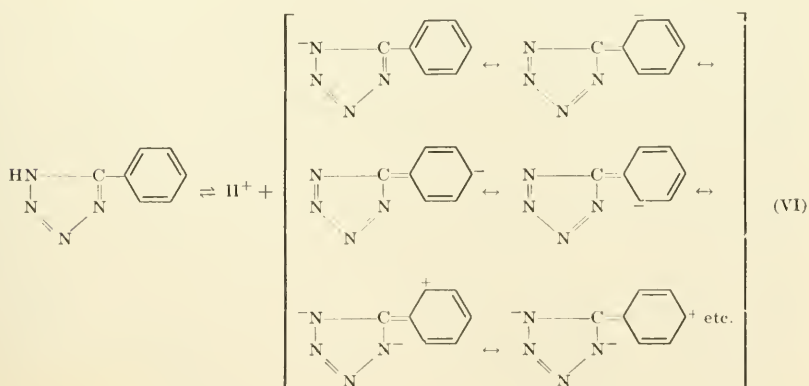
a charge separation, a number of forms in which the benzene ring accepts a charge and effectively withdraws electrons from the tetrazole ring may be written. Although a series of contributing forms may be

written for the benzoate ion (scheme VII), it will be noted that the phenyl group can participate in the resonance picture only when a charge separation is invoked.
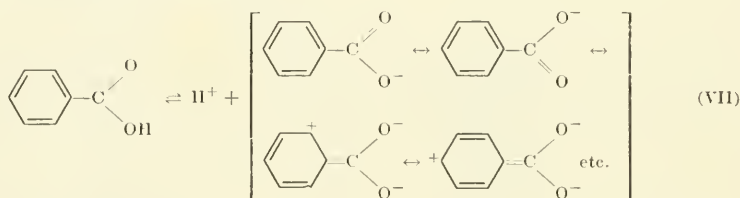
If it is assumed that forms involving a charge separation make only a minor contribution to the respective anions, it becomes apparent that there are more forms contributing to the resonance hybrid of the 5-phenyltetrazolyl anion than to the benzoate ion. On this basis it is suggested that the resonance stabilization of the 5-phenyltetrazolyl anion is sufficiently great to overbalance the lesser electronegativity of nitrogen as compared with oxygen and cause 5-phenyltetrazole to be a stronger acid than benzoic acid. It is also conceivable that resonance phenomena due to the withdrawal of electrons from the tetrazole ring by the phenyl group may cause a reversal of the direction of the inductive effect ordinarily associated with the phenyl group, thereby increasing the electronegativity of the tetrazolyl group and effectively lowering its proton affinity. Following the same reasoning the 5-aryltetrazoles should generally be stronger acids than the correspondingly substituted benzoic acids.

Particularly striking is the effect of orientation of substituent groups upon the apparent dissociation constants of the 5-aryltetrazoles. In the several series for which data are available the dissociation constants decrease in the order *meta > ortho > para*. Furthermore, in the aryltetrazole series the *ortho* and *para* substituted compounds are of approximately the same strength. One exception must be noted: 5-*p*-methoxyphenyltetrazole is about ten times as strong an acid as the *ortho* isomer, a reversal of the situation existing with the methoxybenzoic acids. An explanation of this observation will be advanced later. Because of their insolubility in 50% methanol it was necessary to determine acidic dissociation constants of 5-*p*-chlorophenyl- and 5-*p*-bromophenyltetrazole in 75% methanol. However, dissociation constants determined in both solvent mixtures in several other instances indicated that the values in 50% methanol were about twice as large as those in 75% methanol. Consequently, it seems reasonable to double the values of the apparent dissociation constants measure in

75% methanol when a rough comparison is made with the values for other compounds determined in 50% methanol.

The data in Table 2 indicate that introduction of the electronegative chlorine or bromine atoms as substituents on the benzene ring of 5-phenyltetrazole increases the apparent dissociation constant as compared with the parent compound, whereas introduction of the electropositive methyl group decreases the apparent dissociation constant. This might be anticipated from the inductive effects associated with these substituent groups. However, from this point of view alone the decrease in apparent dissociation constant observed for the methoxyl-substituted compounds is not compatible with the slightly electronegative character of this group. Ordinarily the position of a substituent group should influence the extent to which its inductive effect is transmitted by the benzene ring; the greatest effect upon apparent dissociation constant might be expected when the substituent is nearest the point of attachment of the carboxyl group. On this basis electronegative substituents should cause the apparent dissociation constants to decrease in the order *ortho* > *meta* > *para;* the opposite order might be anticipated for electropositive substituents. Since the observed order of dissociation constants for the 5-aryltetrazoles is *meta* > *ortho* > *para,* it is likely that factors other than inductive or field effects are also influencing the magnitude of the dissociation constants. A combination of the resonance concept and the inductive or field effects of substituent groups will serve to explain the greater strength as acids of the *meta* isomers as compared with the *ortho* and *para* isomers. If we consider the 5-tolyltetrazoles first, the methyl group may be assumed to exert its normal inductive effect which should cause the 5-tolyltetrazoles to be weaker acids than 5-phenyltetrazole. Furthermore, the electropositive methyl group would favor the formation of a field of relatively high electron density around the carbon atom to which it is attached (scheme VIII). Such a field of high electron density in the *ortho* or *para* positions would oppose the development of a formal negative charge at these points and, in effect, reduce the contribution to the resonance hybrid of forms involving negative charges at these points. With the methyl group in the *meta* position, although the inductive effect is in the same direction, an increase in electron density at this point would offer less opposition to resonance phenomena involving formal *ortho* or *para* negative charges. Such an explanation is in accord with the observation that both *o*-tolyl- and *p*-tolyltetrazole are weaker acids than *m*-tolyltetrazole. Since resonance effects involving the benzene ring are not com-

(VIII)
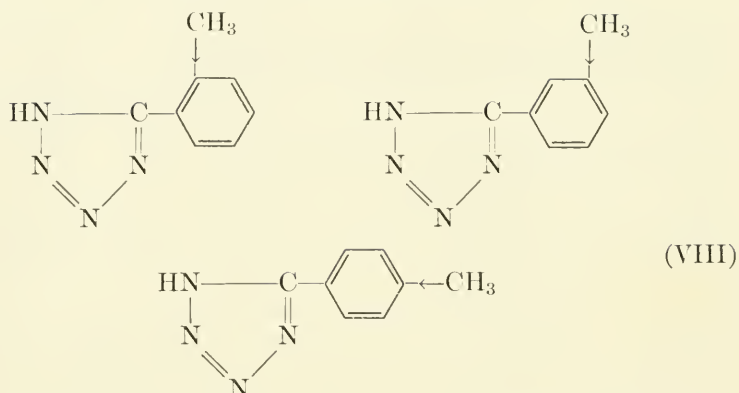
pletely eliminated, the 5-tolyltetrazoles are still appreciably stronger acids than the corresponding toluic acids.

In the halogen-substituted 5-aryltetrazoles the inductive effects associated with the electronegative halogen atoms favor electron displacements that should cause these compounds to be stronger acids than the parent 5-phenyltetrazole. The greater acid strength of the *meta* isomer as compared with the other two may be explained if we again assume that resonance phenomena are more pronounced with the *meta* isomer than with the *ortho* or *para* isomers. Possibly the development of a formal negative charge at the *ortho* and *para* carbon atoms is opposed by the high concentration of electrons in the outer shell of the halogen atoms attached at these positions. This would result in a decrease in apparent dissociation constant of the *ortho* and *para* isomers due to the lesser contribution of such forms to the resonance hybrid (scheme IX, X = Cl or Br).

(IX)
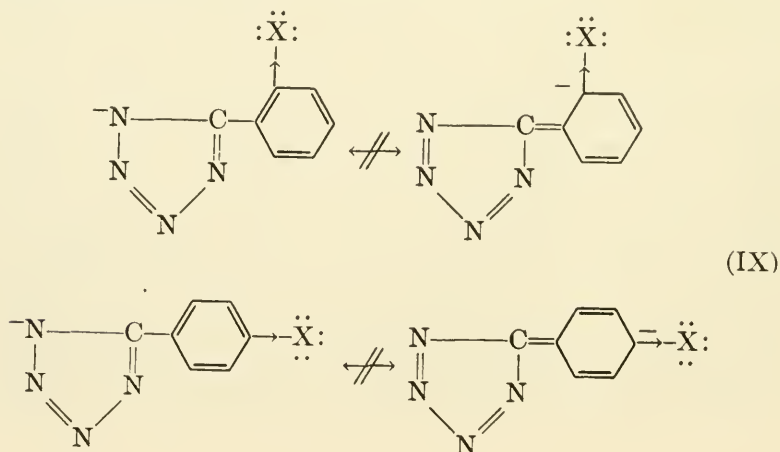
Although the *o*-halobenzoic acids are many times stronger than the *p*-halobenzoic acids, the 5-*o*-halophenyl- and 5-*p*-halophenyltetrazoles are of about the same strength as acids. This probably represents a marked reduction of the strength of the *ortho*-substituted compound rather than a large increase in the strength of the *para* isomer. Conceivably the relatively low dissociation constants of the 5-*o*-halophenyltetrazoles are manifestations of the bulk effect of the *ortho* substituents. The tetrazole ring structure may be sufficiently rigid to cause a moderately bulky group in the *ortho* position of the 5-phenyl substituent to interfere with the formation of structures in which the two rings are coplanar. Resonance forms involving both the tetrazole and the benzene rings simultaneously require coplanarity of the rings.

The small dissociation constant of 5-*o*-methoxyphenyltetrazole as compared with the *para* isomer may be due to hydrogen bonding between the methoxyl oxygen and the acidic hydrogen of the tetrazole ring (scheme X). This effect is much more pronounced in the 5-aryl-

(X)
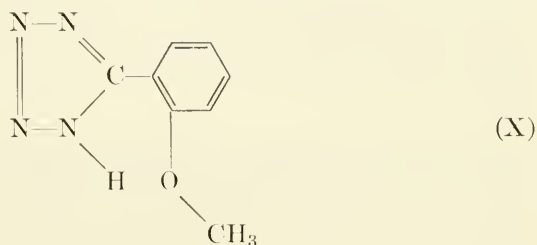
tetrazole series than in the benzoic acid series. In the undissociated forms of the 5-substituted tetrazoles the acidic hydrogen is held in the plane of the ring at a rather fixed angle in position 1 or 2 owing to the rigidity of the tetrazole ring structure. When the acidic hydrogen is located at position 1, the rigidity of the ring would force the hydrogen into a position favorable for hydrogen bonding with a substituent in the *ortho* position of a 5-phenyl group. Such hydrogen bonding would serve to increase the stability of the undissociated molecule and thus decrease the apparent dissociation constant of the compound.

The effect of an amino group or a substituted amino group as substituents in the 5-position of the tetrazole ring is particularly interesting. Such compounds are analogous to the carbamic acids in the carboxylic acid series. The instability of the latter precludes comparisons. The substitution of an amino group in the 5-position of the tetrazole nucleus causes a marked decrease in acid strength (Table 3). Furthermore, it may be noted that the basic function of the amino

Table 3. Apparent Acidic Dissociation Constants of Some 5-amino-tetrazole Derivatives in 50% Methanol *

$$R_2N—CN_4H$$

NR$_2$	$K_a \times 10^8$ 25°C.	NR$_2$	$K_a \times 10^8$ 25°C.
Amino	36 (120) †	Diallylamino	33
Dimethylamino	38 (120) †	Methylamino	21 (87) †
Diethylamino	11 (47) †	Ethylamino	22 (76) †
Diisopropylamino	6	Benzylamino	30
Di-n-butylamino	10	Phenylamino	320
Diisobutylamino	7	o-Nitrophenylamino	8300
Di-n-amylamino	8	m-Nitrophenylamino	1400
Diisoamylamino	7	p-Nitrophenylamino	4600
Dibenzylamino	36	Acetylamino	(2950) †
Benzylmethylamino	38	Nitramino	‡
Benzylethylamino	25		

* All values taken from refs. 3, 10, 11, 12, 13.

† Determined potentiometrically in water at 25°C.

‡ The first dissociation is as a strong organic acid, and K_1 is approximately 1×10^{-1}; in the second dissociation, K_2 is 9×10^{-7}.[11] Both in aqueous solution at 25°C.

group is also very weak. Both of these observations may be attributed to the type of resonance invoked for aniline.[8] The free pair of electrons of the amino nitrogen may be involved in the resonance of the attached ring system. In such a resonating system, the electronegativity of the ring would be decreased and dissociation of a proton from the ring would become more difficult causing a decrease in acidity. Since charge separation imposes ammonium ion character on the amino group, the basicity of this group should also be decreased. Several forms of this type that may contribute to the resonance hybrid are illustrated in scheme XI.

(XI)
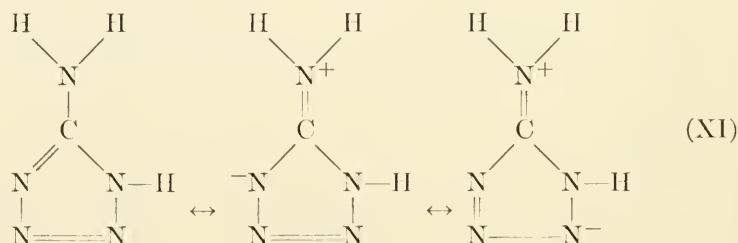
The effect upon the acidity of replacing the hydrogens of the amino group with alkyl groups appears to be primarily steric in nature. The

inductive effect noted when the alkyl groups were attached directly at the 5-position is not transmitted by the amino nitrogen. 5-Amino- and 5-dimethylaminotetrazole have the same apparent acidic dissociation constants. Neither steric nor inductive effects are apparent in this instance. Other groups which may exert little steric influence likewise have essentially no effect on the magnitude of the dissociation constant. Thus, 5-dibenzylamino-, 5-benzylmethylamino-, and 5-dialkylaminotetrazole are as strongly acidic as 5-aminotetrazole and 5-dimethylaminotetrazole (Table 3). On the other hand, there is a marked decrease in acid strength on going from 5-dimethylaminotetrazole to 5-diethylamino-, 5-diisopropylamino-, 5-di-n-butylaminotetrazole, and other larger dialkylaminotetrazoles. Since the inductive effects of most alkyl groups are comparable, their steric influence apparently predominates. A similar decrease in acid strength accompanies the change from 5-benzylmethylamino- to 5-benzylethylaminotetrazole. There is a nice correspondence in the occurrence of a steric effect in this group with that observed in certain hindered carboxylic acids and described by Newman in terms of the "six-number." [9] Those compounds having the largest six-number are the weakest acids (Table 4).

Table 4. Steric Factors and Apparent Dissociation Constants of Some 5-Dialkylaminotetrazoles

R	$K_a \times 10^8$	Six-Number
CH_3	38	0
C_2H_5	11	6
C_3H_7-iso	6	12
C_4H_9-n	10	6

Apparently other factors play a part in determining the acid strength of the 5-monoalkylaminotetrazoles. 5-Methylaminotetrazole is a weaker acid than the corresponding dimethylamino compound. A similar relationship exists between 5-benzylamino- and 5-dibenzylaminotetrazole. On the other hand in 5-ethylaminotetrazole, where the steric effect of a single ethyl group should be appreciably less than

that of two ethyl groups as in 5-diethylaminotetrazole, the anticipated increase in acid strength is realized.

As has been pointed out, the enhanced acidity of 5-phenyltetrazole arises from increased stabilization of the anion by virtue of the conjugation of the tetrazole nucleus and the phenyl group. When the two groups are separated by an amino group, the resulting 5-phenyl-aminotetrazole is a weaker acid than 5-phenyltetrazole because the conjugation of the phenyl group and the tetrazole nucleus is interrupted by the amino group. (It is interesting to note that separation of the phenyl group and the tetrazole nucleus by a methylene group produces very nearly the same quantitative effect.) The fact that 5-phenyl-aminotetrazole is still more acidic than 5-aminotetrazole and its alkyl derivatives leads one to speculate that resonance forms such as those illustrated in scheme XII may be responsible. Such forms would be
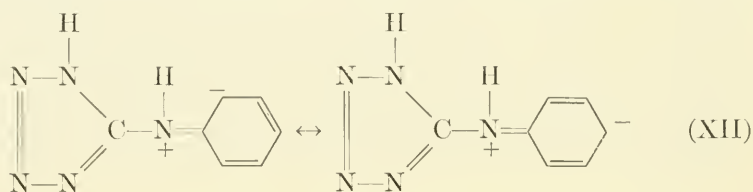
expected to increase the electronegativity of the tetrazole nucleus inductively and result in a decrease in proton affinity. This speculation is given some weight by the relative acidities of the isomeric 5-nitro-phenylaminotetrazoles. The inductive effect just cited should be augmented in decreasing order by the *ortho*, *meta*, and *para* nitro groups. Superimposed on this effect is a resonance reinforcement in the case of the *ortho* and *para* isomers. This combination of effects should cause the *o*-nitrophenylaminotetrazole to be the strongest acid, the *para* intermediate in strength, and the *meta* the weakest. All of them should be distinctly stronger acids than 5-phenylaminotetrazole. This is also the observed order. In scheme XIII several possible contributing forms for the resonance hybrid of 5-*p*-nitrophenylaminotetrazole are illustrated.
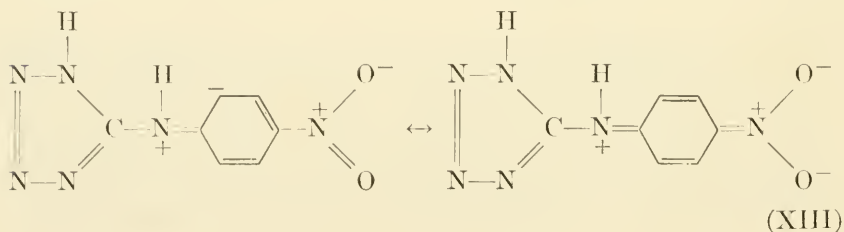
Acylation of the amino group causes some striking changes in the acidity of 5-aminotetrazole. Acetylation brings about a 25-fold increase in acid strength; [10] the resulting compound is almost twice as strong an acid as the unsubstituted tetrazole. Nitration, which in a sense may also be considered as acylation, causes at least a 10,000-fold increase in the acidity of 5-aminotetrazole.[11] 5-Acetylaminotetrazole still behaves as a monobasic acid in aqueous media, but 5-nitramino-tetrazole is a dibasic acid. The presence of the moderately electro-negative acetyl group might be expected to favor tautomerism of the lactam-lactim type or resonance forms involving the amino nitrogen and the carbonyl group (scheme XIV). Resonance of the aniline type involving the tetrazole ring and the amino group is likely to be repressed. Of the several resonance and tautomeric forms shown in scheme XIV those on the left would presumably make the smallest contribution to the hybrid. The forms on the right would cause the amino nitrogen to exert a strong inductive effect in such a direction as to increase the electronegativity and decrease the proton affinity of the ring; a stronger acid should result.

(XIV)
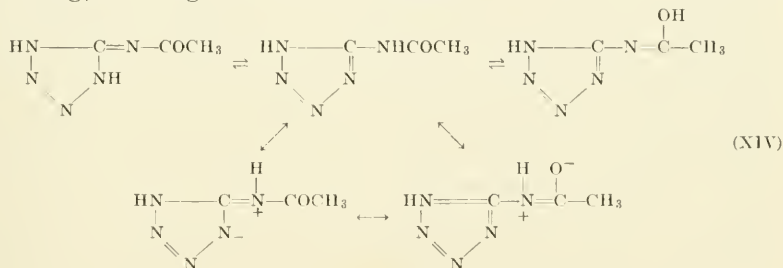
A large variety of tetrazole derivatives have been prepared for investigation of their pharmacological properties. Most of these compounds have carried a nitrogen substituent in addition to the carbon substituent. The group is of interest because pharmacological activity has been associated with the large majority of compounds studied. They have almost uniformly exhibited effects upon the central nervous system that varied from purely depressive to highly stimulatory types. In some instances certain centers of the central nervous system have been rather selectively affected. In addition new types of structures having highly bactericidal and fungicidal action have been encountered.

The close analogy in acidic properties between the 5-tetrazolyl group and the carboxyl group suggests that analogies might be found in the physiological actions of compounds in which the carboxyl group is

replaced by the 5-tetrazolyl group. This thought suggests that a series of antimetabolites in which the 5-tetrazolyl group replaces the carboxyl group as an acidic function is conceivable. For instance, amino acid analogs in which the tetrazolyl group replaces the carboxyl group might serve as effective amino acid antimetabolites. Such antagonists could serve a useful purpose in the study of the effects of interference with the utilization of various amino acids normally occurring in plant and animal proteins. It is also conceivable that malfunctions in the utilization of specific amino acids produced by such antimetabolites could lead to a better understanding of protein and amino acid metabolism. Furthermore, such antimetabolites could be useful in controlling pathological states associated with hyperactivity of amino acid and protein metabolism. At this time only one such compound is known, the 5-β-aminoethyltetrazole which was prepared as a histamine analog by Ainsworth.[14] It failed to exhibit either histaminelike properties or histamine antagonism, observations that are not surprising since the compound should more properly be considered as a β-alanine analog, an analogy which was not recognized at the time.

Beyond this only simple aliphatic and aromatic carboxylic acid analogs have been studied. Although the 5-alkyltetrazoles have shown some interesting pharmacological properties as rather potent anticonvulsant agents as measured by their antagonism towards Metrazole, no compounds have been prepared with long-chain alkyl groups which would make them comparable to fatty acids such as palmitic and stearic. Such long-chain compounds could be of interest in their relationship to fatty acid metabolism in the living organism. Although the biological implications associated with the 5-substituted tetrazoles are purely speculative at this time, the preparation and study of such compounds in the future could lead to interesting organic and biochemical results.

This discussion is based upon experimental work done by Drs. Joseph S. Mihina, William L. Garbrecht, and James A. Garrison, and Mr. Kenneth R. Wilson. The literature concerning tetrazoles has been reviewed by Benson [1] to whom reference should be made for the earlier work.

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The Structural Basis for the
Differentiation of Identical Groups
in Asymmetric Reactions

HANS HIRSCHMANN

Classical organic chemistry defined asymmetric syntheses [1] as processes which convert symmetrical molecules into optically active ones by the *intermediate* use of asymmetric agents, provided the methods employed take no recourse to processes of resolution. Numerous examples have demonstrated the reality of the phenomenon. The asymmetric agent used to bring about this change can either be an "asymmetric form" of energy such as circularly polarized light or an asymmetric form of matter such as an asymmetric molecule. The success of the operation occasioned little inquiry into its structural requirements since the essential features appeared to be trivial ones, i.e., the symmetry of the starting compound and the asymmetry of the product.

Quite a different situation arose, however, with the discovery of closely related phenomena which could not be detected by testing the product for optical activity. If one of the substituents a in compound I is converted preferentially to a substituent d which is different from the three others, the product II is optically active. On the other hand
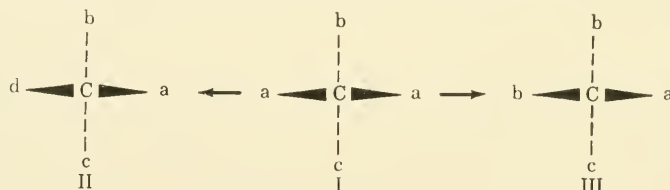
if the reaction involves the selective conversion of one group a into a group b identical with one already present, the product (III) has a plane of symmetry and hence is optically inactive. However, if one of the groups to be substituted is isotopically labeled, it is possible to demonstrate the asymmetry of the process in either case.

The difference between these two types of sterically selective reac-

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tions, therefore, appears not to be an intrinsic one but rather a reflection of the diagnostic tool employed for demonstrating the asymmetry of the process. It can be shown that the selective reactivity of one of a set of identical substituents is possible in certain compounds, but not in others. It, therefore, becomes a problem of both theoretical and practical interest to determine the structural basis of such differentiation. Several criteria [2-4] have been proposed in answer to this question. However, one of these rules [3] is unreliable,[4] and they all fail in providing information about situations that are likely to be encountered. It is the purpose of this discussion to propose a *general* criterion and to compare it with those which have been advanced by others.

The possibility that two identical substituents a in a molecule Caabc can be distinguished from each other in a process catalyzed by an enzyme was postulated by Ogston.[5] The reality of the phenomenon has been amply demonstrated in the case of citric acid and other substances. As is well known, the mechanism envisaged by Ogston assumes an attachment of three groups of the substrate to three sites of the enzyme, of which only one is catalytically active. If the sites α' and α'' can combine specifically with the groups * a' or a'', and the sites β and γ with the groups b and c, respectively, if the reaction proceeds only if three groups are simultaneously engaged, and if only α' but not α'' can catalyze the reaction, then an enzyme constituted as IVa or V will cause a reaction at a' but not at a''. These stipula-
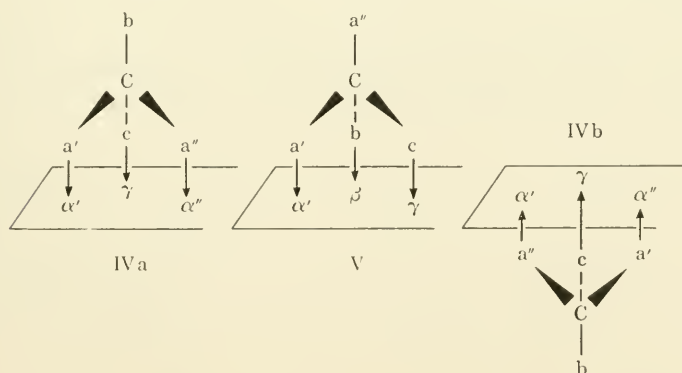
* Throughout this discussion atoms or groups of atoms designated by a', a'', a''' are defined as being identical and are designated by different symbols merely to facilitate discussion. Furthermore, unless stated otherwise, all substituents a, b, c, etc., are assumed to be symmetric. Bonds drawn in solid triangles are directed towards the observer; those in broken lines towards the rear.

tions have been made more stringent by Wilcox et al.,[6] who pointed out that the Ogston scheme is strictly correct only if a fourth condition such as steric hindrance prevents the approach of the substrate from the opposite side, since an interaction as in IVb would place the group a″ at the reactive site α′.

Since the Ogston scheme provides a very satisfactory explanation for the *complete* asymmetry of most enzymatic processes, it apparently has been assumed by some investigators that a three-point attachment between the symmetric substrate and the asymmetric agent is essential for any differentiation of identical groups even if the selectivity is only partial. It is very doubtful that Ogston entertained such thoughts, and a clear statement to the contrary was made by Wilcox.[2] A most emphatic denial can be found in a very lucid analysis by Schwartz and Carter,[4] which may be summarized as follows: Although the substituents a in Caabc are identical and although they are located in a symmetrical molecule, their locations within that molecule nevertheless are sterically non-equivalent. If one views from one substituent a the three other substituents of the central carbon atom, the groups a, b, c appear in a clockwise sequence; whereas a counterclockwise order prevails if one views from the other a group. If one orients the molecule in a predetermined way (e.g., as in I with groups b and c to the rear and with b above c), invariably the same group a will be directed to the right or to the left, respectively. Since the two a groups can be distinguished by inspection, their location cannot be sterically equivalent.

A plane through the central carbon atom which bisects substituents b and c cuts the molecule into halves which are related to each other as object to mirror image but which are not superimposable as long as b and c are different. Since this situation resembles in some respects that prevailing in a *meso* compound which often can be cut into mirror-image halves that are not superimposable, Schwartz and Carter proposed the term *meso* carbon atom for one substituted with two identical and two different substituents (Caabc). It should be noted that the last argument for the steric non-equivalence of the two a groups must be used with caution, since such a molecule as Caaab in which all a groups are sterically equivalent also can be cut into mirror-image halves that cannot be superimposed upon each other. Another failure of this criterion was noted by the authors themselves.[4]

If we consider the approach of a molecule E to one or the other substituent a in Caabc leading to a one-point attachment (either by covalence, electrovalence, hydrogen bonding, or some other force) the

result to be expected will depend on the symmetry or asymmetry of E. If E is symmetrical, the products VIIa and VIIb are optical antipodes

VIa   VIb

VIIa   VIIb
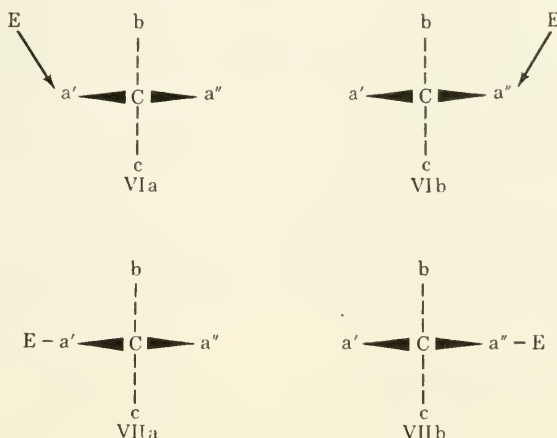
and hence possess equal stability. Similarly, any approach of E to a′ can be matched by a mirror-image approach to a″ possessing equal probability. Hence no differentiation of a′ and a″ is possible. If E is asymmetric, the products VIIa and VIIb are no longer optical antipodes but diastereoisomers and hence would be expected to possess different stabilities. Even if the direction of motion of E towards a′ is the mirror image of that of E towards a″, the corresponding situations during any stage of such an approach (e.g., VIa and VIb) are related like diastereoisomers and not like mirror images and hence can be expected to possess different probabilities. The final proportion of VIIa and of VIIb may be determined by the probability of a success-

VIIIa   VIIIb
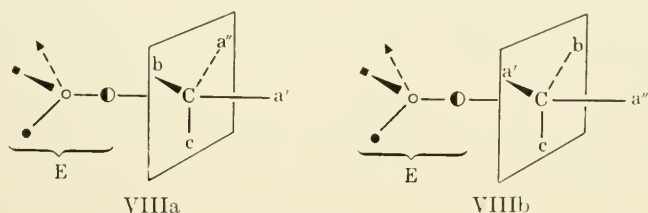
ful collision between E and a′ or a″ (kinetic control), or by the relative stabilities (or solubilities) of the products (thermodynamic control) if the process is reversible. In either case the ratio of reaction products would be expected to be different from unity. If the reaction does not

involve some form of complexing between the a groups and the asymmetric E, differentiation of the a groups can still be expected.

If the process consists in an attack of E on the central carbon atom which replaces either a′ or a″, the transition states [7] (VIIIa or VIIIb, respectively) are not identical but related like diastereoisomers. Hence the heights of the energy barriers separating the starting compounds from the products as well as the energy contents of the latter would be expected to differ for the substitution of a′ and of a″.

If the reaction of the a groups is initiated by some combination of E with the b group as in IXa, the steric relationship of a′ to E is not identical with that of a″ to E nor will it become identical by any rotation around a single bond as in IXb. It is clear then that a

IX a   IX b
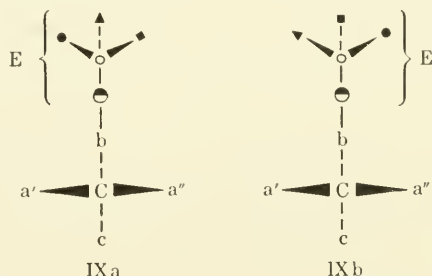
variety of reasonable mechanisms can be conceived which do not require a three-point attachment and which nevertheless permit the differentiation of identical substituents. Although this is to be expected if the optically active species E participates directly * in the steps which alter the a group in Caabc, it must be noted that this differentiation often is too small to be detected or too transitory to be preserved.

Since the common feature of all these reactions is the diastereomeric character of some intermediate or product, it seems justified to neglect these mechanistic details and to seek the basis for the differentiation of identical groups in some structural characteristic of the substrate. To define it, the following rules have been proposed:

1. Wilcox: [2] "In a molecule which has a plane of symmetry or a point of symmetry, if one of the atoms which does not lie in any plane

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* This stipulation seems quite essential. If, for instance, the reaction can be represented by an ideal unimolecular substitution [7] in the a group by E, the rate-determining dissociation proceeds in the virtual absence of E and hence should proceed with equal facility in the a′ and a″ group. Similarly, if E attacks b changing it to the symmetric substituent d which subsequently in the absence of E causes a secondary change affecting the a groups, no differentiation is to be expected.

or point of symmetry is replaced by an isotopic atom, the molecule becomes asymmetric with respect to the labelled atom. In any reaction with an asymmetric reagent, this labelled atom (or group) may react at a rate which is different from that of its counterpart through the plane or point of symmetry, and the difference in rates will be expressed in the distribution of the isotope in the products. This asymmetric behaviour would be superimposed on any difference in the rates of reaction which would result from the different masses of the isotopic atoms."

2. Racusen and Aronoff: [3] "Discrimination of identical groups or atoms by an asymmetric agent (enzyme, optical antipode, etc.) is possible only in molecules which do not possess a twofold (or greater) axis of symmetry." *

3. Schwartz and Carter: [4] "In any molecule containing one (or more) *meso*-carbon atoms, reaction of the two (a) groups with an asymmetric reagent will proceed at different rates, yielding unequal amounts of diastereoisomeric products."

4. We should like to propose the following criterion: A three-dimensional representation of a molecule containing two (or more) identical groups or atoms a designated as a′ and a″ is moved so that the position of a″ will coincide with the original position of a′. If this can be done

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* Two kinds of axes of symmetry are being distinguished. An object is said to possess an n-fold simple axis of symmetry if a rotation around this axis through an angle of $360°/n$ yields an arrangement indistinguishable from the original. An object is said to possess an n-fold alternating axis of symmetry if rotation around this axis through an angle of $360°/n$ followed by a reflection in a plane perpendicular to this axis produces an arrangement indistinguishable from the original. It can readily be seen that a onefold alternating axis is equivalent to a plane of symmetry and that a twofold alternating axis is equivalent to a center (point) of symmetry. A compound which possesses no alternating axis cannot be superimposed on its mirror image and is termed asymmetric in the usual language of organic chemistry. However, such a compound may still possess a simple axis greater than one, and if it does it is often designated as dyssymmetric. A compound which has only onefold simple axes is not considered to possess symmetry of any kind and is termed asymmetric, since every object, no matter how irregular, has an infinite number of such axes. Numerous synonyms are in use. An alternating axis is also called a "rotation-reflection axis," "mirror axis," "improper axis," "axis of the second order," etc., whereas a simple axis has also been called a "rotation axis," "axis of the first order," or merely an "axis of symmetry." The last term, however, unless defined is ambiguous.[8] A few examples of axes of both kinds and a brief discussion of their significance will be given below, but for further information reference is made to the classical treatise by Schoenflies[9] and to accounts given by others.[10-12] Reference 12 discusses the subject without the aid of mathematics.

in such a way that the second arrangement is indistinguishable from the first, the groups a′ and a″ cannot be differentiated from each other in any reaction. However, if such superposition is impossible, the groups a′ and a″ can react with an asymmetric reagent at different rates.*

The validity of this rule can be demonstrated as follows: If the representation of the molecule A is indistinguishable from one showing a″ in place of a′, any product resulting from the change of a′ to d must also be superimposable on the product resulting from the change of a″ to d. This will be true regardless of the symmetry or asymmetry of d. The products must be superimposable even if the reaction is not confined to the a groups but involves additional changes at other substituents. Similarly, any approach of the reagent E (which again may be symmetric or asymmetric) towards a′ or some other atom is indistinguishable from the corresponding approach of E towards a″ or the corresponding other atom. Furthermore, if the products of the direct reaction at (or near) a′ and a″ are indistinguishable from each other, there can also be no differentiation in any successive process. If the reaction should involve several products, the same argument applies to each one of them. We conclude then that the a′ and a″ groups cannot be differentiated from each other in any process if the superposition specified is possible. If, on the other hand, such superposition is not possible, mechanisms exist which permit the differentiation of the a groups. For instance, the product resulting from the combination of a′ with the optically active agent E cannot be superimposed on that resulting from the combination with a″. These products cannot be antipodes if E, as is ordinarily the case, retains its asymmetry during the reaction. Hence the thermodynamic properties of the two products are expected to differ. If the outcome of the reaction of E with A depends on other factors, analogous arguments apply as were outlined above for the specific case of Caabc. Since a possibility of differentiation exists whenever the a groups do not meet the superposition test, the validity of the rule is considered to be fully established. To illustrate its application and utility a few specific examples will be discussed.

Example 1, Caabb (X). Rotation of Xa through 180° around an axis passing through the central carbon atom C and perpendicular to the plane of the paper results in Xb which is indistinguishable from Xa. Hence the two a groups cannot be differentiated from each other. The

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* The application of this rule to compounds which cannot be dealt with adequately by a single representation is illustrated in examples 8–10.

same applies of course to the b groups. The molecule contains two planes of symmetry bisecting the a and b groups respectively, a twofold

$$b' \qquad\qquad\qquad b''$$
$$a' \longleftarrow C \blacktriangleright a'' \qquad\qquad a'' \longleftarrow C \blacktriangleright a'$$
$$b'' \qquad\qquad\qquad b'$$
$$\text{Xa} \qquad\qquad\qquad \text{Xb}$$
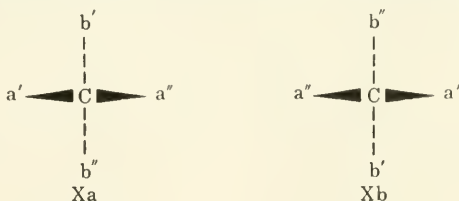
simple axis and no *meso* carbon atom. The result therefore is predicted by rule 2 and not inconsistent with rules 1 and 3.

Example 2, Caaab (XI). Successive rotations of XI*a* through 120° around the axis C*b* convert it into XI*b* and XI*c* which are indistin-

$$b \qquad\qquad b \qquad\qquad b$$
$$C \qquad\qquad C \qquad\qquad C$$
$$a' \quad a''' \quad a'' \qquad a'' \quad a' \quad a''' \qquad a''' \quad a'' \quad a'$$
$$\text{XIa} \qquad\qquad \text{XIb} \qquad\qquad \text{XIc}$$
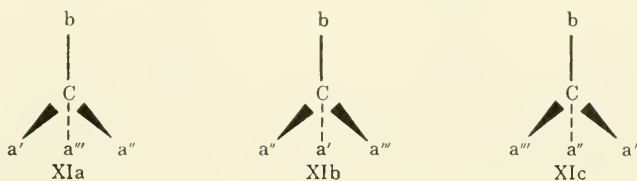
guishable from the original and which contain either a″ or a‴ in place of a′. Hence the three a groups cannot be differentiated. All a groups lie in planes of symmetry, the molecule contains a threefold simple axis and no *meso* carbon atom. The result therefore is again predicted by rule 2 and is not inconsistent with rules 1 and 3.

Example 3, Caabc. (XII). The two a groups can react at different rates with an asymmetric agent since it is impossible to superimpose simultaneously a″ with a′, b with b, and c with c (see, e.g., XII*a*, XII*b*,

$$b \qquad\qquad\qquad b \qquad\qquad\qquad c$$
$$a' \longleftarrow C \blacktriangleright a'' \qquad a'' ---C--- a' \qquad a'' \longleftarrow C \blacktriangleright a'$$
$$c \qquad\qquad\qquad c \qquad\qquad\qquad b$$
$$\text{XIIa} \qquad\qquad \text{XIIb} \qquad\qquad \text{XIIc}$$
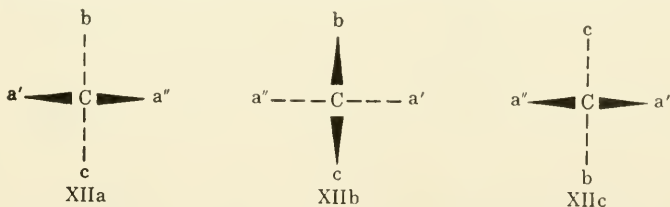
and XII*c*). The molecule has one plane of symmetry but the two a groups do not lie in it; it possesses no axis greater than one and contains a *meso* carbon atom. Hence the result is predicted by rules 1 to 3.

Example 4, Caa (+b)(−b) (XIII). In this case the two b groups are assumed to be structurally identical asymmetric substituents that

are mirror images of each other and hence not superimposable. There-
fore any attempt to place a″ into the position of a′ results in an
arrangement different from the original (e.g., XIIIa and XIIIb). The

XIIIa   XIIIb
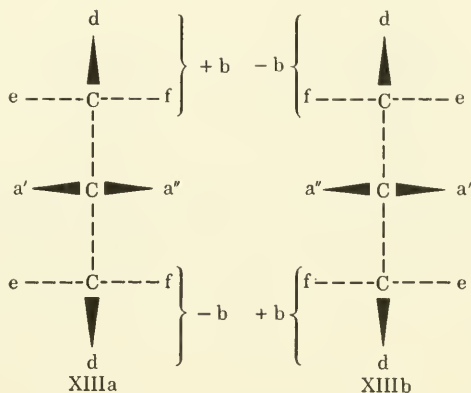
two a groups therefore can react at different rates with an asymmetric
reagent. This result *cannot* be foreseen by applying rule 1 since both
a groups lie in the plane of symmetry. It is predicted, however, by
rule 2, since there is no axis greater than one. The definition given
for the *meso* carbon atom did not refer specifically to such structures
but could quite logically be interpreted to include them. If we do this
we should realize, however, that we make a choice different from that
usually made in defining an asymmetric carbon atom, since the struc-
ture $Ca(+b)(-b)e$ gives rise to two stereoisomers which are *not*
optical antipodes.

Example 5, Caa(+b)(+b) (XIV). If the group $+b$ is defined as
in the preceding example, the compound is not superposable on its
mirror image. In optically active compounds differential reactivity
of identical substituents even in reaction with symmetrical reagents
occurs with such frequency that this fact hardly requires comment.
Example 5 shows, however, that this is not invariably the case and that
even optically active compounds may possess substituents that cannot
be differentiated from each other by any process. Rotation of structure
XIVa through 180° around an axis which passes through the central
carbon atom and is perpendicular to the plane of the paper yields XIVb.
As both arrangements are indistinguishable, the two a groups cannot
be differentiated from each other nor can the b groups. [This conclu-
sion of course should not be construed to mean that the reactivity of
the a groups in this compound, in reaction with an asymmetric reagent,

would be the same as that of the a groups in the optical antipode Caa(−b)(−b).]   Previous discussions have not dealt with dyssym-

XIVa   XIVb
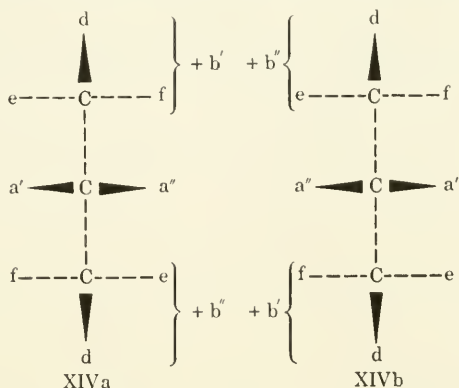
metric structures.   Rule 1 in particular is limited to symmetric compounds.   It may be noted, however, that our finding is consistent with rule 2 since the compound, although non-superposable on its mirror image, possesses a twofold simple axis.   As the central carbon is not a *meso* carbon atom the result is not inconsistent with rule 3.

Example 6, Caab-Caab (XV).   Internal rotation around the central bond permits two symmetric arrangements (XVa and XVc).   If XVa is rotated 180° around an axis which is perpendicular to the plane

XVa   XVb

XVc   XVd
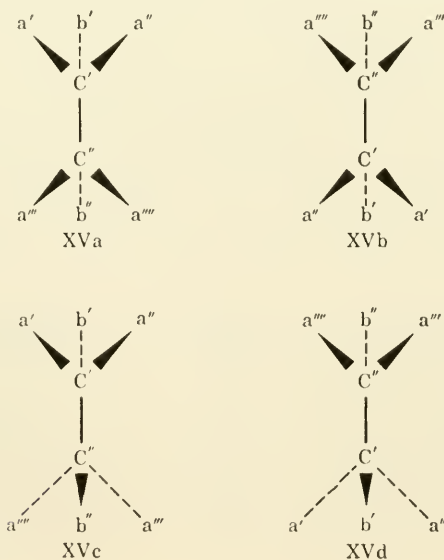
of the paper and which passes through the center of the C-C bond, XV*b* results. Similarly, rotation of XV*c* around an axis which lies within the plane of the paper and which bisects and is perpendicular to the C-C bond yields XV*d*. The conclusions which can be drawn from these two operations are identical since both demonstrate the steric equivalence of C′ and C″, of b′ and b″, of a′ and a⁗, and of a″ and a‴, respectively. As no other motions produce indistinguishable arrangements we can conclude that the following pairs can be differentiated: a′ from a″, a′ from a‴, a″ from a⁗, and a‴ from a⁗. The steric inequalities could have been deduced also by considering example 6 a special case of example 3, which shows that a′ and a″ can be differentiated as well as a‴ and a⁗. The remaining relationships between the a groups follow, then, from consideration of the steric equalities already established. The molecule arranged as in XV*a* possesses two planes of symmetry, as in XV*c* a plane and a center of symmetry. As the a groups lie outside these symmetry elements, the prevailing steric inequalities are predicted also by rule 1 which, however, gives no information about the groups which cannot be differentiated unless further symmetry considerations are applied. Structure XV*a* has a twofold simple axis, and XV*c* a twofold simple axis as well as twofold alternating axes. The results obtained, therefore, are clearly inconsistent with rule 2. The compound contains two *meso* carbon atoms. Rule 3, therefore, predicts correctly two of the steric inequalities of the a groups but fails to disclose the two others. Moreover, it does not indicate the presence of identical substituents which cannot be differentiated.

Example 7, Cabc-Cabc (XVI and XVII). Again, as in examples 4 and 5, the results to be expected depend on the configurations of the asymmetric centers. Application of our criterion to the *meso* compound XVI shows that all pairs of identical substituents as well as the two central carbon atoms can be differentiated, whereas this is impossible with the optically active forms since XVII*a* on rotation

XVI   XVIIa   XVIIb
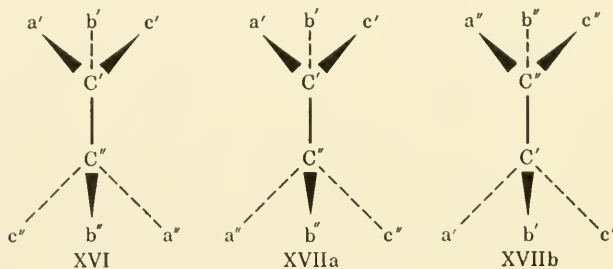
gives XVII*b*, which is indistinguishable. The result with the *meso* compound is predicted by rule 1. However, as structure XVI has two-fold alternating axes, the differentiation of the substituents is an exception to rule 2 unless its authors intended to exclude alternating axes. The result is not predicted by rule 3 since the compound contains no *meso* carbon atoms but two enantiomorphic asymmetric carbon atoms. Our results present an interesting paradox which may be exemplified as follows. If tartaric acid is considered as an intermediate in an enzymatic process which results in differential labeling of the carboxyl groups, the symmetric *meso* compound can qualify as a possible intermediate but the dyssymmetric optically active forms cannot.

If one considers the types of structures which permit the differentiation of identical substituents and those which do not, one can think of innumerable reactions which link the two types in either direction. Since the conversion of a structure which does not permit differentiation by any process into one which admits this possibility might be considered a contradiction, it is perhaps not superfluous to show that this is not the case. For example, the olefinic carbon atoms as well as their identical substituents in structure XVIII*a* cannot be differentiated since the structure is indistinguishable from XVIII*b* which results from it by rotation. However, if this substance undergoes *cis* addition of two *c* groups, a *meso* structure XIX results which as set forth in
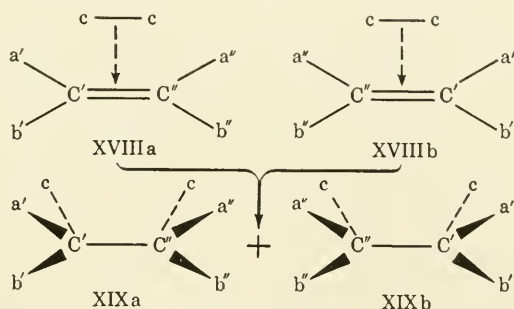
example 7 permits the differentiation of the central carbon atoms and their substituents. Since arrangements XVIII*a* and XVIII*b* are indistinguishable, there will be an equal chance that the molecule presents itself either way to an asymmetric reagent. Hence even a unidirectional *cis* addition (broken arrow) will produce the superposable structures XIX*a* and XIX*b* in equal amounts. Therefore, even if a subsequent reaction at the left of the two central carbon atoms in XIX*a* and XIX*b* proceeds at a rate different from that at the right, this

cannot lead to a differentiation of the carbon atoms labeled C′ and C″ as these have become randomly distributed over the two positions.

Since even a single intermediate which does not permit the differentiation of identical substituents suffices to bring about this result in a long chain of reactions, it seems of particular importance to recognize the structural characteristics which *prevent* selective reactions of identical groups. To our knowledge this question has not been answered previously. Rules 1 and 3 describe structures which *permit* differentiation. Since we have shown that neither criterion covers all situations where this may occur, these rules clearly are not convertible and hence give no reliable information about structures which do not permit differentiation. Rule 2 tried to answer this question, but the criterion was found to have exceptions. One may conclude, therefore, that these three rules are no more than partial solutions of the problem. As one should expect to find the general principle that prevents differentiation of identical substituents in some structural regularity, we shall attempt to link rule 4 to molecular symmetry.

Mathematical analysis has shown that two rigid objects, so related that the distance between any two points in one of them equals the distance of the corresponding points in the other, can be brought into coincidence by a combination of at most three operations, a translational motion, a rotation around an axis, and a reflection in a plane perpendicular to this axis. If the two objects have one point in common, the rotation and the reflection will always suffice.[9, 10] A finite rigid object is said to possess symmetry if two or more indistinguishable arrangements exist that can be interconverted by these two types of operations.[11] We therefore can distinguish two kinds of symmetry: If a rotation suffices to produce another indistinguishable arrangement, the object is congruent with itself in more than one way and the axis of rotation is termed a simple axis of symmetry. If the conversion to another indistinguishable arrangement is possible by a reflection or by a rotation and reflection, the object is congruent with its mirror image and the axis of rotation is termed an alternating axis of symmetry.*

Organic chemistry has concerned itself almost exclusively with symmetry of the second kind. It is quite clear, however, that this mirror-image symmetry has no bearing on our problem, since the operations considered in rule 4 are motions and not reflections and any superposition which cannot be achieved without a reflection is of no concern.

* See footnote on p. 161.

On the other hand, rotations around finite * simple axes through the angles specified by their multiplicity are motions which meet all criteria specified in rule 4. In fact, in the examples given, superposition of identical groups, if this was possible, was achieved by rotation around simple axes. This can be done for any rigid structure meeting this superposition test, since two identical objects which have one point in common [11] can always be brought into coincidence by means of a rotation.[9,10] We have therefore two complementary situations. If a structure has an alternating axis of symmetry, it can be superposed on its mirror image and therefore cannot be resolved into optical antipodes. If a structure has a finite simple axis greater than one, it contains identical substituents which meet the superposition test specified in rule 4 and which therefore cannot be differentiated from each other in any reaction. It is clear, then, that the symmetry elements which prevent the differentiation of identical groups differ in kind from those preventing resolution into enantiomorphs, although both can be found in the same molecule. This dichotomy of symmetry elements is well illustrated by example 7 and fully resolves the paradox presented.

It is of interest to trace the reason for the failure of rule 2 in example 6, since this structure possessed a twofold simple axis. The compound contained four identical substituents, and rotation around this axis permitted the superposition of the members of two pairs of identical substituents but not the mutual superposition of all four. Hence, to exclude the possibility of differentiation of all identical groups in any structure, one cannot set a fixed limit for the multiplicity of simple axes but must relate in some manner the required number, multiplicity, and orientation of simple axes to the number and disposition of identical groups. The so-called symmetry number, which equals the number of indistinguishable positions into which a molecule can be turned by simple rigid rotations,[13] appears to be a useful characteristic for the general solution of this problem. This number has been related to the so-called symmetry group, which is determined by the combination of all symmetry elements which are found in a given structure. The symmetry number must equal, at least, the number of identical substituents to prevent their differentiation. Although the relationship between symmetry group and symmetry number is available in tabular form, the operation of this criterion seems certainly

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* Infinite simple axes are found in linear molecules like hydrogen cyanide or acetylene and lie in the direction of their bonds. Rotation around such an axis does not achieve the superposition of different atoms.

no simpler than the application of rule 4. Moreover, the information so obtained is frequently less detailed. In example 6 we find a symmetry number of two for either XVa or XVc, which is too low for the superposition of four identical groups. This result provides no information about the disposition of the a groups which can or cannot be differentiated. Finally we shall find that the use of symmetry numbers encounters difficulties from yet another source.

We have thus far assumed, quite unrealistically, that molecules can be represented by rigid bodies. The rotation around single bonds, in particular, has presented a problem also in classical stereochemistry, where it has been met by various devices, including the suggestions that the symmetry properties of a molecule be determined for a properly selected conformation or for a time average of all conformations.[12] The first of these devices has been used in this discussion thus far; the second can be applied to example 8. In examples 9 and 10, however, it is no longer possible to derive the correct answer if we merely consider the symmetry properties of some rigid representation of the whole molecule. We shall still find it possible, however, to apply rule 4.

Example 8, cyclohexane derivatives (XX). If one tests the chair form XXa for the steric equivalence of the two carbon atoms labeled a′ and a″, one obtains on rotation arrangement XXc which is obviously different. However, the original compound is accompanied by an equal amount of XXb which yields XXd, when subjected to the

XXa   XXb   XXe

XXc   XXd   XXf
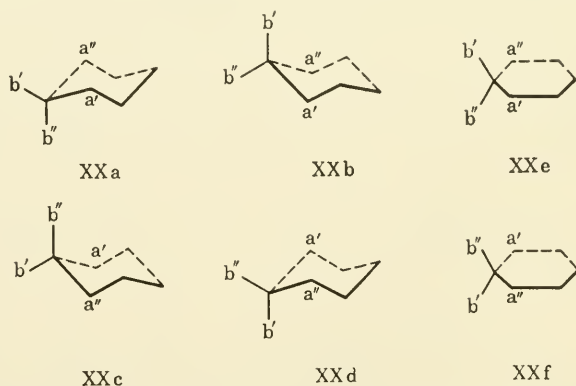
corresponding motion. Since XXd can be superposed on XXa, and XXc on XXb, the actual compound which contains equal parts of XXa and XXb meets the superposition test even if the individual molecules do not. It follows that the two a groups or b groups, re-

spectively, will react with equal rates under any condition which permits the equilibration of conformations. The chair forms, nevertheless, possess no simple axis greater than one. If we consider, however, not the prevalent conformations but the averaged positions of their nuclei, we obtain the planar form XX*e*, which on rotation yields XX*f*. This representation, therefore, meets rule 4 and contains a twofold simple axis.

Example 9, Caaa(+*b*) (*XXI*). This case differs from example 2 in having an asymmetric substituent +b instead of the symmetric b. Such a molecule has no simple axis of symmetry >1. Unless rotation around the central C-C bond is severely restricted, every conformation (e.g., XXI*a*) is accompanied by two others (XXI*b* and XXI*c*) which

d }
e   f } + b
    C
    |
    C
a' a''' a''
XXIa

d
e   f
    C
    |
    C
a'' a' a'''
XXIb

d
e   f
    C
    |
    C
a''' a'' a'
XXIc
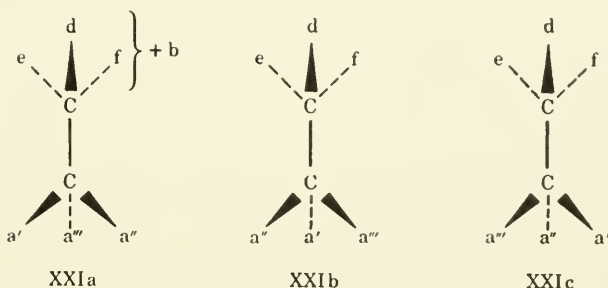
are equally stable and mutually superposable. The prevailing mixture, therefore, meets the superposition test jointly even if its individual members do not. The three a groups therefore cannot be differentiated from each other.

Example 10, RCOOH (*XXII*). Tautomerism ordinarily refers to an equilibrium of non-equivalent structures which is of no concern in this discussion. If we consider, however, a reaction such as the prototropic shift of an acid, the question of the steric equivalence of the two oxygen atoms arises. A mixture of XXII*a* and XXII*b* will yield by

$$XXIIa \quad R-C{\overset{O'}{\underset{O''H}{\lessgtr}}} \quad \Bigg\} \longleftrightarrow \Bigg\{ \quad R-C{\overset{O'H}{\underset{O''}{\lessgtr}}} \quad XXIIc$$

$$XXIIb \quad R-C{\overset{O''H}{\underset{O'}{\lessgtr}}} \quad\quad\quad R-C{\overset{O''}{\underset{O'H}{\lessgtr}}} \quad XXIId$$
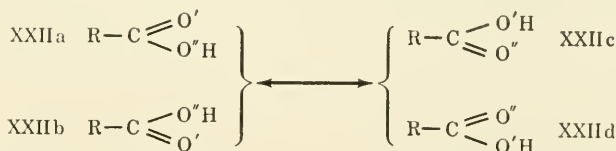
prototropy the superposable mixture XXII*c* and XXII*d*. Hence differentiation is not possible under ionizing conditions.

An alternative and somewhat simpler way of analyzing situations arising from rotational isomerism or tautomerism (examples 8–10) is

available if we recognize that the motions to be used for the super-position of identical substituents include not only those applicable to rigid bodies but also all internal motions of nuclei that proceed readily under reaction conditions. Obviously there is nothing in the derivation of rule 4 which would preclude such an interpretation of permissible motions.*

As set forth above, numerous mechanisms can be conceived to explain the differentiation of identical substituents in suitably constituted molecules. The efficacy of these mechanisms, however, is often quite small, and the question arises whether any process besides three-point attachment to an asymmetric reagent can be expected to lead to a differentiation of a high order. Studies of relatively simple systems afford at least a tentative answer to this question. Only a few demonstrations of discrimination of identical substituents in symmetric molecules have been recorded in which an asymmetric reagent of known structure is employed. Although these experiments have clearly demonstrated the occurrence of the phenomenon, they have left some doubt as to the exact role of the asymmetric agent in the differentiation.[4] The example reported most recently [4] appears to be no exception.[8] In order to observe the differentiating powers of thermodynamic and kinetic factors separately, it seems best, therefore, to turn to related phenomena. If a racemic mixture of a compound containing a labile asymmetric center is permitted to interact with an optically active reagent, the 1:1 proportion of optical antipodes is frequently disturbed.[1] The extent of such asymmetric transformations can be quite substantial even in homogeneous systems (62% excess in the case of chlorobromomethanesulfonic acid as a salt of (−)-hydroxyhydrinda-mine), but essentially complete conversion to one of the diastereoisomers occurred only if the transformation was aided by selective precipitation. Although the asymmetry so induced is frequently lost again by racemization after removal of the optically active reagent, such transformations can be looked upon as a demonstration of the efficacy which thermodynamic factors can possess in asymmetric processes.

Good examples for kinetic control of asymmetric processes can be

* Although the definition of symmetry number has also been adjusted to meet the problem of internal rotation (ref. 13, p. 510), such symmetry numbers are no longer applicable to our problem. For example, n-butane which has a "rigid symmetry number" of two and a "free-rotation symmetry number" of 18 contains six equivalent primary hydrogens but two non-equivalent pairs of secondary hydrogen atoms.

found in certain addition reactions to carbonyl groups. An analogy between asymmetric additions and the differentiation of identical substituents has been pointed out by Schwartz and Carter,[4] who suggest that the "two bonds" linking C and a in a = Cbc (or perhaps more appropriately the two layers of high electron density occupied by the π electrons) could be considered to correspond to the two a groups of the ordinary *meso* carbon atom. The following example has been studied most thoroughly. The symmetric α-keto acid $R_1COCOOH$ is esterified with the optically active alcohol $H(OH)Cab$; the resulting ester XXIII is treated with the Grignard reagent R_2MgX and then

XXIII   XXIV   XXV
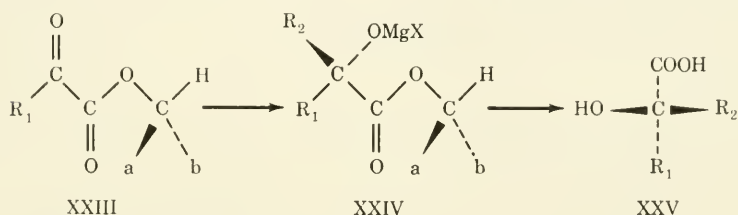
hydrolyzed to furnish the hydroxy acid XXV, which generally is found to consist of an unequal mixture of the two antipodes. Their proportion does not depend on the relative stabilities of the intermediate Grignard complexes XXIV, since an exchange of the alkyl groups between the keto acid and the Grignard reagent alters the sign of rotation of the resulting hydroxy acid. The steric result, therefore, is determined not by the nature of the product but by the mechanism of the addition reaction. Prelog et al.[14] were able to relate the directing influence of the alcohol $H(OH)Cab$ to the bulk and orientation of the alkyl substituents a and b and therefore presumably to their differential ability to block the approach of the Grignard reagent to the keto group. Although the distance between the blocking group and the site of the reaction is rather large, the steric selectivity went as high as 69% excess. Even greater predominance of one isomer may result if the keto group and the directing asymmetric center are adjacent to each other.[1,15]

These results seem sufficiently encouraging to warrant the view that even a single linkage between an enzyme and its substrate might explain a substantially complete differentiation of identical groups. This seems conceivable also in terms of a modified Ogston scheme if one assumes that the steric hindrance effects near the catalytic center are so graded and so distributed that only one of the two a groups can approach. As these mechanistic details may well be quite variable

and at the moment, at least, are largely a matter of conjecture, it seems fortunate that a knowledge of substrate structure alone suffices to foresee when differentiation is possible and when it is not.

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The Nitrogen-Sparing Effect
of Glucose

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The utilization of dietary nitrogen in the synthesis of body proteins is more efficient when starch, sucrose, or some other readily metabolized carbohydrate is present in the diet. This action of carbohydrates on the metabolism of nitrogen is commonly called the "nitrogen-sparing" effect. The retention of nitrogen induced in this way is a consequence of interactions in the intermediary metabolism of carbohydrates and amino acids and not of proteins. This follows from the fact that, when a small amount of N^{15}-glycine or N^{15}-aspartic acid is administered to fasting rats and to rats ingesting a solution of 30% glucose, 40% less N^{15}-urea and 40% less N^{14}-urea are excreted by the glucose-fed than by the fasting animals.[1] The fall in the total urea-nitrogen output is quantitatively accounted for in terms of reactions at the amino acid level, for almost all of the excreted N^{15}-urea was formed from the administered amino acid concurrently with and not after the incorporation of N^{15} into the body proteins. In similar experiments performed with N^{15}-ammonia it was shown that the amount of N^{15} appearing in the urinary urea of animals receiving N^{15}-ammonium citrate was 35% less when a solution of 30% glucose was ingested than when the animals were fasted.[2] These results are interpreted to mean that in the sharing of common pathways of metabolism glucose suppresses the oxidative deamination of amino acids and accelerates the synthesis of amino acids from ammonia, and/or that glucose inhibits the formation of urea by interfering with the operation of the Krebs-Henseleit cycle. In the following discussion these hypotheses will be examined in relation to our present knowledge of the reactions leading from the oxidative deamination of amino acids to the synthesis of urea.

In mammalian liver the oxidative deamination of amino acids results from the activities of two enzymatic processes, i.e., direct oxidative

deaminations catalyzed by the flavoproteins, L-amino acid oxidase and glycine oxidase,[3, 4] and indirect oxidative deaminations catalyzed by aminophorases and DPN-dependent glutamic dehydrogenase.[5] Whereas the deaminations brought about by the flavoproteins appear to be irreversible, this is not true of the reactions of the aminophorase— glutamic dehydrogenase system. Indeed with present evidence of the broad scope of transamination reactions [6] it may reasonably be assumed that in general the synthesis of amino acids from ammonia and α-keto acids occurs by way of the aminophorase-glutamic dehydrogenase system. Conditions are thus provided for a competition for ammonia between the glutamic dehydrogenase system and step I of the urea cycle, i.e., the citrulline-synthesizing system. This is indicated more clearly in the sequence of reactions shown.

$$
\begin{array}{ccc}
\text{Step II} & & \text{Step I} \\
\uparrow & & \uparrow \\
\text{Aspartic acid} & & \Big| \\
\Updownarrow & & \Big| \\
\text{L-Amino acids} \rightleftharpoons \text{Glutamic acid} \xrightleftharpoons[\text{DPNH}]{\text{DPN}^+} \text{Ammonia} \leftarrow \text{L-Amino acids}
\end{array}
$$
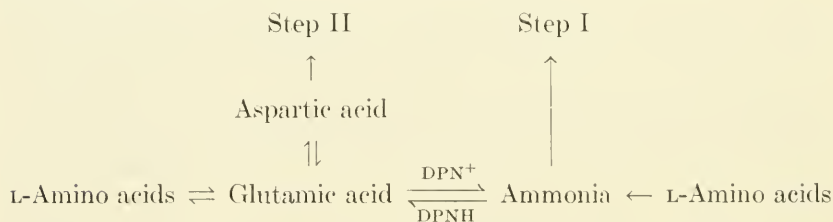
In accordance with the fact that the specific enzymatic activity of crystalline beef-liver glutamic dehydrogenase is 10 times greater when the enzyme is present in the reduced than when in the oxidized form,[7] it may be expected that a relatively small increase in the ratio of reduced to oxidized DPN may accelerate the formation of glutamic acid, diminish the concentration of hepatic ammonia, and thus reduce the rate of synthesis of urea. In the liver the synthesis and not the oxidation of glutamic acid predominates. Accordingly, the larger proportion of glutamic dehydrogenase is present in the reduced form and is maintained in this state by the operation of DPN-dependent coupled oxidations. Under conditions of low carbohydrate intake the coupling of the glutamic dehydrogenase system to endogenous oxidations, principally the oxidation of fat, may lead to a lower ratio of reduced to oxidized DPN than that which is established in the presence of exogenous glucose. Support for this hypothesis comes from the finding that fat, unlike glucose, does not evoke nitrogen retention in adult animals ingesting a protein meal.[8] If the above concept is correct, we may explain the nitrogen-sparing effect of carbohydrates by assuming that, in the oxidative metabolism of ingested carbohydrates, the ratio, DPNH/DPN, coupled to the glutamic dehydrogenase system, is so

increased as to promote further than before the synthesis of glutamic acid from ammonia and α-ketoglutaric acid.

The alternative hypothesis proposes that the nitrogen-sparing action of carbohydrates is a consequence of the inhibition of one or more reactions of the Krebs-Henseleit cycle. In step I of the urea cycle the synthesis of citrulline is accomplished in the enzyme-catalyzed reaction between ornithine and carbamyl phosphate, the latter compound being formed enzymatically from stoichiometric amounts of NH_3, CO_2, and ATP.[9] Siekevitz and Potter [10] have observed that the synthesis of citrulline by washed rat-liver mitochondria may be completely inhibited in the presence of a sufficiently high concentration of hexokinase and glucose. Since it was also noted that the concentration of ATP in the medium declined to low levels, the authors concluded that the hexokinase reaction competes for ATP with the citrulline-synthesizing system. It is conceivable also that step II of the urea cycle is similarly blocked in the competition for ATP between the arginine-synthesizing system and the hexokinase reaction. Clearly, serious consideration must be given to the competition for ATP as a factor of physiological significance in regulating the rate of synthesis of urea.

In rat-liver homogenates the synthesis of urea from ammonia and α-ketoglutaric acid is not as rapid as from glutamic acid.[11] To account for this finding it has been suggested that a high concentration of α-ketoglutaric acid inhibits transaminations from glutamic acid, thus restricting the amount of aspartic acid available to the arginine-synthesizing system. This is still another way in which the formation of urea may be regulated by interactions between the Krebs-Henseleit cycle and the intermediary metabolism of carbohydrates.

Referring once more to the sequence of reactions leading to the synthesis of urea from amino acids, it may be seen that, if the concept of interaction between the intermediary metabolism of carbohydrates and the aminophorase-glutamic dehydrogenase system is correct in accounting for the nitrogen-sparing effect of carbohydrates, it may be expected that the specific rate of utilization of ammonia in the formation of amino acids will be increased in the presence of dietary carbohydrate and that the concentration of hepatic ammonia will decline. On the other hand, if nitrogen retention results from the blocking of step I of the urea cycle, it may be anticipated that the specific rate of utilization of ammonia in the synthesis of citrulline will decline and the concentration of ammonia in the liver will increase. In the event that the intermediary metabolism of carbohydrates invokes a

nitrogen-sparing action by inhibiting step II of the urea cycle, one may anticipate a decline in the specific rate of utilization of aspartic acid in the synthesis of urea and no change, or possibly a rise, in the concentration of hepatic ammonia. However, in view of the fact that the specific rate of step II would also decline in the event of the slowing of step I, only the anticipated effect on the ammonia concentration is of interpretive value in this case.

In order to arrive at the correct interpretation we will consider the kinetics of utilization of N^{15}-ammonia in steps I and II of the urea cycle. In the scheme shown, a is the amount of N^{15} originally present as $N^{15}H_3$; s_1 is the fraction of $N^{15}H_3$ which, per unit time, is used in the synthesis of citrulline via step I; s_2 is the fraction of N^{15}, appearing in aspartic acid, which, per unit time, enters the urea cycle via step II; s_3 is the fraction of $N^{15}H_3$ which, per unit time, is transformed to aspartic acid; and s_4 is the fraction of N^{15} of aspartic acid which, per unit time, is incorporated into body proteins. U_1 and U_2 are the amounts of N^{15} which are ultimately utilized in steps I and II, re-

$$\text{Step I} \qquad\qquad \text{Step II}$$
$$U_1 \qquad\qquad\qquad U_2$$
$$\overset{s_1}{\nearrow} \qquad\qquad\qquad \overset{s_2}{\nearrow}$$
$$\text{Ammonia} \overset{s_3}{\longrightarrow} \text{Aspartic acid} \overset{s_4}{\longrightarrow} \text{Proteins}$$
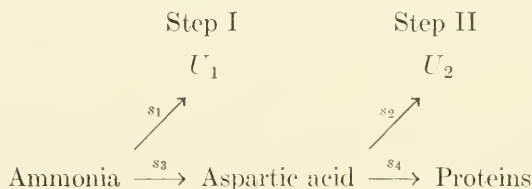
spectively. The assumed irreversibility of the utilization of ammonia in the formation of aspartic acid is in conformity with the view, previously stated, that the reaction between ammonia and α-ketoglutaric acid stongly favors the formation of glutamic acid, as well as with the observation that aspartic-glutamic aminophorase of rat liver favors the formation of aspartic acid in the ratio of 2 to 1.[12] Theory shows that when all of the administered N^{15} is completely distributed between urea and the body proteins (neglecting the relatively slow return of isotope from labeled tissue proteins):

$$(U/a)_{\text{Am}} = \frac{U_1 + U_2}{a} = \frac{s_2 s_3}{(s_1 + s_3)(s_2 + s_4)} + \frac{s_1}{(s_1 + s_3)} \tag{1}$$

$$\frac{U_1}{a} = \frac{s_1}{(s_1 + s_3)} \tag{2}$$

$$\frac{U_2}{a} = \frac{s_2 s_3}{(s_1 + s_3)(s_2 + s_4)} \tag{3}$$

Since $s_2/(s_2 + s_4)$ is readily evaluated by measuring the fraction of isotopic urea formed from a given amount of N^{15} aspartic acid,[13] the ratio s_3/s_1 may be calculated by substituting the appropriate data in the following equation:

$$s_3/s_1 = \frac{1 - (U/a)_{Am}}{(U/a)_{Am} - (U/a)_{As}} \tag{4}$$

where $(U/a)_{Am}$ is the fraction of N^{15} given as ammonia which appears in the urinary urea in an arbitrary time (2 days) and $(U/a)_{As}$ is the fraction of N^{15} given as aspartic acid which appears in the urinary urea during an equal interval. In Table 1 are the results of experiments

Table 1. Influence of Fasting and Glucose on the Utilization of N^{15}-Labeled Precursors in the Synthesis of Urea and Amino Acids

Conditions	$(U/a)_{Am}$	$(U/a)_{As}$	s_3/s_1	U_1/a	U_2/a	Rate of Excretion of Urinary Urea (mg. N/100 gm./hr.)
Fasting	0.70	0.35	1	0.50	0.18	2.2
Ingesting glucose	0.46	0.20	2	0.33	0.13	1.3

For definition of symbols see text.

carried out, as indicated, on fasting rats and on animals ingesting a solution of 30% glucose.

Attention is directed first to the results of calculations which show that glucose induces a substantial shift in the utilization of ammonia from step I of the urea cycle to the synthesis of amino acids. Theoretically the proportion of amino acids formed from ammonia is $s_3(s_3 + s_4)$. The calculated utilization of ammonia in the synthesis of amino acids in animals ingesting glucose is thus two-thirds, and in fasting animals one-half so that one-third more ammonia is transformed to amino acids and one-third less is used in the formation of urea. Although this accounts in large measure for the observed fall in the output of the total urea nitrogen of glucose-treated animals, the question which must be raised here is whether the increase in the ratio of s_3/s_1 resulting from the ingestion of glucose represents an increase in the absolute value of s_3, or whether s_1 is decreased in relation to s_3. In accordance with earlier considerations, an increase in the absolute value of s_3 may be expected to lead to a decrease in the concentration of ammonia in the liver whereas a decline in the absolute value of s_1 may be expected to have the reverse effect. It was found that the concentration of ammonia in the liver of the glucose-fed rats was approximately 30% less than in fasting animals. This was indicated by the

observation that the isotope concentration of the total urea nitrogen of the rats receiving glucose was 30% higher than that of the total urea nitrogen of fasting animals. The experimental results therefore support the view that the nitrogen-sparing effect of carbohydrates is a consequence of the coupling of the oxidation of carbohydrates to the aminophorase-glutamic dehydrogenase system.

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The Metabolism of Inositol
in Microorganisms

A STUDY OF MOLECULAR CONFORMATION
AND BIOCHEMICAL REACTIVITY

BORIS MAGASANIK

Inositol * was discovered in muscle extract by Scherer a little over 100 years ago and identified as a hexahydroxycyclohexane by Ma-
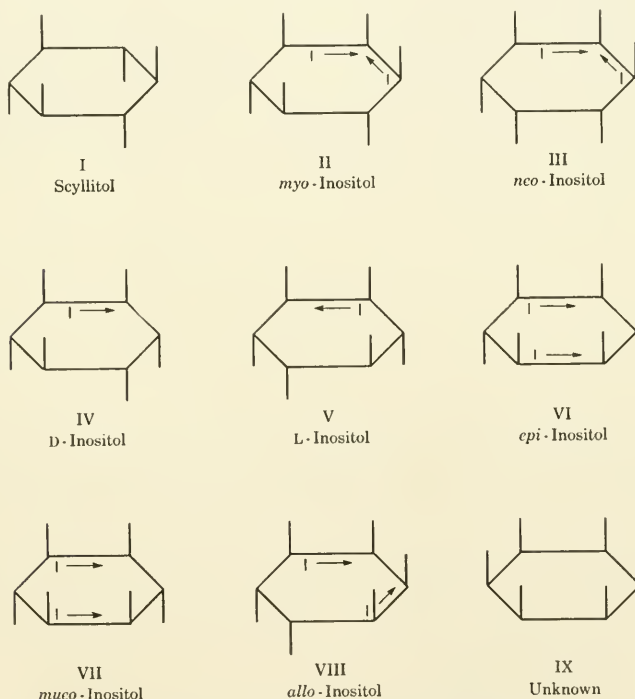
FIG. 1. The configurations of the inositols.

* Two reviews of the chemistry and biological activity of the inositols have appeared in recent years.[1,2] In this paper the nomenclature proposed by Fletcher, Anderson, and Lardy (see ref. 2) is used.

quenne 37 years later. Bouveault pointed out that theoretically nine stereoisomers of inositol could exist, two of which would be optical enantiomorphs. Four of these isomers are known to occur in nature. Scherer's muscle sugar, now called *myo*-inositol, an ubiquitous cell component, has been recognized as a growth factor for certain yeasts and molds and as a vitamin necessary for the health of rats and mice. Scyllitol, originally discovered in the organs of fish, occurs in trees and in human urine. D-inositol and L-inositol are found as monomethyl ethers in a variety of plants. Similarly, two of the sixteen possible stereoisomeric deoxyinositols have been isolated from plants. Of the remaining inositols, four have been synthesized, so that at present only one of the nine isomers remains unknown. The configurations of the inositols (Fig. 1) were determined by Posternak and by Dangschat and Fischer through the conversion of derivatives of the inositols to known saccharic acids.

The studies described here were begun in collaboration with Dr. Erwin Chargaff at the Department of Biochemistry, Columbia University, in 1946 and have their origin in the observation of Kluyver and Boezaardt[3] that *Acetobacter suboxydans* oxidizes *myo*-inositol (II) to a monoketone, subsequently identified by Posternak as 2-keto-*myo*-inositol (X).[4]

$$\frac{1}{2} O_2 \quad \text{A. suboxydans}$$

II　　　　　　　　　　　　　　　　　　X
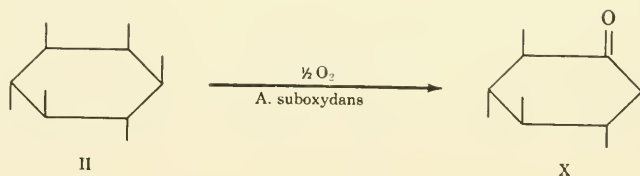
The microorganism had thus singled out the central one of the three vicinal *cis*-hydroxyl groups of *myo*-inositol for oxidation. The ability of *A. suboxydans* to carry out partial oxidations of polyhydroxy compounds to monoketones had long been known to be subject to certain steric limitations, which were formulated by Bertrand and by Hudson as the following rule: Only a secondary hydroxyl group located between a primary hydroxyl group and another secondary hydroxyl group in *cis* position is oxidized.

However, since this rule could obviously not be applied to cyclic compounds, it seemed of interest to study the specificity of the enzymatic attack of *A. suboxydans* on the hydroxyl groups of cyclitols, particularly as the rigidity of these cyclohexane derivatives, owing to the

lack of free rotation around carbon to carbon bonds, would permit a clearer correlation between reactivity and the spatial arrangement of the reactive groups.

The measurement of the oxygen uptake of resting cell suspensions of *A. suboxydans* with several isomers of inositol as substrates revealed that these compounds differed greatly in their susceptibility to enzymatic attack. *Myo*-inositol and *epi*-inositol were oxidized with the uptake of 1 gram atom of oxygen per mole and L-inositol, D-inositol, and L-2-deoxy-*muco*-inositol (*d*-quercitol) were oxidized with the uptake of 2 gram atoms of oxygen per mole, whereas scyllitol was not attacked at all.[5]

The oxidation product of *epi*-inositol was isolated and found to be a levorotatory ketoinositol which on reduction with sodium amalgam yielded *myo*-inositol, indicating that the hydroxyl group in either position 2 or 4 of *epi*-inositol had been oxidized.[5] Posternak isolated the same keto compound and converted it by oxidation with permanganate to a mixture of D-talomucic and D-glucosaccharic acid.[6] These reactions identified the *Acetobacter* oxidation product as D-2-keto-*epi*-inositol (XI).
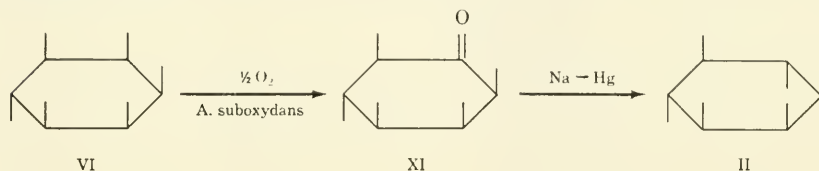
The final products of the oxidation of D- and of L-inositol were isolated by means of phenylhydrazine. Both products proved to be bisphenylhydrazones of diketoinositols; they had identical melting points, identical absorption spectra characteristic for osazones, and optical rotations equal but opposite in sign. The consumption of periodic acid corresponded to 3 moles of the oxidant per mole of bisphenylhydrazone. The products of this reaction were cyclic derivatives of the dialdehydes expected in the periodic acid oxidation of bisphenylhydrazones of α-diketoinositols. The racemic mixture of the two enantiomorphic α-bisphenylhydrazones was found to be identical with the osazone obtained by the treatment of the phenylhydrazone of 2-keto-*myo*-inositol (X) with phenylhydrazine. These reactions, shown on the accompanying flow sheet, identified the oxidation products of D- and of L-inositol as L-1,2-diketo-*myo*-inositol (XII) and D-1,2-diketo-*myo*-inositol (XIII), respectively.[5]

Phenylhydrazone of X
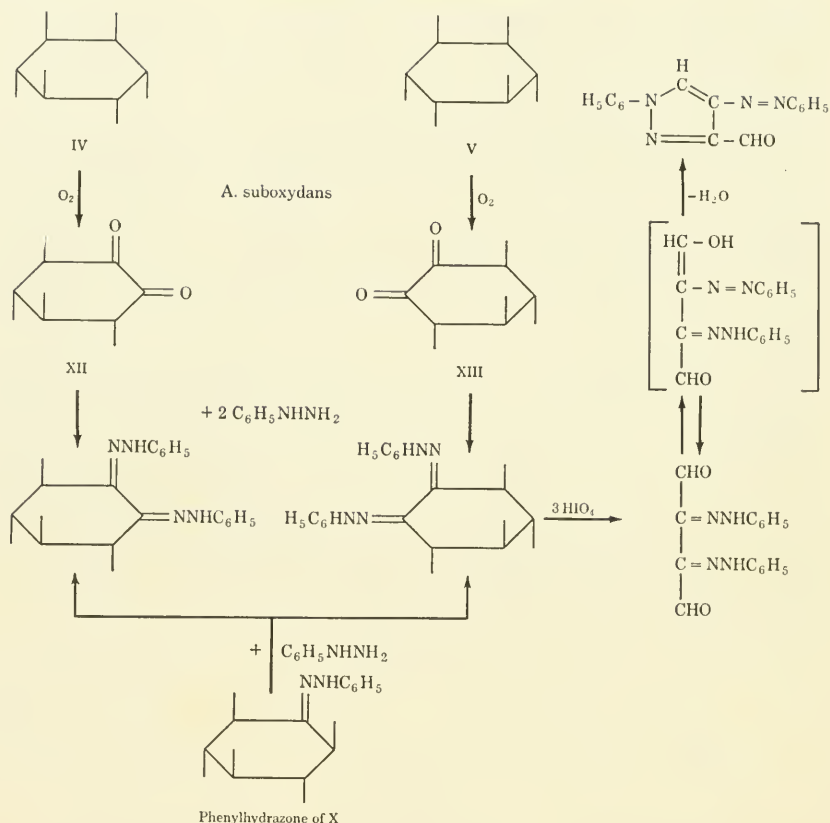
It was possible to isolate the monoketoinositol which is the initial product of the action of *A. suboxydans* on D-inositol (IV). The compound was identified as L-1-keto-*myo*-inositol (XIV) by its oxidation with resting cells of *A. suboxydans* to the diketone XII and by its reduction with hydrogen catalyzed by platinum to *myo*-inositol (II).[7]

The product formed by the enzymatic oxidation L-2-deoxy-*muco*-inositol (*d*-quercitol) (XV) was found to react with phenylhydrazine to form a bisphenylhydrazone which had an absorption spectrum characteristic of osazones and which reacted with periodic acid with the uptake of 2 moles of oxidant. These observations indicated that the keto groups were vicinal and that the three hydroxyl groups were located on adjacent carbon atoms. The choice between the two possible structures was made by comparing the rate of oxidation by periodic acid of this α-bisphenylhydrazone and the one prepared from
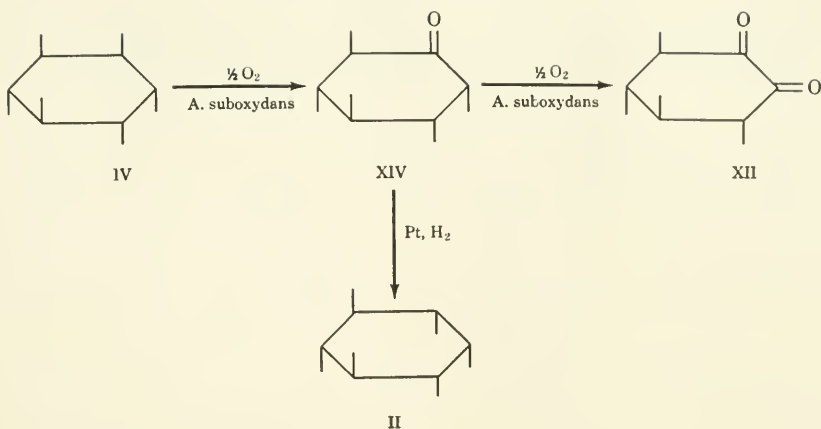
D-inositol (XII). The deoxyinositol derivative was not attacked more rapidly than the D-inositol derivative in which all hydroxyl groups are in *trans* position. The oxidation product of XV was therefore identified as D-2,3-diketo-4-deoxy-*epi*-inositol (XVI), in which the three adjacent hydroxyl groups are in *trans* position.[8]
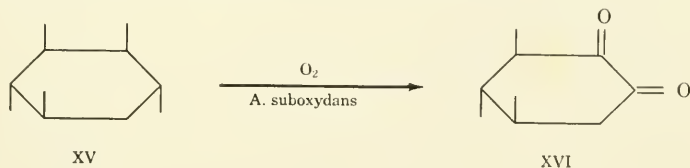
The action of *A. suboxydans* on these cyclitols seemed at first to fit no easily discernible pattern. In the case of the inositol isomers, one hydroxyl group of a pair of adjacent *cis* hydroxyls had been attacked, suggesting a specificity of oxidation similar to the one found in straight-chain compounds, but the oxidation of the hydroxyl group on carbon 3 of L-2-deoxy-*muco*-inositol (XV) was totally unexpected. However, it must be borne in mind that the structural formulas (Fig. 1) do not describe the actual position of the hydroxyl groups in space but represent merely planar projections based on the conventions introduced into stereochemistry by Emil Fischer. It had long been known that the cyclohexane molecule was not planar but could exist as a strainless ring in the "boat" and "chair" forms; later evidence obtained in studies using electron diffraction and infrared spectroscopy had led to the recognition of the chair form as the stable conformation of the cyclohexane ring (Fig. 2). Inspection of a model of cyclohexane

in the chair form reveals that the carbon atoms are equidistant from a plane passing through the center of the molecule. Six of the free
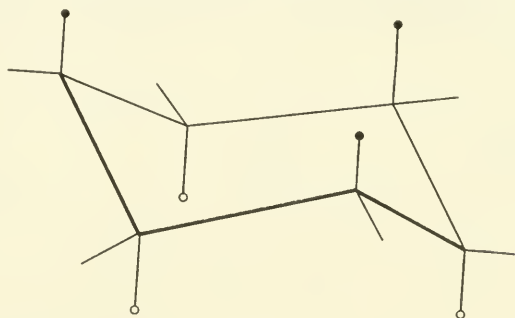
FIG. 2. The cyclohexane ring in the chair form. Equatorial bonds are shown as lines, north polar bonds as lines ending in solid circles, and south polar bonds by lines ending in open circles.
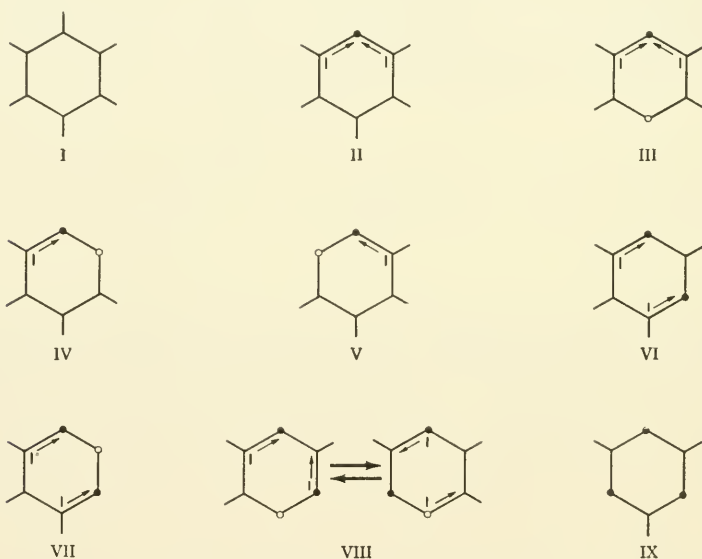
FIG. 3. The conformations of the inositols. Equatorial hydroxyl groups are represented by lines, north polar hydroxyl groups by solid circles, and south polar hydroxyl groups by open circles. The hydrogen atoms are not shown.

bonds on the carbon atoms are located in that plane projecting out-wards and are called equatorial bonds. The other six bonds are per-pendicular to the plane being directed alternately upwards (north

polar) and downwards (south polar).* Each carbon atom possesses one equatorial and one polar bond and can therefore carry substituents in either position. There are two possible chair forms, one being formed from the other by a movement of the ring atoms through the central plane which changes polar bonds to equatorials and vice versa.

In substituted cyclohexanes, the substituent groups occupy preferentially the equatorial positions in which more space is available than in the crowded polar regions. Consequently, when the two chair forms are not identical, the molecule will be found to exist in the conformation having the smallest number of polar substituents.

On the basis of these considerations the conformations shown in Fig. 3 could be assigned to the inositol isomers. It was reasonable to expect that the susceptibility of the inositol molecule to chemical and enzymatic attack would depend on the actual shape of the molecule as shown by its conformation; and indeed, on correlation of the action of *A. suboxydans* on the inositols with their conformations (Table 1),

Table 1. The Action of *A. suboxydans* on Inositols and Their Deoxy and Keto Derivatives

	Compound	Polar Hydroxyls, Positional Numbers *	Action of A. suboxydans		Ref.
			O₂ Taken Up, gram atoms per mole	Hydroxyls Oxidized, Positional Numbers *	
I	Scyllitol	None	None		5
II	*myo*-Inositol	2	1	2	4
III	*neo*-Inositol	2, 5	None		
IV	D-Inositol	2, 3	2	2, 3	5
V	L-Inositol	2, 3	2	2, 3	5
VI	*epi*-Inositol	2, 4	1	2	5,6
VII	*muco*-Inositol	2, 3, 4	2	Not determined	9
XVIII	2-Deoxy-*myo*-inositol	None	None		9
XXII	D-1-Deoxy-*myo*-inositol	2	1	2	10
XXI	L-1-Deoxy-*myo*-inositol	2	1	2	10
XIX	D-2-Deoxy-*epi*-inositol	4	None		9
XX	L-2-Deoxy-*epi*-inositol	4	1	4	9
XV	L-2-Deoxy-*muco*-inositol	3, 4	2	3, 4	8
X	2-Keto-*myo*-inositol	None	None		4
XI	D-2-Keto-*epi*-inositol	4	None		5
XVII	L-2-Keto-*epi*-inositol	4	1	Not determined	5
XIV	L-1-Keto-*myo*-inositol	2	1	2	7

* In *meso* compounds alternative numbering sequences are possible (see Fig. 1); for presentation in this table these compounds have been numbered clockwise.

a striking regularity became at once apparent: *only polar hydroxyl groups had been oxidized.*

* The use of the term "axial" instead of "polar" has been suggested to avoid ambiguity (D. M. R. Barton et al., *Science, 119,* 49 (1954).

However, it was also apparent that not *all* polar hydroxyl groups were attacked by the microorganism. Only one of the two polar groups of *epi*-inositol (VI) had been oxidized to yield the optically active D-2-keto-*epi*-inositol (XI), whose polar hydroxyl group in position 4 was resistant to further enzymatic oxidation. L-2-Keto-*epi*-inositol (XVII), on the other hand, could be attacked by *A. suboxydans,* and presumably converted to a diketone, as shown by the observation that racemic DL-2-keto-*epi*-inositol (XI + XVII) (produced from *myo*-inositol by oxidation with nitric acid) was oxidized with the uptake of 0.5 gram atoms of oxygen per mole.

In order to define the requirements for oxidation by *A. suboxydans* with greater stringency, three deoxyinositols were prepared and subjected to the action of the microorganism. 2-Deoxy-*myo*-inositol (XVIII), prepared by the catalytic reduction under acid conditions of 2-keto-*myo*-inositol (X), was not attacked. DL-2-Deoxy-*epi*-inositol (XIX + XX), prepared in a similar manner from DL-2-keto-*epi*-inositol (XI + XVII), was oxidized with the uptake of 0.5 gram atoms of oxygen per mole. The isomer oxidized was identified as L-2-deoxy-*epi*-inositol (XX) by the isolation of unchanged XIX from the reaction mixture, as well as by the demonstration that XIX prepared from D-2-keto-*epi*-inositol (XI) was resistant to the action of *A. suboxydans*. The point of the enzymatic attack on XX was identified as the polar hydroxyl group in position 4 by the reduction of the resulting monoketone with sodium amalgam to 2-deoxy-*myo*-inositol (XVIII).[9]
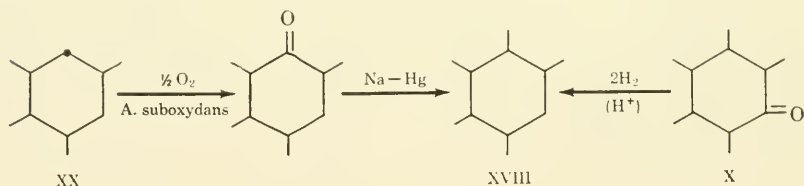
Inspection of the conformations of those pairs of enantiomorphs of which one member only is oxidized by *A. suboxydans* revealed that the isomers susceptible to attack all possess an equatorial hydroxyl group in position *d* relative to the location of the oxidizable polar hydroxyl group.
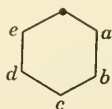
Replacement of this equatorial hydroxyl by a polar hydroxyl (VI*b*),*
by oxygen (XI) or by hydrogen (XIX) prevents enzymatic oxidation;
corresponding changes in position *b* are without effect.

VIb*   XI   XIX   Not oxidized

VIa*   XVII   XX   Oxidized
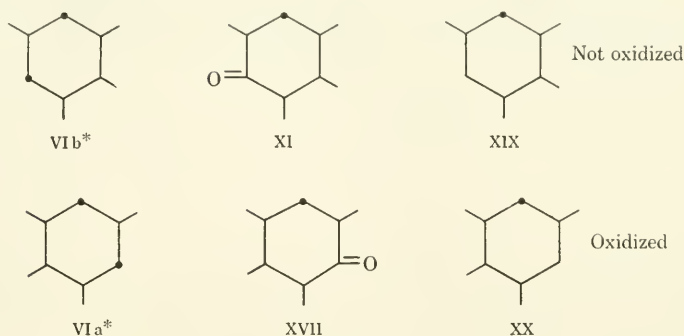
The validity of this generalization was confirmed and its scope ex-
tended by considering the other cyclitols that had been studied. *Myo*-
inositol (XI) has five equatorial hydroxyls. The replacement of the
equatorial hydroxyl in position *a* by a polar hydroxyl group (IV), by
hydrogen (XXI), or by oxygen (XIV) does not interfere with the
oxidation of the north polar hydroxyl group.

IV   XXI   XIV   Oxidized
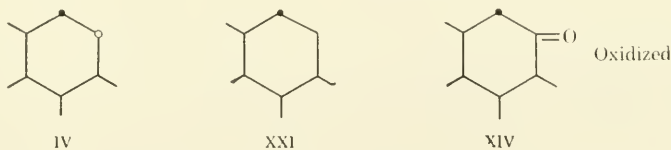
Similarly, oxidation of the polar hydroxyl group occurs when the
equatorial hydroxyl in position *e* is replaced by a polar hydroxyl (V)
or by hydrogen (XXII).

V   XXII   Oxidized
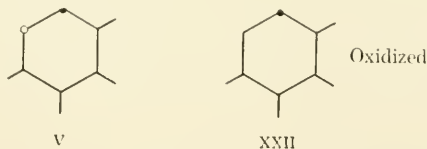
The importance of the equatorial hydroxyl group in position *c* could
at that time not be ascertained, as no compound without an equatorial

* The structures are so arranged as to place the polar hydroxyl group under
comparison at the top of the hexagon. For this reason *epi*-inositol (VI) is shown
in two arrangements (VI*a* and VI*b*).

hydroxyl in this position was available. Recently *neo*-inositol (III) which carries a polar hydroxyl group in position *c* was synthesized by Angyal;[11] his kind gift of a small amount of this isomer permitted its use as a substrate for *A. suboxydans*. It was not attacked; apparently the presence of an equatorial hydroxyl group in position *c* is required for oxidation by the microorganism.

Not oxidized

III
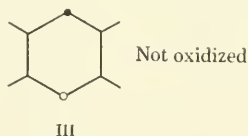
The results of these studies can be summarized in three rules defining the steric requirements for the oxidation of inositols, deoxyinositols, and ketoinositols by *A. suboxydans* (Fig. 4).
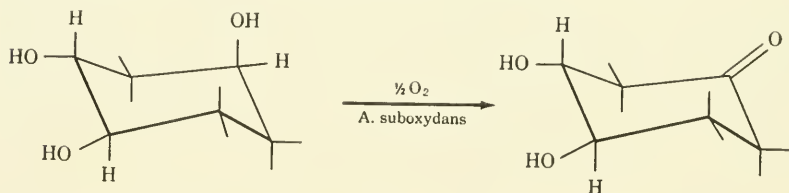
Fig. 4.   The steric requirements for oxidation by *A. suboxydans*.

1. Only polar hydroxyl groups are oxidized.

2. The carbon in *meta* position to the one carrying the polar hydroxyl group (in counterclockwise direction if north polar, clockwise if south polar) must carry an equatorial hydroxyl group.

3. The carbon in *para* position to the one carrying the polar hydroxyl group must carry an equatorial hydroxyl group.

These structural requirements demonstrate three points of contact between substrate and enzyme and can account for the oxidation of *epi*-inositol (VI) to D-2-keto-*epi*-inositol (IX), an asymmetric synthesis which theoretically demands a three-point attachment of substrate to enzyme. The carbon atom carrying the polar hydroxyl group seems to be particularly susceptible to dehydrogenation, as shown by the recent report that platinum catalyzes the specific conversion of *myo*-inositol (II) to 2-keto-*myo*-inositol (X) with oxygen as hydrogen acceptor.[12] The initial attack of the *A. suboxydans* enzyme is presumably directed against the equatorial hydrogen atom located on the carbon carrying the polar hydroxyl group; the enzyme-substrate com-

plex may be held together by bonds between the enzyme or a metal ion associated with the enzyme and the two required equatorial hydroxyl groups which occupy the same plane as the hydrogen atom (Fig. 4).

The rules which have been presented apply to *hexahydroxycyclohexanes, pentahydroxycyclohexanes,* and their *keto* derivatives. *Trihydroxycyclohexanes* are oxidized by a different enzyme and one subject to rules which have as yet not been elucidated; it is possible that this enzyme is identical with the one responsible for the oxidation of straight-chain polyhydroxy compounds.

The biological significance of the inositol dehydrogenase of *A. suboxydans* is not known. The enzyme is not adaptive but is present in cells grown in the absence as well as the presence of inositol. The organism obtains useful energy but no building blocks for the synthesis of its protoplasm by the incomplete oxidation of the inositols. In other species of microorganisms, however, *myo*-inositol can be metabolized with the production of energy and building blocks. This can be inferred from the observation that seven bacterial species of a group of fourteen can grow on *myo*-inositol as the only source of carbon.[13] One of these species, *Aerobacter aerogenes*, was chosen for a study of this extensive degradation of the inositol molecule.

It was found that in addition to *myo*-inositol (II), four other cyclitols [D-inositol (IV), 2-keto-*myo*-inositol (X), L-1-keto-*myo*-inositol (XIV) and L-1,2-diketo-*myo*-inositol (XII)] could support the growth of capsulated strains of *A. aerogenes* as sole sources of carbon. Scyllitol (I) was not attacked by the microorganism but inhibited specifically and reversibly the dissimilation of these five compounds. The other cyclitols tested (V, VI, XIX, XX, XV, XI, XVII, and XIII) showed neither growth-supporting nor inhibitory activity.[14]

II   IV   X   XIV   XII   I
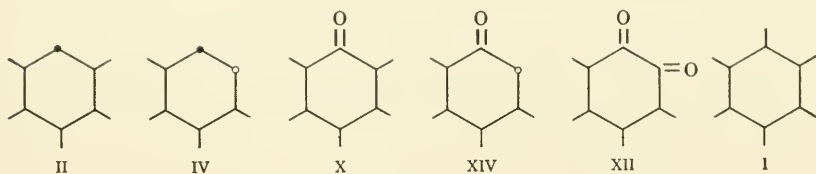
Suspensions of cells grown on glucose did not oxidize the five cyclitols immediately but only after a period of lag of about 1 hour, although suspensions of cells grown on *myo*-inositol oxidized this compound as well as the other four cyclitols without lag. The process occurring during the period of lag could be shown to require energy by its susceptibility to the inhibitory action of dinitrophenol. The energy was

apparently used for the biosynthesis of protein from small precursors, since the ability of glucose-grown amino acid-deficient mutants of this organism to attack *myo*-inositol was stimulated by the amino acid required for growth.[15] These experiments indicated that the attack on the cyclitols was mediated by adaptive enzymes whose synthesis could be induced by *myo*-inositol. The validity of this assumption was confirmed by the demonstration that extracts of *myo*-inositol-grown cells possessed two enzymes (later identified as a *dehydrogenase* acting on inositols II and IV and a *dehydrase* acting on the ketoinositols X and XIV), which were not found in extracts of glucose-grown cells.[16] The enzymes could be separated by treatment with protamin sulfate. The dehydrogenase was precipitated and could be redissolved by the dissociation of the insoluble protamin salt with polymethacrylate.

The dehydrogenase purified in this fashion was found to catalyze the reduction of diphosphopyridine nucleotide (DPN), but not that of triphosphopyridine nucleotide, by *myo*-inositol, and more slowly by D-inositol, in the presence and absence of inorganic phosphate. The equilibria of these reactions were too unfavorable for dehydrogenation (even at a relatively high *p*H) to permit the isolation of the products; therefore the reverse reaction, the oxidation of DPNH by ketoinositols, was investigated. 2-Keto-*myo*-inositol (X) reacted with DPNH in the presence of the enzyme to give equimolar amounts of DPN and of *myo*-inositol (II) (determined by bioassay with *inositol-less Neurospora crassa*); L-1-keto-*myo*-inositol (XIV) reacted with DPNH to give DPN but was itself not converted to *myo*-inositol. These results indicated that the enzyme catalyzes the conversions of *myo*-inositol (II) and of D-inositol (IV) to monoketones by removing the

Reaction 1a   II   + DPN$^+$ ⇌ X   + DPNH + H$^+$

Reaction 1b   IV   + DPN$^+$ ⇌ XIV   + DPNH + H$^+$
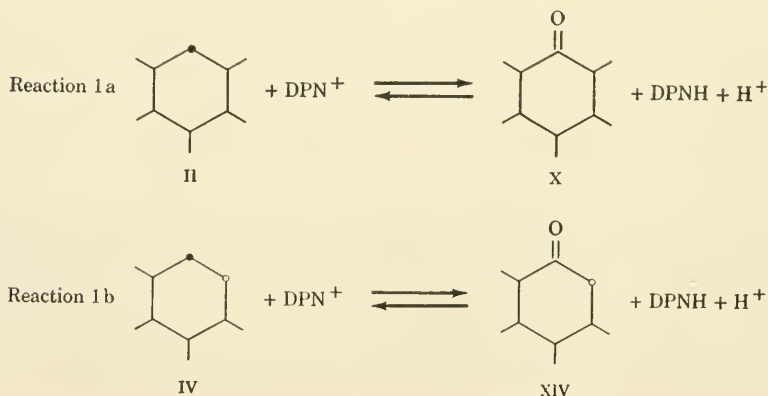
hydrogen atom from carbon atoms carrying a polar hydroxyl group (reactions 1a, b).

The *Aerobacter* enzyme had thus singled out the same carbon atom of *myo*-inositol for dehydrogenation as the *Acetobacter* enzyme. Apparently, a carbon atom carrying a polar hydroxyl group is more susceptible to dehydrogenation than one carrying an equatorial hydroxyl group; this concept is in good agreement with the observation mentioned earlier that platinum similarly catalyzes specifically the attack on the carbon atom of *myo*-inositol which carries the polar hydroxyl group. The inability of the *Aerobacter* enzyme to act on most of the cyclitols which are attacked by the *Acetobacter* enzyme shows that the steric requirements of the two enzymes other than their specificity for polar hydroxyl groups are not the same.

A. suboxydans does not possess the enzymes necessary to carry the attack on *myo*-inositol beyond 2-keto-*myo*-inositol. *A. aerogenes,* on the other hand, possesses an enzyme found in the supernatant fluid after treatment with protamin sulfate, which acts on 2-keto-*myo*-inositol (X) in the absence of added cofactors. The strong absorption band of the product (*E*, 4000) at 261 mμ and its reducing properties suggested it to be an α,β unsaturated ketone. This assumption was confirmed by the isolation of this compound by means of phenylhydrazine. The hydrazone was identified by its reaction with periodic acid and its absorption spectrum as the bisphenylhydrazone of 2,3-diketo-4-deoxy-*epi*-inositol (XVI); the same compound had previously been obtained through the oxidation of L-2-deoxy-*muco*-inositol (XV) by *A. suboxydans*. These results show the enzyme to be a *dehydrase* which converts 2-keto-*myo*-inositol to the enol XXIII by attacking one of the equatorial hydroxyl groups in *meta* position to the keto
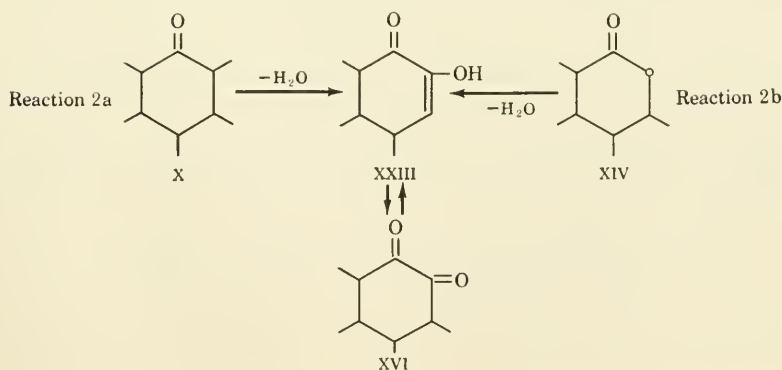
group (reaction $2a$). A similar attack converts L-1-keto-*myo*-inositol to the same product at a slower rate (reaction $2b$).

The presence of the dehydrogenase and the dehydrase in extracts of *myo*-inositol-grown cells indicates that the initial attack of *A. aerogenes* on *myo*-inositol is a dehydrogenation (reaction $1a$), followed by dehydration (reaction $2a$). The ability of inositol-adapted cells to attack 2-keto-*myo*-inositol is in agreement with its role as an intermediate in this reaction sequence. The simultaneous adaptation to D-inositol and L-1-keto-*myo*-inositol finds its explanation in the fact that these compounds are attacked by the same enzymes as *myo*-inositol and 2-keto-*myo*-inositol and converted to a common product, the enol XXIII.

The further steps in the degradation of *myo*-inositol have as yet not been demonstrated in a cell-free system. The rapid oxidation and fermentation of L-1,2-diketo-*myo*-inositol (XII) by suspensions of *myo*-inositol-grown cells suggests that this compound is an intermediate in the degradation of *myo*-inositol. It could presumably arise by oxidation of the enol XXIII (reaction 3).

Reaction 3

XXIII   XII
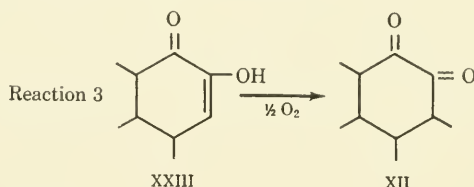
The broad outlines of the metabolic pathway leading to the complete degradation of *myo*-inositol were elucidated by the study of the products of inositol dissimilation under aerobic and anaerobic conditions.[17] The oxidation of *myo*-inositol, 2-keto-*myo*-inositol, or 1,2-diketo-*myo*-inositol by resting cell suspensions of *A. aerogenes* yielded the same products as the oxidation of glucose. The amount of CO_2 produced and of O_2 taken up was sufficient for the complete oxidation of one-half of the molecule to CO_2 and H_2O. The other half of the molecule was apparently assimilated as material of the composition $C_3H_6O_3$. In the presence of dinitrophenol, an agent known to inhibit oxidative assimilation, the oxygen uptake approached the theoretical values for complete oxidation. In the presence of As_2O_3, an inhibitor of pyruvate degradation, glucose was degraded largely to 3-carbon compounds (pyruvate and lactate) whereas *myo*-inositol and the keto-inositols were dissimilated to a mixture of 3-carbon compounds (pyruvate and lactate), 2-carbon compounds (acetate and ethanol), and

CO_2. Similar patterns were observed in the anaerobic degradations of glucose and the cyclitols. Glucose yielded acid, but no CO_2; in the presence of As_2O_3 glucose was converted to 2 moles of lactic acid per mole. On the other hand, *myo*-inositol, as well as its keto derivatives, was fermented with the production of 1 mole of CO_2 per mole of substrate. In the presence of As_2O_3 *myo*-inositol was converted to an
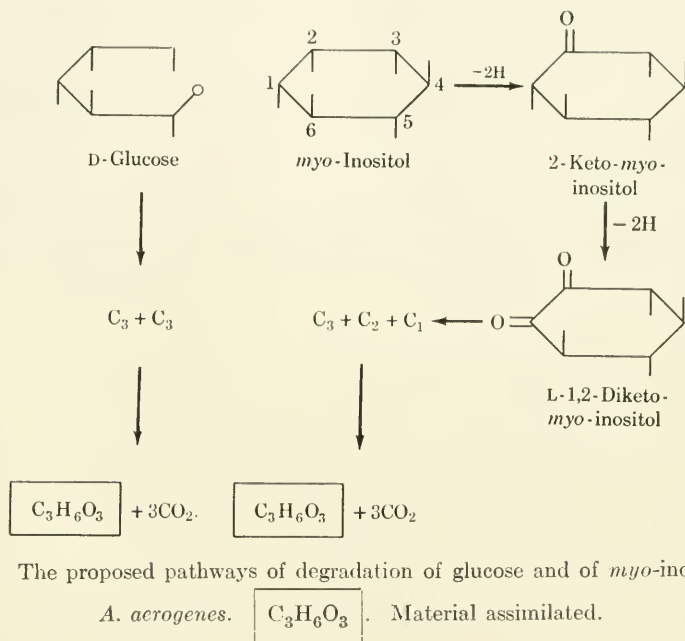
FIG. 5. The proposed pathways of degradation of glucose and of *myo*-inositol in *A. aerogenes.* $\boxed{C_3H_6O_3}$. Material assimilated.

equimolar mixture of CO_2, ethanol, and lactic acid; apparently pyruvate and acetate (or an active form of acetate) can serve as hydrogen acceptors in the dehydrogenation steps (reactions 1*a* and 3).

Thus glucose and *myo*-inositol are metabolized by *A. aerogenes* by different pathways to the same final products (Fig. 5). The direct conversion of *myo*-inositol to glucose by cleavage of the bond between carbon atoms 3 and 4 which has frequently been postulated does not occur in this system. However, the production of pyruvate from *myo*-inositol by a pathway corresponding to the one described in *A. aerogenes* could well explain the antiketogenic effect of *myo*-inositol, and the conversion of stably bound deuterium in *myo*-inositol to stably bound deuterium in glucose which have been observed in the rat.[2]

The pathway of inositol degradation is superficially similar to the

"oxidative pathway" of glucose dissimilation, in that in both the carbon chain is split with the production of CO_2 and a 3-carbon compound. However, the inositol pathway has the distinguishing characteristic that the initial enzymatic attack is a dehydrogenation and not a phosphorylation. The degradation proceeds at least as far as the diketone XII without the introduction of a phosphate group. It is not known by what mechanism the energy generated in the dehydrogenation steps (reactions 1 and 3) can be utilized by the cell. The observation that the microorganism can grow on *myo*-inositol in the absence of oxygen suggests that a process other than oxidative phosphorylation can serve to harness the reactions of inositol degradation to the production of useful energy.

The ability to initiate the degradation of a polyhydroxy compound with the dehydrogenation of a secondary hydroxyl group seems to be characteristic of certain microorganisms. *A. suboxydans* converts *myo*-inositol to 2-keto-*myo-inositol* and glycerol to dihydroxyacetone, but it does not possess the enzymes for the rapid degradation of these keto compounds. Capsulated strains of *A. aerogenes* can carry out not only the complete degradation of *myo*-inositol but also that of glycerol.[18] The direct dehydrogenation of glycerol by an enzyme whose synthesis is specifically induced by glycerol yields dihydroxyacetone which is rapidly dissimilated via pyruvic acid. On the other hand, acapsulated strains of *A. aerogenes* cannot attack *myo*-inositol at all and initiate the degradation of glycerol with its phosphorylation to L-α-glycerophosphate.

The pathways of dissimilation in which dehydrogenation is the initial step of the enzymatic attack are as efficient as the classical Embden-Meyerhoff pathway in providing capsulated *A. aerogenes* with energy and building blocks for growth, since the growth rate and the total cell crop are nearly the same whether glucose, glycerol, or *myo*-inositol is the sole carbon source. However, the formation of adaptive enzymes, such as the biosynthesis of histidase, which is induced by histidine, proceeds during growth on *myo*-inositol or glycerol but not during growth on glucose.[19] Thus the ultimate source of energy may have a profound influence on the enzymatic constitution of the bacterial cell.

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The Biochemistry
of Ferritin

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An interpretation of biological phenomena in terms of chemical structure or chemical interaction remains a basic goal of biochemistry. Progress along these lines has been made with compounds of low molecular weight and relatively simple structure. Examples are the reactions involved in metabolism of carbohydrates, fatty acids, and amino acids; these reactions can now be written in great detail and their mechanisms have been revealed. Such advances have been aided in no small measure by the relative ease with which these compounds can be obtained in a pure state and by the simplicity of the criteria for their purity.

The difficulties are greatly multiplied for macromolecules such as proteins, where the criteria for purity are themselves often in question. Progress in relating function to chemical structure is currently in evidence among one group of proteins, the enzymes; their activities are measured by methods which are relatively simple, quick, and quantitative. However, for proteins endowed with hormonal activity, our understanding is more limited. Because of the nature of the tests for physiological activity, the isolation of protein hormones in a pure state has been slow and studies relating their activity to structure are hampered by lack of sufficient amounts of the protein, lack of accuracy of the determination, and the length of time required to obtain an adequate measure of activity. An additional factor which complicates the study of protein hormones is the fact that the observed activity in an animal, organ, or tissue is usually the end result of a series of reactions which occur between the administration of the protein and the final observation of functional activity, with no evidence concerning the nature of the initial or intermediate reactions.

Methods of study relating the activity of protein hormones to their chemical structure can be patterned after those used so successfully with enzymes. The early suggestions concerning enzyme-substrate interaction were brought to a focus by the Michaelis-Menten formulation of enzyme-substrate compound formation and subsequently to the implication in the reaction of specific groups on the enzyme surface.

More difficult to study from a dynamic point of view are those cellular proteins which we loosely term "structural" or "storage" proteins. Certainly such proteins have a biological function and can undergo chemical alterations in addition to those of biosynthesis and degradation, but the problem of measuring such subtle changes or attempting to relate the structure of these proteins to function is obviously very difficult. The present report is concerned, nevertheless, with attempts to study the relationship of protein structure to biological activity where the protein is a "storage" protein, ferritin.

Work in our laboratory, involving alterations in the circulation of animals in hemorrhagic shock led to the identification of the iron protein ferritin with a substance liberated in very small quantities into the circulation during this state. The physiological activities which our early work had associated with ferritin seemed unrelated at that time to its well-known iron-storage function. These activities, which might be termed hormonal if they could be shown to operate under physiological rather than pathological conditions, are twofold:

(a) The intravenous injection into a normal rat of very small quantities of ferritin results in a temporary inhibition of the constrictor response to the topical application of adrenaline on the part of the muscular capillary blood vessels in the mesentery. This has been called its "vasodepressor" effect.

(b) The injection of ferritin into the circulation of the hydrated rabbit or dog stimulates the neurohypophysis to the secretion of its antidiuretic hormone, which in turn acts on the kidney tubules to bring about an increased resorption of water, i.e., an oliguric or "antidiuretic" effect.

With these two activities, non-quantitative and time-consuming as their measurements may be, we were better able to investigate the relationship of the functional groups in ferritin to activity than if we had known only about its iron-storage activity. As will be seen, our present findings make it likely that all three physiological activities of ferritin are related to the same chemical groups and to similar alterations in their structure.

The Bulk of Ferritin Iron

Ferritin is found mostly in the liver and spleen and to a lesser extent in bone marrow, kidneys, and placenta, and in much smaller quantities in skeletal muscle, testes, and pancreas. It has also been reported to be present in the intestinal mucosa of the anemic guinea pig in response to iron feeding.[1] Table 1 lists quantitative data for the ferritin

Table 1. Ferritin Content of Various Tissues

Species	Hemo-globin, gm. per cent	R.B.C. $\times 10^6$	Ferritin N, μg./gm. wet tissue							
			Liver	Spleen	Bone Marrow	Kidney Cortex	Pan-creas	Skeletal Muscle	Cardiac Muscle	Pla-centa
Dog	16.2	6.1	134	90	16	18	6	2	1	—
Dog	14.1	6.2	65	99	18	—	8	5	1	—
Dog	14.1	6.1	75	166	12	14	3	9	2	—
Dog	14.1	6.1	115	42	—	22	7	2	1	—
Human										6
Human										10
Human										12

A. Mazur and E. Shorr, *J. Biol. Chem.*, *182*, 607 (1950); determined by the quantitative immuno-chemical method.

content of dog tissues [2] and human placenta, obtained by the quantitative immunochemical method of Heidelberger. The best source for the isolation of crystalline ferritin is horse spleen, from which it is prepared by the method of Granick [3] using $CdSO_4$ for crystallization of the protein as first recommended by Laufberger.[4] Concentrated ferritin solutions free of inorganic ions are obtained by dialysis and can be stored in sterile bottles in the refrigerator after filtration through a Seitz filter. Such ferritin preparations have a low cadmium content and are quite stable.

The bulk of iron in ferritin appears to exist in the form of colloidal ferric hydroxide, since the visible absorption spectrum is identical at equivalent concentrations of iron with that of solutions of colloidal ferric hydroxide. The iron is tightly held by the protein but can be removed by prolonged dialysis of ferritin solutions in the presence of sodium hydrosulfite at pH 4.6 in concentrated acetate buffer and α,α'-dipyridyl. This treatment results in the reduction of ferric to ferrous iron and the removal of the latter by complexing with di-pyridyl. After several such treatments and dialysis against water, the protein is undenatured and essentially colorless. Addition of $CdSO_4$ produces crystals of apoferritin, almost entirely free of iron, and identical in form with those obtained from ferritin. Ferritin is

also associated with phosphate which is removed together with the iron during this procedure (Table 2). Granick has assigned $(FeOOH)_8$-

Table 2. Elementary Analysis of Ferritin and Apoferritin

	Total N, %	Total Fe, %	Total P, %
Ferritin	11.0	20.7	1.29
Apoferritin	16.2	0.0	0.05

A. Mazur, I. Litt, and E. Shorr, *J. Biol. Chem.*, *187*, 473, 1950; analytical data reported on the basis of dry weight. Various preparations of ferritin vary in their ratio of N:Fe:P.

$(FeOOPO_3H_2)$ as the formula for the colloidal micelles in ferritin.

As a result of measurements of the paramagnetic susceptibility of ferritin as well as of the ferric hydroxide-ferric phosphate prepared from ferritin by alkali treatment, Michaelis [5] reported that the iron atoms in this protein have an orbital arrangement corresponding to 3 unpaired electrons. Ferric iron may also exist with 1 or 5 unpaired electrons. This type of orbital arrangement in ferritin iron makes it unique among biological iron compounds and stresses the highly specific nature of the iron-incorporation reaction which takes place during ferritin biosynthesis, since the iron compounds ingested with food represent all types of iron with respect to paramagnetic susceptibility. It also points to a specific type of iron binding to the protein, the nature of which is still unknown.

The ultracentrifugal pattern obtained with ferritin solutions [6] indicates that it is a mixture of molecules consisting of approximately 20 to 25% iron-free apoferritin together with a series of ferritin molecules of varying total iron content and presumably in varying states of aggregation. Apoferritin, however, behaves as a single component during ultracentrifugation with a sedimentation constant corresponding to a molecular weight of 465,000 (horse apoferritin). If considered as an ellipsoid, apoferritin has an axial ratio of 3:1.

In contrast to its behavior in the ultracentrifuge, ferritin behaves as a single component on electrophoresis. Apoferritin has mobilities identical with those of ferritin over a range of pH from 4 to 8.6,[2] indicating that the large quantity of iron in ferritin does not affect the surface charge density of the protein in solution at these pH's. Other evidences of similarities of these two proteins from the point of view of surface properties are: identical viscosities [7] calculated on the basis of nitrogen content, and identical quantitative immunochemical re-

actions of ferritin and apoferritin when added to the antibody directed against either of these proteins.[2] These findings, together with direct electron microscopic studies by Farrant [8] appear to offer conclusive evidence that *most* of the iron lies inside the protein molecule.

Little information can be offered concerning the nature of bonding of the iron micelles to the protein. However, differences between ferritin and apoferritin can be demonstrated with regard to ease of denaturation. As a rule globular proteins must be denatured before they can be digested by pepsin or trypsin. This is also true for ferritin and apoferritin.[9] Our studies show, in addition, that ferritin is less easily denatured by acid (at the same pH) than apoferritin (Table 3)

Table 3. Effect of pH on Peptic Hydrolysis and Protein Denaturation

| pH | Ferritin | | Apoferritin | | Hemoglobin |
	Hydrol-ysis	Dena-turation	Hydrol-ysis	Dena-turation	Hydrolysis
1.6	67	69	99	99	96
2.0	16	35	79	47	89
2.5	3	3	17	17	19
3.0	0	0	4	4	18

A. Mazur, I. Litt, and E. Shorr, *J. Biol. Chem.*, *187*, 473, 1950. Denaturation determined by degree of insolubility at the isoelectric point.

and that the greater the iron content of ferritin the less easily it is denatured and, therefore, digested by pepsin (Table 4). After de-

Table 4. Effect of Iron Content on Peptic Hydrolysis of Ferritin

Ferritin Sample	$\dfrac{\text{mg. Fe}}{\text{mg. N}}$	Per cent Hydrolysis
Original	1.55	11
Fraction A	1.04	27
Fraction C	0.0	52

A. Mazur, I. Litt, and E. Shorr, *J. Biol. Chem.*, *187*, 473, 1950. Fractions A and C prepared from original ferritin by partial and complete removal of iron, respectively, by reduction with hydrolsulfite and dialysis.

naturation by urea-alkali, both proteins are digested by trypsin. The protective effect of ferritin-bound iron against acid denaturation of the protein may be due to the existence of strong bonds between the iron micelles and groups within the protein which are necessary for maintenance of its native state.

Amino acid analyses of apoferritin and ferritin indicate that no nitrogen components are present other than amino acids. The content of dicarboxylic and basic amino acids (Table 5) agrees with the observed

Table 5. Amino Acid Nitrogen Distribution in Apoferritin and Ferritin

| | Apoferritin | | Ferritin, |
| | | | |
Amino Acid	gm. per 100 gm. protein	% of total N	% of total N
Ammonia N		10.0	
Humin N		3.4	
Glutamic acid	17.2	10.1	9.9
Aspartic acid	6.8	4.4	
Lysine	7.8	9.2	
Arginine	9.1	18.0	
Histidine	4.8	8.0	
Cystine	1.7	1.2	
Methionine	1.9	1.1	1.0
Tyrosine	5.0	2.4	
Phenylalanine	6.1	3.2	3.0
Leucine	19.1	12.6	12.5
Isoleucine	1.4	0.9	
Glycine	3.4	3.9	
Valine	4.3	3.2	3.1
Alanine	1.9	1.8	
Threonine	4.3	3.1	
Proline	1.5	1.1	
Tryptophan	1.2	1.0	

A. Mazur, I. Litt, and E. Shorr, *J. Biol. Chem.*, *187*, 473, 1950. Serine has also been shown to be present. Analyses reported above were done in most cases by the microbiological assay technique; several by specific colorimetric methods. The total S was 0.89%, of which 98% was accounted for by the cystine and methionine content. The amino N was 5.0% of the total N.

isoelectric point for horse ferritin, 4.4, determined electrophoretically. Crystalline dog ferritin has an isoelectric point of 5.2; that from the human is 5.5. Evidence was obtained from quantitative immunochemical studies [2] that these three ferritins are related but not identical; they cross-react, but the heterologous ferritin antigen reacts to a lesser extent than the homologous ferritin with its antibody. However, ferritins from different tissues of the same animal (liver and spleen) appear to be immunochemically identical.

Sulfhydryl Groups and Ferritin Activity

Our studies attempting to relate the structure of ferritin to its biological activities made use of the rat test for vasodepressor effect [10]

and the antidiuretic action in hydrated dogs as indices of physiological activity.[11]   Inactivation of these ferritin activities by aerobic liver slices (rat, rabbit, or dog) and activation by anaerobic liver slices suggested the presence of groups in ferritin capable of undergoing reversible oxidation-reduction.   Inactive ferritin could be activated by treatment with cysteine or reduced glutathione, whereas active ferritin was inactivated by treatment with iodoacetamide, o-iodosobenzoate or p-chloromercuribenzoate, all sulfhydryl-reacting reagents.   These data appeared to fit the hypothesis that sulfhydryl groups were involved in these biological activities of ferritin.

Chemical estimation of sulfhydryl groups yielded elevated values for active ferritin and decreased values for inactive ferritin.   The choice of a method for measurement of SH content was a difficult problem because of the intense color of the protein.   Amperometric titration with Ag^+ or Hg^{++} gave poor results with ferritin as it does with some proteins.   The method of Rosner using iodoacetic acid was modified so as to use iodoacetamide followed by precipitation of the ferritin with trichloracetic acid.   The extent of reaction of sulfhydryl groups with iodoacetamide (for a 10-minute period at pH 7.4) was measured in the clear supernatant solution by oxidizing the HI formed, with H_2O_2, to yield free iodine.   The iodine color was read in a photocolorimeter before and after treatment with thiosulfate.   If adequate blanks are used, this method gives reproducible values and is quite specific for sulfhydryl groups since addition of p-chloromercuribenzoate prior to reaction with iodoacetamide reduces the extent of reaction of ferritin with iodoacetamide, under these conditions, to zero.

The conclusion, based on the data described above, that sulfhydryl groups were directly related to ferritin activity, was brought into question by an experimental finding contradictory to this hypothesis. When inactive iodoacetamide-treated ferritin is incubated with cysteine, reduced glutathione or ascorbic acid, the resulting ferritin becomes quite active.   Since the reaction of sulfhydryl groups with iodoacetamide is known to be irreversible, we had to consider the possibility that another group capable of undergoing oxidation-reduction might be responsible for activity of this protein.

Iron and Ferritin Activity

In our earlier studies, iron was not seriously considered as related to ferritin activity for two reasons:

(a) Apoferritin, essentially free of iron, is as active as ferritin containing 23% iron, when tested in the rat for vasodepressor activity

and when administered intravenously to the dog for study of its anti-diuretic effect.

(*b*) Ferritin can be separated into a number of fractions which have varying total iron:total nitrogen ratios, all of which, however, are equally active on the basis of nitrogen content.

The first of these results could be explained without eliminating iron as a participant if it could be shown that apoferritin, on intravenous injection, combines with iron in the plasma to yield active ferritin. The second could be explained if the various fractions obtained by fractionating ferritin could be shown to consist of two forms of iron, one of which was present in these fractions in a constant ratio to the nitrogen content. This second form of iron would fulfill the requirements of oxidation-reduction reactions if it were ionic and therefore capable of existence either in the ferric or ferrous state.

Iodoacetamide-treated ferritin, with no measurable sulfhydryl content was incubated with cysteine, ascorbic acid, or reduced glutathione. The reagents were then removed by dialysis and the ferritin analyzed for sulfhydryl groups. No sulfhydryl groups were regenerated by cysteine or ascorbic acid, but a definite though small increase appeared after treatment with glutathione. The new sulfhydryl groups were, however, part of the added glutathione which had been bound to the ferritin molecule. This was established by incubating iodoacetamide ferritin with S^{35}-labeled glutathione followed by extensive dialysis against water. The radioactivity associated with the ferritin was found to be approximately equal to the sulfhydryl content as measured chemically by reaction with iodoacetamide and could be removed by treatment with trichloracetic acid. These results make it unlikely that ferritin activity is *directly* concerned with sulfhydryl groups.

Active ferritin solutions were now treated with α,α'-dipyridyl and the ferritin precipitated by addition of an equal volume of saturated ammonium sulfate. The clear protein-free supernatant solution had the typical pink color of the ferrous-dipyridyl complex even when the reaction was carried out at pH 7.4. At a constant concentration of dipyridyl and varying concentrations of ferritin the data showed that, as the ferritin was diluted, a larger fraction of its total iron was bound by dipyridyl in the form of ferrous iron. At a constant concentration of both dipyridyl and ferritin the quantity of ferrous iron bound by dipyridyl increased with decrease in pH. These results were consistent with the hypothesis of a competition between dipyridyl and ferritin for its ferrous iron, which was therefore capable of some dissociation and probably at or near the surface of the protein.

Although this method does not yield data for the absolute amount of ferrous iron in ferritin it was found useful for comparative purposes. From this data it can be calculated that active ferritin can contain at least 0.2% of its total iron in the form of ferrous iron.

Repetition of our earlier experiments to include ferrous iron analyses indicated that whenever sulfhydryl groups increased ferrous iron also increased, and vice versa (Table 6). Further, iodoacetamide ferritin

Table 6. Effect of Liver Slices on Sulfhydryl Groups and Ferrous Iron of Ferritin

Ferritin Treatment	SH	Fe^{++}
	μM per 100 mg. ferritin N	
Original ferritin	25.0	1.7
(a) Ferritin + liver slices in N_2	33.7	6.5
(b) (a) + liver slices in O_2	14.9	2.3

A. Mazur, S. Baez, and E. Shorr, *J. Biol. Chem.*, March, 1955.

after treatment with cysteine, ascorbic acid, or glutathione showed marked increases in ferrous iron content (Table 7 and 8). Finally,

Table 7. Effect of Reducing Agents on Ferrous Iron of Ferritin

Treatment	Ferrous Iron / Total Iron
	μM per mM
Original ferritin	0.86
Ferritin + GSH	9.3
Ferritin + ascorbate	27.6
Ferritin + cysteine	105.0

A. Mazur, S. Baez, and E. Shorr, *J. Biol. Chem.*, March, 1955.

Table 8. Effect of Reducing Agents on Iodoacetamide-Treated Ferritin

Treatment	SH / Total N	Ferrous Iron / Total N
	μM per 100 mg. total N	
(a) Original ferritin	16.5	2.0
(b) Ferritin + iodoacetamide and dialyzed	6.5	1.2
(b) + cysteine	5.2	11.1
(b) + ascorbate	5.7	4.9
(b) + glutathione	8.7	8.4

A. Mazur, S. Baez, and E. Shorr, *J. Biol. Chem.*, March, 1955.

after treatment of ferritin with iodoacetamide, o-iodosobenzoate, or p-chloromercuribenzoate, a decrease in ferrous iron content was noted. Since iodoacetamide and p-chloromercuribenzoate are known to react only with sulfhydryl groups and not with iron, the result may be explained if we assume that the function of the sulfhydryl groups in active ferritin is to stabilize the ferrous iron against autoxidation, a reaction which occurs spontaneously when ferrous iron is added to water or to most proteins at neutral pH. Alteration of the sulfhydryl groups would thus lead to autoxidation of ferrous iron and inactivation of ferritin. It should be noted that chelation of metals like ferrous iron or cuprous copper tends to stabilize the lower valence state of these metals.

The finding, mentioned previously, of equal vasodepressor activity of ferritin fractions with varying total iron content was now reinvestigated. Fractions were prepared from ferritin by serial precipitation using increasing concentrations of ammonium sulfate. In this way a number of fractions were obtained with decreasing ratios of total iron: total nitrogen as the concentration of ammonium sulfate needed to precipitate these fractions increased (Table 9). The sulfhydryl con-

Table 9.   Relationship of Total Iron, Ferrous Iron, and SH groups in Ferritin Fractions

Concentration of $(NH_4)_2SO_4$, % of saturation	Total Iron / Total N	Ferrous Iron / Total N	SH / Total N	Ferrous Iron / Total Iron
		μM per mM		
(Original)	(454)	(0.8)	(4.1)	(1.7)
0–27	549	0.7	3.2	1.2
27–31	454	0.6	3.3	1.4
31–34	361	0.6	3.4	1.6
34–50	251	0.8	5.1	3.2

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tent among these fractions was fairly constant with the exception of that fraction containing the least total iron; it had a higher sulfhydryl content and would correspond to a mixture of molecules relatively rich in apoferritin. In contrast, the ratio of ferrous iron:total nitrogen was constant for all fractions, a result to be expected if ferrous iron were more specifically associated with ferritin activity.

The other difficulty with the argument for iron participation in ferritin activity is the equivalent activity of iron-free apoferritin. There is no direct evidence for the reaction of apoferritin with plasma

iron to form a ferrous apoferritin compound. However, some indirect evidence is available to indicate that apoferritin needs to be in contact with plasma before it can become active as an antidiuretic, whereas ferritin does not. A study of its antidiuretic action has shown that ferritin acts by stimulating the neurohypophysis. When injected into the circulation via the femoral vein ferritin is a potent antidiuretic in amounts of 150 to 250 μg. ferritin N per kg; the same is true of apoferritin. Both soon disappear from the circulation, presumably due to inactivation by the liver. However, when injected directly into the carotid artery in order to reach the neurohypophysis immediately, ferritin was found to be active in amounts of 10 to 30 μg. ferritin nitrogen per kg., whereas at these concentrations apoferritin was not active at all. This result would be consistent with the apparent inability of apoferritin to combine with plasma iron in the brief interval between injection and arrival at the site of action.

Our data therefore lead us to postulate the following structure for ferritin: a protein, apoferritin, containing varying quantities of colloidal micelles or clusters of ferric hydroxide-ferric phosphate internally situated and held to the protein by unknown bonds. The iron is probably in equilibrium with surface ionic iron which may exist in the ferric or ferrous state, the state being dependent on the presence of free sulfhydryl groups, which help to chelate the ferrous iron and thus stabilize it against autoxidation. Blocking or oxidation of the sulfhydryl groups leads to a spontaneous oxidation of ferrous to ferric iron, this change representing a change from a physiologically active to inactive ferritin. The activity of ferritin is primarily due to the presence of stably bound ferrous iron which nevertheless is capable of dissociation for combination with any avid iron-binding agent.

Transport of Iron from Liver to Plasma

Our earlier experiments with anaerobic liver indicated that all of the ferritin in such a liver exists in the sulfhydryl form. We were interested in determining whether the presence of an hypoxic liver would result in an increase in plasma iron derived from the more easily dissociable hepatic ferritin-ferrous iron. Dogs, subjected to prolonged hypotension by graded hemorrhage to the state of shock, are known to have hypoxic livers; that is, livers in which the ferritin is in the sulfhydryl state and can now be assumed to contain a maximum of ferrous iron at its surface. Plasma samples were withdrawn from a series of such animals and analyzed for total iron and iron-

binding capacity. From these two values the degree of saturation of the plasma iron-binding protein could be calculated.

Fourteen dogs were bled by a standard procedure for inducing hemorrhagic shock. Of these, 9 died within 24 hours after terminating the experiment by retransfusion of all the blood previously withdrawn. Analyses of the last blood sample gave the following values:

(a) Total iron: *287* (173–534) % of the original.

(b) Iron-binding capacity: *15* (6–25) % of the original.

(c) Saturation of iron-binding protein: *90* (80–97) % as contrasted with 20 to 40 % for control values.

Of the remaining 5 dogs in this series, one survived beyond 24 hours but died subsequently, with values for the last blood sample similar to those noted above. The remaining 4 dogs which survived showed less marked changes for final plasma samples:

(a) Total iron: *171* (142–216) % of original.

(b) Iron-binding capacity: *46* (38–58) % of original.

(c) Saturation of iron-binding protein: *70* (55–84) %.

In contrast to these alterations, changes were barely evident in another series of dogs pretreated with the adrenergic blocking agent Dibenzyline * and then subjected to hemorrhagic hypotension of an equivalent degree and duration. Dogs pretreated with Dibenzyline usually survive this hemorrhagic procedure. There is evidence that Dibenzyline exerts its protective effect by virtue of its blunting action on the intense peripheral vasoconstriction which normally accompanies hemorrhage, thus maintaining a better blood flow through the liver and other splanchnic organs. Six dogs treated in this manner had plasma values for the last sample as follows:

(a) Plasma iron: *100* (50–150) % of original.

(b) Iron-binding capacity: *86* (54–133) % of original.

(c) Saturation of iron-binding protein: *40* (18–67) %.

These results confirm the reality in vivo of the more labile ferrous linkage to ferritin and emphasize the fact that in the reduced state ferritin can liberate its iron for passage into the plasma, to be bound by the plasma iron-binding protein. This process was readily demonstrated in vitro by dialysis of partially purified plasma iron-binding protein against several ferritins, each treated in such a way that they contained differing quantities of ferrous iron (Table 10). The greatest quantity of ferritin iron was transported for binding by the iron-binding

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* N-Phenoxyisopropyl-N-benzyl-β-chlorethylamine, manufactured by Smith, Kline and French, Philadelphia.

Table 10. Extent of Binding of Ferritin Ferrous Iron by Plasma Iron-Binding Protein

	SH Content	Fe^{++} Content (c)	Fe^{++} Bound by Plasma Iron-Binding Protein	
Ferritin Treatment	μM/100 mg. ferritin N		μM/100 mg. ferritin N	Per cent of (c)
Original Ferritin	25.0	1.7	0.8	47
(a) Ferritin + liver slices in N$_2$	33.7	6.5	5.7	88
(b) (a) + liver slices in O$_2$	14.9	2.3	0.2	9

A. Mazur, S. Baez, and E. Shorr, *J. Biol. Chem.*, March, 1955.

protein from that ferritin which had been incubated under anaerobic conditions with rat-liver slices. The least ferritin iron was transported from that ferritin which had been treated aerobically with liver slices. Intermediate binding occurred with an untreated ferritin solution.

Mechanism of Iron Transport

Since ferritin occurs in the bone marrow we next determined whether marrow could convert ferric ferritin to ferrous ferritin. It was demonstrated that rabbit-bone-marrow suspensions were able under anaerobic conditions to increase the ferrous iron content of ferritin. As a result of these studies we can postulate that all three physiological activities of ferritin—its iron-storage and release property, its vasodepressor activity, and its antidiuretic activity—are related to the same functional groups in ferritin and that the same mechanisms operate for their alteration. A scheme is shown in Fig. 1 which attempts to demonstrate this idea. Although this scheme is not novel,[12] it does give mechanisms which are substantiated by experimental data for almost all reactions:

1. Inactive ferric disulfide ferritin in the liver is changed to active ferrous sulfhydryl ferritin under hypoxic conditions. Glutathione, which is present in the liver in relatively high concentrations, can perform this reaction. The hypoxia can be mild and local under normal physiological conditions, thus allowing a small amount of iron to be present in the ferrous state.

2. Ferrous iron from reduced ferritin is transferred into the plasma to be bound by the plasma iron-binding protein. This reaction occurs in vivo as an acute response to severe blood loss and is probably a reflection of a less marked transfer of iron under physiological conditions.

3. The iron-binding plasma protein is presumed by many workers to contain ferric iron only, although Laurell provides evidence [13] which

makes it likely that some of the iron is in the ferrous state and that the complex is dissociable in vivo. The iron could thus be made available for transport to the bone marrow for storage as ferritin.

4. In response to lowered oxygen tensions, such as occur during anoxia or at high altitudes, some ferritin iron is reduced in the marrow to the ferrous state so that it can combine with protoporphyrin for
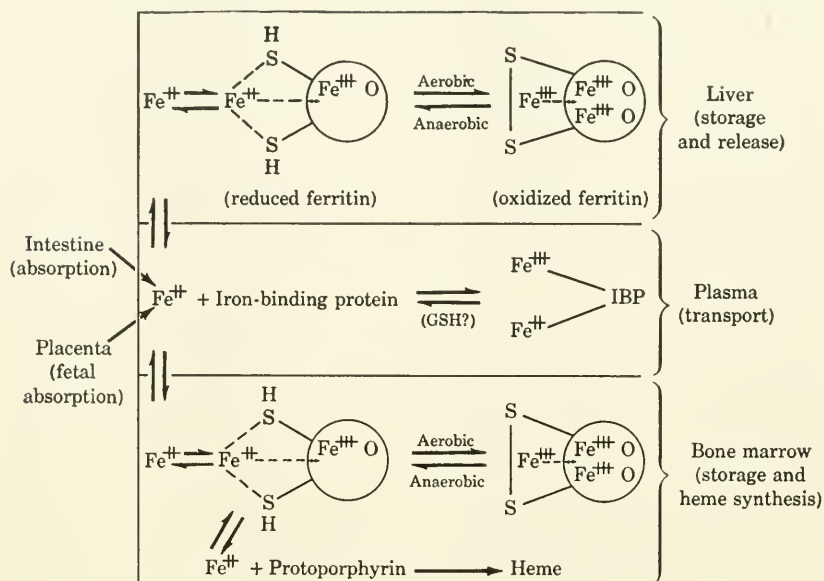
Fig. 1. Scheme illustrating the participation of ferritin in iron transport.

heme synthesis. The latter reaction has been reported by Granick in hemolyzates of chick red cells under anaerobic conditions, after addition of inorganic ferrous iron and protoporphyrin.[14]

5. The presence of ferritin in the placenta and the presence there as well of lowered oxygen tensions [15] makes this tissue an ideal one for storage of iron for purposes of transfer from maternal to fetal plasma. Ferritin in the placenta would exist in the ferrous state, and the transfer of iron would take place regularly across the placental barrier to the fetal plasma iron-binding protein. This hypothesis is supported by the observation [13] that in the fetus serum iron is normal or slightly increased and iron-binding capacity is low, whereas in the pregnant woman serum iron is somewhat low or normal and iron-binding capacity greatly increased.

Biochemical Activity of Ferritin

The activity of ferritin in vivo as demonstrated by its "vasodepressor" effect suggests the possibility that it, or its iron, might react with endogenous adrenaline in the smooth muscle cells of the precapillary blood vessels and catalyze its oxidative inactivation. In this way the smooth muscle cells would now become less reactive to the topical application of a threshold concentration of exogenous adrenaline, an effect which occurs during the rat mesoappendix assay. Some of the preliminary results obtained from experiments concerned with the interaction of ferritin with adrenaline may be pertinent to the mechanism of action of ferritin in vivo.

At pH 4.5 ferritin itself has little effect on adrenaline oxidation. The same is true for inorganic Fe^{++} or Fe^{+++}, although the latter forms a colored complex with adrenaline. Such colored complexes are known to form with many o-dihydroxyphenols and their colors vary with pH. Adrenaline forms similar compounds with Fe^{+++} which are green at pH 4.5, purple at pH 6.0, and wine red above 7.0. The different colors are due to varying ratios of Fe^{+++}:phenol in the complex. On the basis of reports with other o-dihydroxyphenols it is likely that at pH 4.5 the ratio of Fe^{+++}:phenol is 1:1 and that at pH 7.4 it is 1:2 or 1:3.

At pH 4.5, in acetate buffer and in the presence of ferritin, the addition of H_2O_2 results in a catalytic oxidation of adrenaline to a series of colored compounds, of which the N-methyl indole quinone, adrenochrome, can be recognized. Adrenochrome then undergoes further oxidative changes which result in the formation of melaninlike pigments, a conversion which is relatively slow at acid pH. The addition of H_2O_2 to inorganic Fe^{++} also results in a catalytic oxidation of adrenaline to adrenochrome. Fe^{+++} has a lower initial activity but does act eventually since it is reduced to Fe^{++} by the H_2O_2 present. These can best be interpreted in terms of the action of Fenton's reagent (Fe^{++} and H_2O_2) which produces the free radicals OH and O_2H which oxidize adrenaline. A similar oxidation of adrenaline takes place in the presence of high-energy X rays which are presumed to act via the formation of similar free radicals.

The pseudoperoxidase action of ferritin at acid pH cannot be used to suggest a mechanism for adrenaline oxidation in vivo. However, at a pH of 7.4, ferritin itself catalyzes the oxidation of adrenaline without the addition of H_2O_2. At this pH, adrenochrome which is formed is rapidly converted to the melanins, a reaction which is not

affected by ferritin. The major difference between the reactions at 4.5 and 7.4 is the fact that, whereas at the more acid pH inorganic Fe^{+++} is less active than Fe^{++}, at pH 7.4 Fe^{+++} is more active than Fe^{++} in catalyzing the oxidation of adrenaline. The activity of inorganic Fe^{++} is due to its autoxidation, at pH 7.4, to Fe^{+++}. One should keep in mind that the addition of inorganic iron salts to solutions at pH 7.4 would ordinarily produce insoluble and highly undissociated hydroxides were it not for the presence in the above systems of adrenaline, which forms soluble complexes with these ions. Ferritin, of course, serves the very useful purpose of carrying both Fe^{++} and Fe^{+++} at pH 7.4 in a soluble and reactive state.

A curious problem now arises with respect to (a) the biological activities of ferritin and (b) its adrenaline oxidation activity, since in (a) it is the ferrous iron which is associated with activity and in (b) it is the Fe^{+++} which appears to be responsible for its activity. It is possible however to suggest an hypothesis for the behavior of ferritin in vivo which would satisfy both of these findings.

The available evidence concerning the passage of iron across a cell-wall barrier—whether it be from intestine across the intestinal mucosa for iron absorption, from placenta across the placental membrane into fetal blood, or from liver ferritin stores across the liver cell wall into the plasma for transport—requires that iron be present in the ferrous state. Since the biologically active form of ferritin contains Fe^{++} in a dissociable state, these facts may hold the clue to the problem: Ferritin which is circulating in the plasma is active because it carries ferrous iron capable of passing across the muscle cell wall into its interior. Once inside the cell the Fe^{++} would be oxidized to Fe^{+++}, complex with adrenaline, and bring about its oxidation.

The reaction sequence for the oxidation of adrenaline by iron derived from ferritin may be expressed along the lines suggested by the work of Nelson and Dawson [16] on the oxidation of catechol by tyrosinase:

Adrenochrome
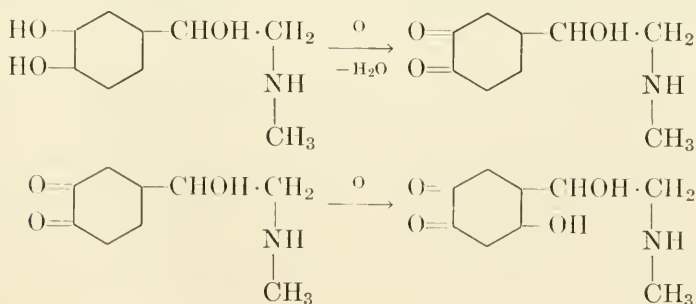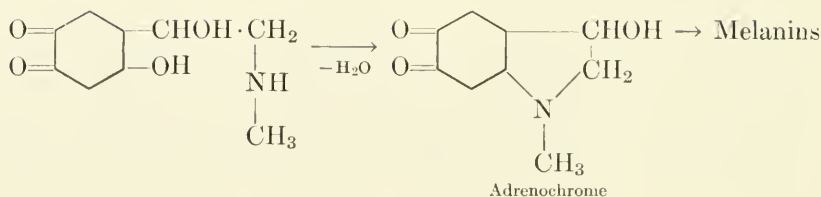
The end result of these reactions is the catalytic destruction of the physiological activity of adrenaline, brought about by the appearance in the circulation of the sulfhydryl ferrous form of ferritin in very small quantities. In addition, during the state of irreversible hemorrhagic shock, because of the change within the liver cell of disulfide ferric ferritin to the reduced state, there is produced an increased quantity of ferrous iron available for transfer into the circulation. Such extra iron tends to saturate the iron-binding protein of the plasma and decreases its effectiveness in reacting with the ferrous iron which is being carried by circulating ferritin. Thus, ferritin is portrayed as the carrier, by virtue of its surface sulfhydryl groups, of the iron which, after penetration of the smooth muscle cell wall, will inactivate adrenaline.

Finally, it is now possible to suggest a reason why the injection of relatively large doses of active ferritin does not bring about a proportionally greater effect on the precapillary blood vessels, an experimental finding which has troubled us for some time. Just as there exists a "mucosal block" that sets a limit to the extent of iron absorption through the intestinal wall, there may also exist a similar block to the transfer of ferrous iron across the smooth muscle cell wall under normal physiological conditions. By this method a limit is soon reached to the intensity of the biological effect of circulating ferritin. Should the permeability of the cell wall be damaged (under pathological conditions), this block to iron transfer would be removed, with consequent deleterious effects on the cell. Could this be the state in irreversible hemorrhagic shock?

The experimental facts and hypotheses which have been presented serve to emphasize that the biological actions of ferritin—its vasodepressor effect, its antidiuretic effect, and its iron-storage and iron-release properties—are affected by changes in oxygen tension which may be local and operative under normal conditions or may be general and acute under pathological conditions. In addition the biological effects which this molecule can exert may be limited by a factor such as permeability. In this respect, ferritin may be regarded as part of

a homeostatic mechanism which can perform useful biological work. These findings also point to the fact that proteins which are usually thought of as relatively inert storage compounds may have wider physiological function. In the present case, all three activities are based on the peculiar ability of ferritin to carry iron in a variety of ways.

This paper has presented some biochemical aspects of a broader study being carried out under the direction of Dr. Ephraim Shorr at Cornell University Medical College and the New York Hospital. Current contributors to this work are Drs. Silvio Baez and Saul Green. It has been aided by grants from The Josiah Macy, Jr., Foundation, Eli Lilly & Co., The National Institutes of Health, U. S. Public Health Service (Grant H-79), The Armour Laboratories, The Office of the Surgeon General, Department of the Army (Contract DA-49-007-MD-388), and The Postley Fund.

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Some Aspects of Nitrogen Transfer in Biosynthetic Mechanisms

SARAH RATNER

The origin of urinary urea has been one of the oldest concerns of biochemistry as well as a major problem of nitrogen metabolism. The attention it has received stems from the biochemist's great interest in mammalian, and particularly human, physiology and pathology. During the last few decades, the novel concepts of Krebs were responsible for bringing the whole problem much closer to solution. By visualizing the formation of urea as a cyclic phenomenon, carried out through the agency of intermediates acting as nitrogen carriers, he opened the experimental approach to urea formation as a process of cellular metabolism.

In the past few years our detailed understanding of the reactions which comprise the ornithine cycle of Krebs, along with general advances in our knowledge of intermediary metabolism, have caused our attention to turn from the significance of these reactions in the formation of urea to their significance in the formation of arginine. The individual reactions which lead to the formation of citrulline and arginine now hold considerable interest. It is growing more evident that these reactions are engaged in synthetic activities far beyond their connection with the formation of two amino acids. A broader metabolic network can be seen in which urea production assumes a place as but one aspect of arginine metabolism. Urea is formed by a circuitous pathway, and it is only through the action of arginase that two nitrogens can be conveniently removed as urea if such a pathway is to be followed. The entire synthetic course, up to the arginase step, is of general use metabolically. From this point of view, it becomes economical for the living organism to employ elaborate mechanisms for the formation of urea when they serve more than one function.

Scheme 1 represents our present concepts of the various ramifications of arginine metabolism with the original participants of the ornithine

Scheme 1 (diagram):

$$CO_2 + NH_3 \quad \textit{Ornithine} \quad \text{Aspartic acid} \quad \textit{Urea} \leftarrow \quad \text{Arginine phosphate}$$

Carbamylphosphate $\longrightarrow$ *Citrulline* $\longrightarrow$ Argininosuccinic $\longrightarrow$ *Arginine* $\longrightarrow$ Proteins

Aspartic acid — Carbamylaspartic acid $\downarrow$ Pyrimidines

Carbamylaspartic acid $\downarrow$ Pyrimidines

Guanidino acetic acid $\downarrow$ Creatine $\longrightarrow$ Creatine phosphate

Glycine
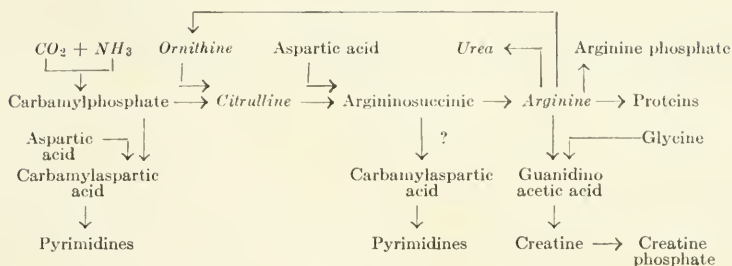
Scheme 1

cycle shown in italics. Before pursuing the various relationships, it is well to examine the reaction mechanisms through which they are interlinked.

In 1932, Krebs found that the respiring liver slice can form large amounts of urea from CO_2 and NH_3 in the presence of a small amount of ornithine. As an explanation of his observations, he proposed [1] the three-step cyclic mechanism shown in scheme 2.
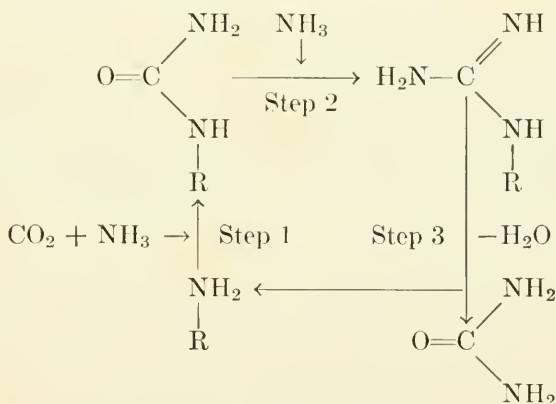
Scheme 2 (RNH_2 = ornithine)

After successive additions of CO_2 and NH_3 to ornithine and citrulline, to form arginine, urea is split off by an arginase-catalyzed hydrolysis. The liberated ornithine again participates in the cycle. The entire process has come to be known as the ornithine cycle. Krebs observed that urea-synthesizing activity disappeared when the cells were disrupted or when respiration was prevented. Permeability barriers, the unrecognized presence of essential substrates, and the inability to interrupt the cycle, owing to the complex dependence on respiration, impeded further exploration with intact cells.

In the past few years, as an outgrowth of the basic observations, it has been possible to obtain synthesis in broken-cell suspensions, to

interrupt the steps of the cycle, to dissociate the respiratory mechanisms to which they are linked, and, finally, through enzyme isolation, to examine the nature of the individual reactions.[2-7]

Phosphate Bond Energy and Formation of C-N Bonds

The synthesis of urea is known to require the expenditure of a large amount of energy, and one of the major questions concerning this problem has been the source of the energy and the manner of its utilization. The energy liberated in oxidative metabolism is made available for synthetic needs as "high-energy" (pyrophosphate) bonds largely in the form of adenosine triphosphate (ATP). It turns out that the energy is utilized as ATP in the synthesis of citrulline and arginine. During their formation, nitrogen becomes attached to carbon with the aid of mechanisms which couple phosphate-bond energy to the formation of certain types of C-N bonds.

Conversion of Ornithine to Citrulline

Citrulline formation involves several enzymatic steps. In reactions 1a and 1b, CO_2 and NH_3 form carbamic acid; this compound then reacts with ATP in the presence of G, which can be any one of several N-acyl derivatives of glutamic acid (acetyl, chloroacetyl, carbamyl, formyl, or propionyl), to form an unstable intermediate. Another

$$NH_3 + CO_2 \rightleftharpoons H_2NCOOH \qquad (1a)$$

$$H_2NCOOH + CO_2 + ATP + G \rightleftharpoons H_2N\overset{\overset{O}{\|}}{C}-O-\overset{\overset{O}{\|}}{P}(OH)_2----G + ADP$$
$$(1b)$$
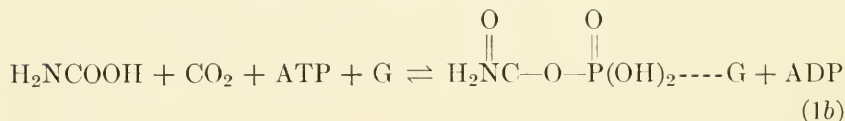
enzyme catalyzes the formation of citrulline from ornithine and the unstable intermediate, as shown in reaction 2. Orthophosphate and the glutamic acid derivative are liberated at the same time.

$$H_2N\overset{\overset{O}{\|}}{C}-O-P(OH)_2----G + \underset{\underset{NH_2CHCOOH}{\overset{\overset{|}{(CH_2)_3}}{|}}}{NH_2} \rightleftharpoons \underset{\underset{NH_2CHCOOH}{\overset{\overset{|}{(CH_2)_3}}{|}}}{HN}-\overset{\overset{O}{|}}{C}-NH_2 + H_3PO_4 + G \qquad (2)$$

Carbamyl phosphate   Ornithine   Citrulline   Glutamic acid
or compound X   derivative
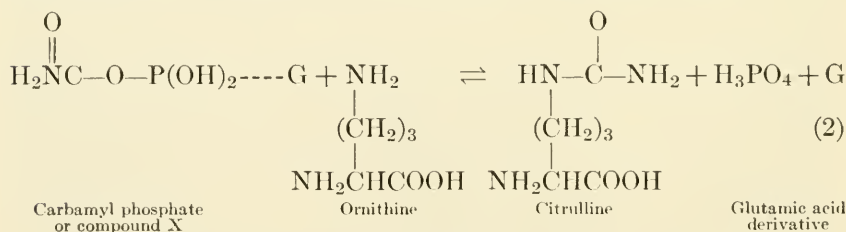
The constitution of the intermediate is not yet known and the compound is therefore referred to as compound X by Grisolia and Cohen

who have conducted extensive investigations of the mammalian enzyme system.[8] Compound X contains phosphate which is bound in an extremely labile form, and the compound readily undergoes spontaneous decomposition to orthophosphate, CO_2, and NH_3.[9,10] Recent experience with the high-energy phosphate-coupled degradation of citrulline in bacterial systems,[11-14] and with the behavior of synthetic carbamyl phosphate in both bacterial and mammalian systems described by Lipmann and his collaborators,[15] makes it extremely likely that the carbamyl phosphate structure is formed in reaction 1b as a part of compound X. However, we know little of the mode of linkage to the glutamic acid derivative.

With the aid of ATP, the carbamic acid formed from CO_2 and NH_3 is transformed to an anhydride of phosphoric and carbamic acids. The energy of the pyrophosphate bond is retained in carbamyl phosphate and can then be utilized in the attachment of the carbamyl group to the terminal nitrogen of ornithine. There are many indications that reactions 1 and 2 are reversible in the mammalian liver.[15,16]

Investigations of bacteria reveal that the same reversible reactions also represent the bacterial mechanisms, possibly without participation of the glutamic acid derivative for no evidence has yet been obtained that this derivative is required in bacteria. It may be seen that the degradation of citrulline to NH_3, CO_2, and ornithine proceeds with the generation of ATP by a reversal of reactions 1 and 2. Prompted by a consideration of the chemistry of carbamyl phosphate, it is tempting to visualize that the acid anhydride structure permits the energy to be retained in the carbamyl group in the direction of synthesis or in the phosphate bond in the direction of degradation. Enzymatic cleavage on one side of the anhydride oxygen would yield high-energy phosphate (anhydrophosphate accepted by ADP), and carbamic acid which breaks down to CO_2 and NH_3. Cleavage on the other side would result in a carbamyl group (accepted by ornithine) and orthophosphate.[15,17]

$$NH_2CO\sim O\!-\!PO(OH)_2 + NH_2R \rightarrow NH_2CO\sim NHR + H_3PO_4$$

$$NH_2CO\!-\!O\sim PO(OH)_2 + ADP \rightarrow NH_2COOH + ADP\sim PO(OH)_2$$
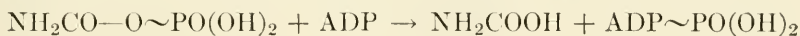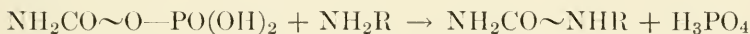
Conversion of Citrulline to Arginine

ATP is also required in the formation of arginine. The generation of ATP accompanies the oxidative processes of respiration and, indeed, it was the recognition that phosphate-bond energy is the driving force of these reactions that finally permitted them to be made experimentally independent of oxidative metabolism.[4,5,7]

Arginine formation can be resolved into two enzymatically distinct steps. The first of these, reaction 3, involves the condensation of citrulline with aspartic acid to form the relatively stable intermediate

$$
\begin{array}{ccc}
\text{NH} & \text{COOH} & \\
\| & | & \\
\text{C--OH} & \text{NH}_2\text{CH} & + \text{ATP} \rightarrow \\
| & | & \\
\text{NH} & \text{CH}_2 & \\
| & | & \\
(\text{CH}_2)_3 & \text{COOH} & \\
| & & \\
\text{NH}_2\text{CHCOOH} & & \\
\text{Citrulline} & \text{Aspartic} & \\
 & \text{acid} &
\end{array}
$$

$$
\begin{array}{cc}
\text{NH} & \text{COOH} \\
\| & | \\
\text{C--NH--CH} & + \text{ADP} + \text{H}_3\text{PO}_4 \quad (3) \\
| & | \\
\text{NH} & \text{CH}_2 \\
| & | \\
(\text{CH}_2)_3 & \text{COOH} \\
| & \\
\text{NH}_2\text{CHCOOH} & \\
\text{Argininosuccinic} & \\
\text{acid} &
\end{array}
$$
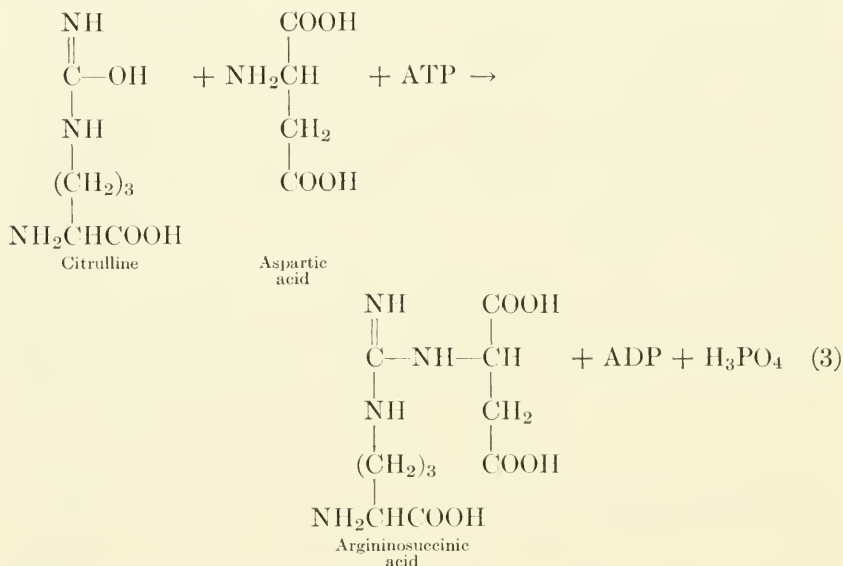
argininosuccinic acid. This is followed, as shown in reaction 4, by a cleavage which liberates arginine by detaching fumaric acid.

$$
\begin{array}{cccc}
\text{NH} & \text{COOH} & \text{NH} & \text{COOH} \\
\| & | & \| & | \\
\text{C--NH--CH} & & \text{C--NH}_2 & \text{CH} \\
| & | & \rightleftharpoons \quad | & \| \quad + \quad (4) \\
\text{NH} & \text{CH}_2 & \text{NH} & \text{CH} \\
| & | & | & | \\
(\text{CH}_2)_3 & \text{COOH} & (\text{CH}_2)_3 & \text{COOH} \\
| & & | & \\
\text{NH}_2\text{CHCOOH} & & \text{NH}_2\text{CHCOOH} & \\
\text{Argininosuccinic} & & \text{Arginine} & \text{Fumaric} \\
\text{acid} & & & \text{acid}
\end{array}
$$
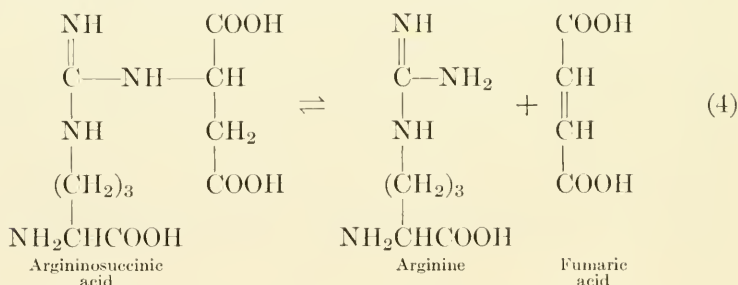
The nitrogen atom acquired by citrulline can be donated only by aspartic acid and not by NH_3 or glutamic acid, as the earlier experiments with slices and homogenates appeared to indicate, nor by any other amino acid.

Argininosuccinic acid is a guanidine derivative, and the reaction by which it is formed is depicted as a condensation between aspartic acid and the tautomeric isourea form of citrulline, analogous to the chemical synthesis of guanidines from amines and S-methylisothiourea or O-methylisourea. The structure of argininosuccinic acid has been sub-

stantiated in considerable detail, and the compound exhibits the chemical properties to be expected of a substituted guanidine. Though much more stable than the intermediate encountered in reaction 1b, when subjected to heat or dilute acid it rapidly undergoes non-enzymatic conversion to a cyclic anhydride (amidine N to carboxyl C). The latter can be converted back to argininosuccinic acid only by exposure to dilute alkali. The two compounds behave like creatine and creatinine in this respect and in solution tend to form equilibrium mixtures governed by pH and temperature. The anhydride appears to be metabolically inert.[18, 19]

Reaction 4 merely serves to convert a disubstituted guanidine to a monosubstituted one. The conversion is reversible and argininosuccinic acid can be readily formed from arginine and fumaric acid by this reaction. It involves but a small net change in free energy. The formation and cleavage of this type of C-N bond cannot therefore account for any significant part of the energy required for the synthesis of urea. The energy utilization is confined to the C-N bond established in reaction 3. The transformation of the ureide group to the guanidine level represents the actual synthesis of the amidine group.

Although phosphate-bond energy is utilized in the condensation and orthophosphate is thereby liberated, it has not been possible to detect a free, phosphorylated intermediate formed prior to condensation. It may, of course, be formed transiently on the enzyme surface, or the equilibrium of the reaction may be too unfavorable to allow detection. For the present, the precise manner of phosphate-bond utilization is a matter of conjecture, and the analogy to chemical guanidination, mentioned above, is the basis for the hypothesis which now appears most attractive. The isourea configuration, represented below in the citrulline structure II, is the more reactive of two tautomeric forms.
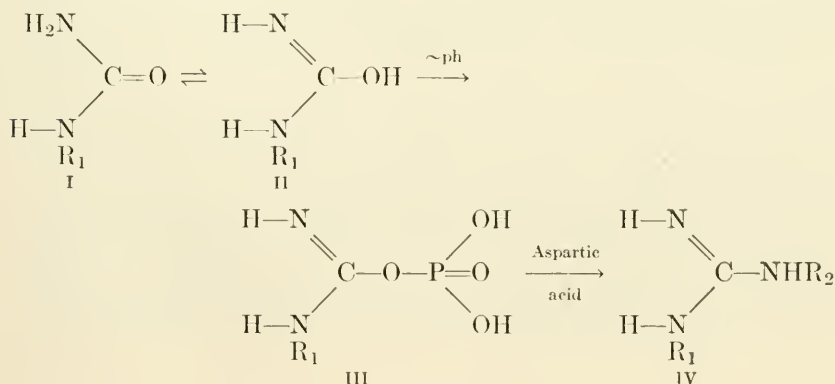
Removal of this tautomer by phosphorylation would tend to displace an unfavorable equilibrium mixture of citrulline tautomers in the direction favorable to condensation. The energy of the pyrophosphate bond is perhaps utilized just to promote this tautomerization.

Whether or not condensation can be further broken down to partial steps, reaction 3 is at present the only one of the four reactions that is not detectably reversible, although a number of procedures designed to detect reversibility have been carried out.[20]

Nitrogen Transfer in the Ornithine Cycle

A condensed representation of the ornithine cycle, as it appears at present, is given in scheme 3. Compound X is replaced by carbamyl phosphate for convenience. Mention has already been made of the highly provisional status of phosphorylated citrulline. The individual

Scheme 3 (RNH$_2$ = ornithine)
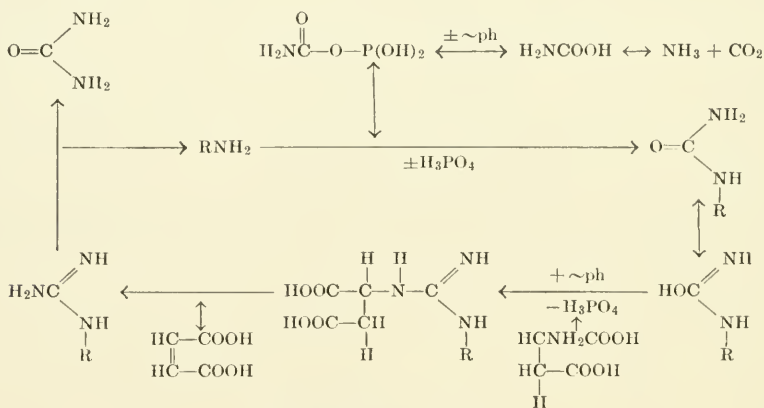
steps include (1) the formation of carbamic acid, (2) the formation of carbamyl phosphate, (3) the transfer of the carbamyl group to the terminal nitrogen of ornithine to form citrulline, (4) the condensation of citrulline with aspartic acid to form argininosuccinic acid, (5) the removal of fumaric acid to form arginine, (6) the hydrolytic cleavage of arginine to form urea and ornithine. It may be seen that each transformation involves a different mechanism of C-N attachment or cleavage. The incorporation of these steps into a cycle by the action of arginase represents, in view of our present knowledge, a diversion from their more general functions toward a special requirement developed by the mammalian liver in connection with nitrogen excretion.

Synthetic Mechanisms Associated with Arginine Formation

As familiarity with these mechanisms increases, it appears that the stepwise manner in which "active" groupings are built up and transferred not only adapts them to the utilization of a common supply of energy but also allows their participation in the synthesis of many compounds that possess the ureide, amidine, or guanidine structure.

Arginine Synthesis in General

The interest in arginine synthesis has in the past been primarily confined to the ornithine cycle. It is perhaps as a result of gaps in our knowledge of nitrogen metabolism that the significance of these mechanisms in providing arginine for cellular protein has been somewhat neglected. The important implications of the isotope experiments carried out by Schoenheimer and his group with respect to the rates of protein synthesis and the origin of the incorporated amino acids are too well known to require discussion here.[21] The incorporation of N^{15} derived from NH_3 or amino acids into the amidine group of tissue arginine is in accord with the operation of an ornithine cycle. Evidence of this type, moreover, obtained by "trapping" arginine in tissue proteins, offers the additional demonstration that the same arginine-forming mechanisms are drawn upon to supply the needs of protein synthesis.

Many unicellular organisms that lack arginase are able to synthesize arginine, and, wherever it has been investigated, citrulline invariably appears to lie in the pathway of arginine synthesis from ornithine. Specific enzymes catalyzing each of the individual reactions have already been detected in a number of these organisms, thus providing more detailed evidence that the same group of arginine-forming mechanisms which operate in the mammalian liver are widely distributed in nature.

Carbamyl Group Transfer

Just as the carbamyl group of compound X or carbamyl phosphate can be transferred to ornithine, there is evidence that this group can also be transferred to aspartic acid to form carbamyl aspartic acid, as shown in reaction 5. Here, as in scheme 3, compound X has been omitted for convenience. The reaction occurs in mammalian liver preparations with compound X, and in bacteria with carbamyl phosphate.[15, 22, 23]

$$H_2N-\overset{\overset{\text{O}}{\|}}{C}-O-\overset{\overset{\text{O}}{\|}}{P}(OH)_2 \; + \; H_2N-\underset{\underset{\underset{\underset{H}{|}}{HC-COOH}}{|}}{\overset{\overset{COOH}{|}}{CH}} \qquad \rightarrow$$

Carbamyl
phosphate

Aspartic
acid

$$H_2N-\overset{\overset{\text{O}}{\|}}{C}-\underset{\underset{H}{|}}{N}---\underset{\underset{\underset{H}{|}}{HC-COOH}}{\overset{\overset{COOH}{|}}{CH}} \qquad + \; H_3PO_4 \quad (5)$$

Carbamyl
aspartic acid
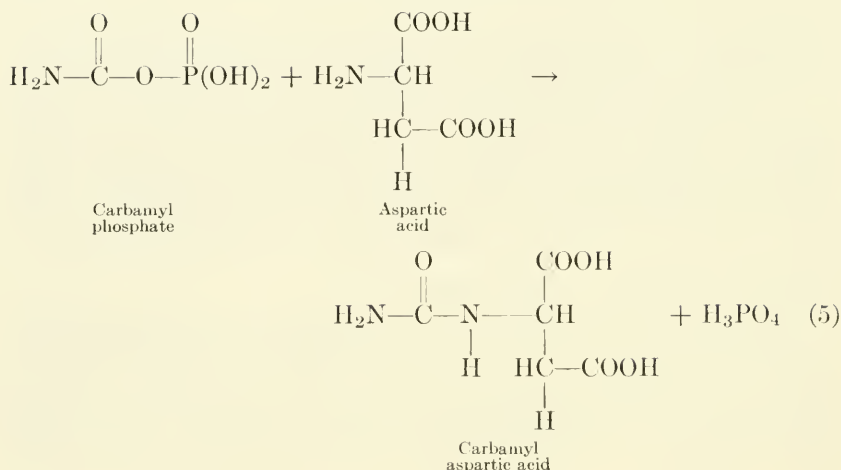
Considerable interest attaches to this reaction, for carbamyl aspartic acid (ureidosuccinic acid) can function as a precursor of pyrimidines, through orotic acid, according to scheme 4.

$$\begin{array}{ccc} \overset{NH_2}{\underset{|}{O=C}} & \overset{COOH}{\underset{|}{CH_2}} \\ \underset{|}{HN} & --- & \underset{|}{CH} \\ & & COOH \end{array} \rightleftharpoons \begin{array}{ccc} \overset{NH-C=O}{\underset{|}{O=C}} & \underset{|}{CH_2} \\ \underset{|}{HN} & --- & \underset{|}{CH} \\ & & COOH \end{array} \rightleftharpoons$$

Ureidosuccinic
acid

Dihydroorotic
acid

$$\begin{array}{ccc} \overset{NH-C=O}{\underset{|}{O=C}} & \underset{|}{CH} \\ \underset{|}{HN} & --- & \overset{\|}{C} \\ & & COOH \end{array} \rightarrow \begin{array}{ccc} \overset{NH-C=O}{\underset{|}{O=C}} & \underset{\|}{CH} \\ \underset{|}{HN} & --- & \underset{|}{CH} \\ & & ribose-PO_4 \end{array}$$

Orotic
acid

Uridylic
acid
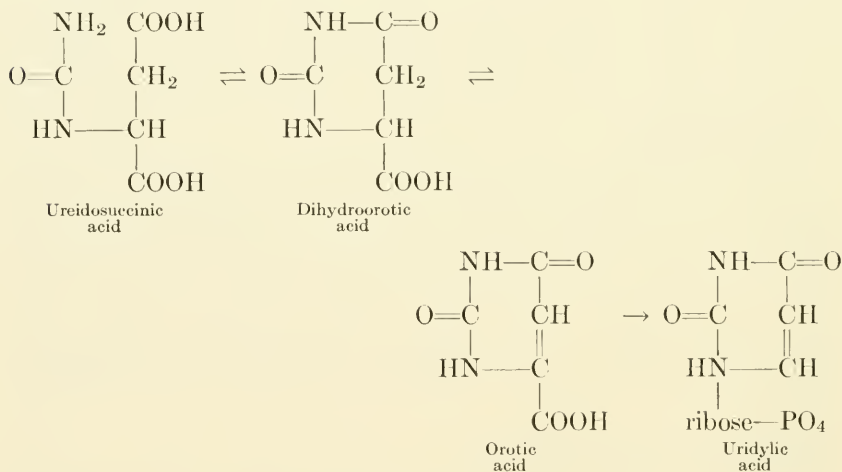
Scheme 4

There is ample evidence for the mammalian and bacterial incorporation of orotic acid into the pyrimidines of nucleic acid,[24-26] and evidence has been obtained for the steps represented in scheme 4.[27,28] According to this pathway, the cyclic ureido configuration, as well as the carbon skeleton incorporated in the pyrimidine structure, will have originated in carbamyl phosphate and aspartic acid.

The possibility exists that argininosuccinic acid may also lie in the pathway to pyrimidines since hydrolytic cleavage can theoretically occur so as to form ornithine and ureidosuccinic acid.

Amidine Group Transfer

Arginine assumes an important role in the formation of guanidino-acetic acid (glycocyamine) and creatine.[29, 30] The former compound is synthesized in the kidney and is subsequently converted to creatine by a methylation which occurs in the liver. Guanidinoacetic acid is formed by the interaction of arginine and glycine, according to reaction 6. The process represented in this reaction involves the transfer of the amidine group (originally formed in reaction 3) from arginine to glycine. The transfer proves to be reversible.[31, 32]

$$
\begin{array}{c}
\text{H—N} \\
\diagdown \\
\text{C—NHR} \\
\diagup \\
\text{H}_2\text{N}
\end{array}
+ \text{HN—C—COOH} \rightleftarrows
$$

Arginine   Glycine

$$
\begin{array}{c}
\text{H—N} \\
\diagdown \\
\text{C—N—C—COOH} + \text{NH}_2\text{R} \quad (6) \\
\diagup \\
\text{H}_2\text{N}
\end{array}
$$

Guanidino acetic   Ornithine
acid
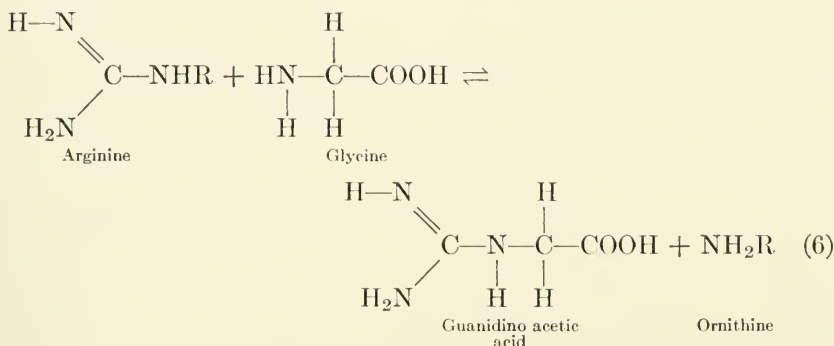
Aspartic Acid and Conversion of Inosinic Acid to Adenylic Acid

The 6-NH_2 group of adenylic acid is replaced in intact animals more rapidly than the nitrogens of the ring.[33] Although it is known that the deamination of adenylic acid to inosinic acid occurs hydrolytically and irreversibly, the mechanism of amination has been obscure. Both of the nucleotides are active in metabolism as the mono-, di-, and triphosphates, for inosine triphosphate, like ATP, acts as a donor in systems requiring high-energy phosphate.

Progress in solving this problem has come from the isolation of adenylosuccinic acid, a new nucleotide that may prove to be an inter-mediate in the amination process. The compound is formed, reversibly, from fumaric and adenylic acid, as shown in the second part of reaction 7, by an enzyme found in yeast.[34] Structurally, adenylosuccinic acid resembles argininosuccinic acid, for the 6-NH_2 group of purines can be looked upon as part of a cyclic amidine configuration. The similarities

$$
\underset{\substack{\text{Inosinic}\\\text{acid}}}{
\begin{array}{c}
\text{OH}\\
|\\
\text{C}\\
\diagup\!\!\diagup\quad\diagdown\\
\text{N}\qquad\text{C--R}\\
|\qquad\ \|\\
\text{H--C}\qquad\text{C--R}\\
\diagdown\!\!\diagdown\diagup\\
\text{N}
\end{array}}
\quad\xrightarrow[\substack{\text{Aspartic}\\\text{acid}}]{\text{ATP}}\quad
\underset{\substack{\text{Adenylosuccinic}\\\text{acid}}}{
\begin{array}{c}
\text{COOH}\\
|\\
\text{HN--CHCH}_2\text{COOH}\\
|\\
\text{C}\\
\diagup\!\!\diagup\quad\diagdown\\
\text{N}\qquad\text{C--R}\\
|\qquad\ \|\\
\text{H--C}\qquad\text{C--R}\\
\diagdown\!\!\diagdown\diagup\\
\text{N}
\end{array}}
\quad\rightleftharpoons
$$

$$
\underset{\substack{\text{Adenylic}\\\text{acid}}}{
\begin{array}{c}
\text{NH}_2\\
|\\
\text{C}\\
\diagup\!\!\diagup\quad\diagdown\\
\text{N}\qquad\text{C--R}\\
|\qquad\ \|\\
\text{H--C}\qquad\text{C--R}\\
\diagdown\!\!\diagdown\diagup\\
\text{N}
\end{array}}
\ +\
\underset{\substack{\text{Fumaric}\\\text{acid}}}{
\begin{array}{c}
\text{COOH}\\
|\\
\text{CH}\\
\|\\
\text{CH}\\
|\\
\text{COOH}
\end{array}}
\qquad(7)
$$
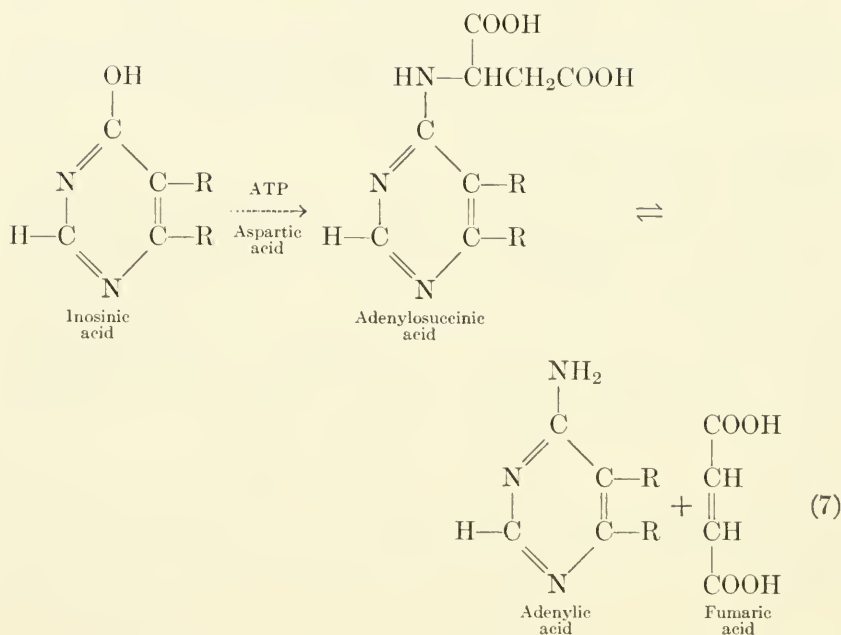
in structure and in enzymatic detachment of fumaric acid suggest that a prior step occurs, similar to reaction 3, in which adenylosuccinic acid is formed by condensation of inosinic acid with aspartic acid.

The mechanisms of group synthesis or transfer discussed in the preceding sections all function in the formation of compounds which possess the ureide, guanidine, or amidine configuration (cf. scheme 1). It is interesting to find that the chemical lability associated with these groupings has a counterpart in their manifold metabolic activities and in what might be called their metabolic lability.

Ammonia and Aspartic Acid

When nitrogen excretion was intensively examined as a problem of evolutionary development and survival, urea formation came to be seen as a means of converting NH_3 into a form which is relatively innocuous and easily excreted, well suited to the physiological limitations of many terrestrial forms of life. Our attention was drawn to this point of view before knowledge of nitrogen metabolism had been extended to its present scope and complexity. Most of the nitrogen is, of course, derived from amino acids, and it had been supposed that the amino acids were directly broken down with the liberation of NH_3.

The whole metabolic burden of maintaining a low concentration of NH_3 within the body was thus assigned to the ornithine cycle.

This view of amino acid breakdown goes back to the early work of Neubauer, Dakin, and Knoop on the oxidative deamination of amino acids and was strengthened by the observations that many natural and unnatural amino acids can undergo oxidative deamination in liver slices and, later, by the isolation of flavoproteins that are capable of catalyzing the interaction with oxygen, as shown in reaction 8.

$$\underset{\underset{\text{H}}{|}}{\overset{\overset{\text{NH}_2}{|}}{\text{R}-\text{C}-\text{COOH}}} + O_2 + H_2O \rightarrow \overset{\overset{\text{O}}{\|}}{\text{R}-\text{C}-\text{COOH}} + NH_3 + H_2O \quad (8)$$
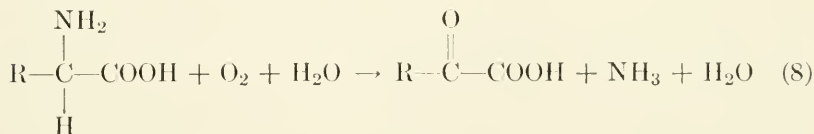
A second mechanism of deamination was later proposed by Braunstein, consisting of transamination coupled to the dehydrogenation of glutamic acid (reactions 9 and 10).

$$\underset{\underset{\text{H}}{|}}{\overset{\overset{\text{NH}_2}{|}}{\text{R}-\text{C}-\text{COOH}}} + \alpha\text{-Ketoglutaric acid} \rightleftharpoons$$

$$\overset{\overset{\text{O}}{\|}}{\text{R}-\text{C}-\text{COOH}} + \text{Glutamic acid} \quad (9)$$

Glutamic acid $+ \text{DPN} + H_2O \rightleftharpoons$

$$\alpha\text{-Ketoglutaric acid} + NH_3 + \text{DPNH} + H^+ \quad (10)$$
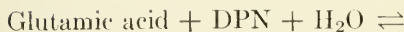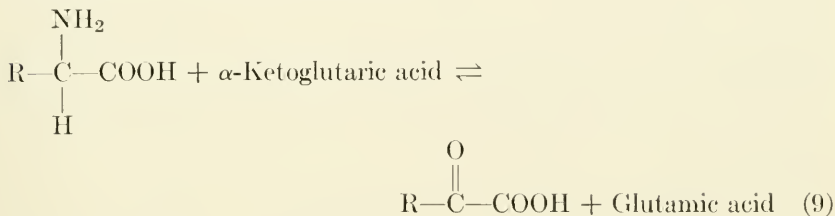
Since both of these reactions are reversible, they can be used in the direction of reductive amination for the synthesis of amino acids. This combination of reactions accounts, in a more satisfactory way than has yet been possible, for the incorporation of N^{15}-labeled amino acids and NH_3 into other amino acids isolated from the proteins of intact animals. We now realize that nitrogen metabolism involves as much synthetic activity as catabolic breakdown. A process such as reductive amination can also be concerned with the intracellular removal of NH_3.

NH_3 is an extremely toxic substance and cannot be tolerated by many organisms above a very low blood concentration. Teleologically, the conversion of NH_3 to urea has the appearance of a detoxication mechanism and has been frequently interpreted as such. As pointed out in preceding sections, the nitrogen atoms of a number of bodily

constituents are in part acquired from aspartic acid, including half of the urea nitrogen, one of the pyrimidine nitrogens, and possibly the $6\text{-}NH_2$ group of purines. Their origin in amino nitrogen actually circumvents the formation of NH_3. Aspartic acid nitrogen has to come, ultimately, from the general pool of amino acids and can do so directly by two successive transaminations involving glutamic acid, as shown in reactions 11 and 12.

Amino acids + α-Ketoglutaric acid ⇌

$$\text{α-Keto acids + Glutamic acid} \quad (11)$$

Glutamic acid + Oxalacetic acid ⇌

$$\text{α-Ketoglutaric acid + Aspartic acid} \quad (12)$$

None of these interconversions and transfers has the appearance of a detoxication mechanism, nor are they primarily concerned with promoting the excretion of nitrogen, yet they reduce the possibility of undue NH_3 accumulation. Many metabolic processes, in effect, collaborate to this end. As aspartic acid assumes an increasing share in the specific donation of nitrogen, the function of the highly active glutamic-aspartic transamination becomes much easier to understand. This transaminating pair appears as a link in the transfer of nitrogen from amino acids to other nitrogenous constituents of the body through aspartic acid.

The formation of glutamine and asparagine, and their ability to undergo transamination with other amino acids,[35] must also exert a control on NH_3 levels. Unlike the amino acids from which they derive, glutamine, and presumably asparagine, are freely permeable to the liver cell, are present in high concentration in the blood, and undoubtedly play an important role in the transport of nitrogen to the liver.[36]

Energetics of NH_3 Formation and Nitrogen Transfer

From an inspection of reactions 3 and 4, it may be seen that their combined effect is to bring about detachment of nitrogen from aspartic acid, incorporation of nitrogen in arginine, and liberation of fumaric acid. In order to estimate the energy change that occurs when one nitrogen atom is moved from the amino acid pool into urea, by way of aspartic acid and citrulline, the shortest pathway is considered.

Of the seven reactions covered in scheme 5, several involve little change in free energy and can be neglected; they are the two transaminations, the detachment of fumaric acid from argininosuccinic acid,

$$\alpha - \text{Keto acids} \xleftarrow{\qquad} \text{Glutamic acid} \longrightarrow \text{Oxalacetic acid} \underset{\pm H_2}{\overset{\pm DPN}{\rightleftharpoons}} \text{Malic acid}$$

Amino acids $\longrightarrow$ α – Ketoglutaric $\longrightarrow$ Aspartic acid $\pm H_2O$
acid

Aspartic acid + Citrulline $\longrightarrow$ Argininosuccinic $\longrightarrow$ Arginine + Fumaric acid
acid
$\downarrow$
Urea + Ornithine
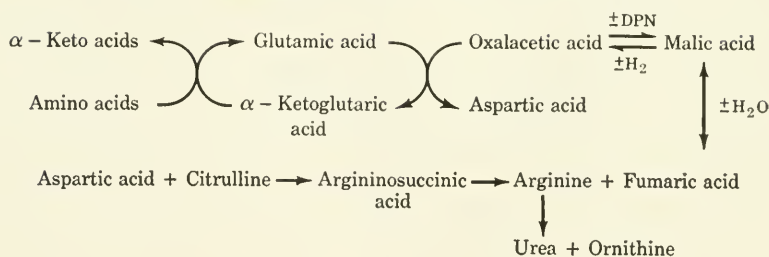
Scheme 5

and the conversion of fumaric acid to malic acid. No utilizable energy is made available in the removal of urea. For the remaining two reactions, the DPN-linked oxidation of malic acid to oxalacetic acid permits three high-energy phosphate bonds ($\sim$ph) to be gained through phosphorylation coupled to the reoxidation of DPNH, and the synthesis of argininosuccinic acid utilizes 1$\sim$ph. The net gain of the entire process will be $+2\sim$ph.

It is of interest to compare this with the net gain obtained when another nitrogen atom comes to ornithine from the amino acid pool by way of NH_3. Assume, for purposes of discussion, that the route of deamination is the most favorable energetically, and that NH_3 is liberated by reactions 9 and 10, which allow energy to be gained from the reoxidation of DPNH. The detachment of nitrogen is then followed by the generation of 3$\sim$ph, and the carbamyl group attachment is associated with the utilization of 1$\sim$ph. The two pathways are thus approximately equal in terms of cell economy, for each incorporates a DPN-linked step.

As far as we know, the energy liberated in flavoprotein catalyzed reactions, which link directly to oxygen, is not made available in a utilizable form. If oxidative liberation of NH_3, according to reaction 8, were to precede the ornithine step, it would have the effect of lowering the energy gain from $+2$ to $-1\sim$ph.

Nitrogen Equilibration

In the early investigations of nucleic acid metabolism with N^{15}-labeled nitrogen, the two ring nitrogens of pyrimidines and all but one of the four ring nitrogens of purines appeared to come from NH_3. It now seems highly probable that half of the pyrimidine nitrogen comes from aspartic acid, and evidence is accumulating that positions 1, 3, and 9 of purines are derived from aspartic or glutamic acids and from the amide group of glutamine.[37] The free amino group of adenylic

acid, once thought to come from NH_3 or amide nitrogen, is probably derived from aspartic acid, and, by analogy, the same may prove to be true of the amino group of cytidylic acid.

The role of the nitrogen of aspartic and glutamic acids, and their amides, has been difficult to interpret for a number of reasons, some of which have become apparent during the investigation of arginine synthesis. In contrast to their amides, or to NH_3, these two amino acids are relatively impermeable to liver cells and usually give the misleading impression of not being precursors when they are investigated in tissue slices or the intact animal. On the other hand, glutamic and aspartic acids can be rapidly synthesized within the respiring cell from NH_3 and their respective keto acids (supplied by the operation of the citric acid cycle) through reactions 10 and 12. NH_3 thus masks their participation in the donation of nitrogen. Difficulties in detecting a precursor, or in determining the actual pathway which a nitrogen atom has taken, also arise whenever N^{15}-labeled NH_3 is compared to glutamic and aspartic acids, or to their amide groups, because rapid isotope equilibration among all the nitrogens takes place through the same reversible reactions in conjunction with reversible amide formation from NH_3. Further experimental difficulties are introduced by the dependence of the synthetic mechanisms on ATP. In respiring systems, just as the citric acid cycle, by facilitating the formation of glutamic and aspartic acids from NH_3, tends to obscure the nitrogen source, it can also obscure the energy source by forming ATP.

Our present problems of nitrogen metabolism come to us from the pioneer accomplishments of earlier decades, when the enormous synthetic potentialities of intracellular metabolism were clearly recognized by investigators who initiated the experimental approach to cellular activity, using surviving tissue or isotopes and the intact animal. Perhaps it will be said of this decade that we are exploring nitrogen metabolism at a higher level of magnification, where the enzyme is both subject and tool.

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On the Bigness of Enzymes

DAVID RITTENBERG

The action of enzymes seems to be dependent on two factors: one geometric, the other energetic. The geometric factor is determined by spatial relationships of the substrate and the corresponding enzyme. Although it is beyond our capabilities to alter the basic structure of an enzyme in any significant manner, it is relatively simple to prepare and to test the interaction of a great number of variants of the natural substrate with the enzyme. In this manner it has become clear that specificity is geometrical in nature. It seems at present that all the data can be explained if we assume that the substrate and the enzyme have such a geometrical configuration as to permit the substrate (generally the smaller of the two interacting particles) to approach closely a portion of the enzyme molecule (the active site).

Were we able to visualize the relative positions of the individual atoms of the enzyme and of the substrate, we would see a mutual complementarity preserved by non-specific Coulombic and van der Waals forces. The Ogston hypothesis offers a simple explanation for stereochemical specificity.[1] The same general concepts have been used by Nachmansohn and Wilson [2] in their synthesis of all the isolated facts concerning the interaction of choline esterase and its variously modified substrates and inhibitors.

Neither the substrate nor the products of the enzymatic reaction should be bound too strongly to the enzyme since if either were the reaction would cease owing to poisoning either by the substrate or the products. Indeed, the explanation of the action of many catalytic poisons seems to be the fact that they are strongly bound to the enzyme surface, and that they do not permit access of the natural substrate molecules, e.g., the inhibition of ferrous iron enzymes by carbon monoxide. Since all enzymatic reaction takes place in water, the reaction between the substrate and the enzyme should be formulated as:

$$E^{\pm} \cdot (H_2O)_n + S \rightleftharpoons E^{\pm} \cdot (H_2O)_{n-y} \cdot S + y \cdot H_2O \qquad (1)$$
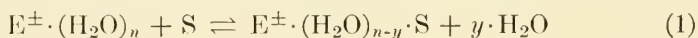
in which we denote the enzyme by $E^{\pm}$ and the substrate by S. Not only does the substrate displace water molecules bound to the enzyme, but it also changes the organization of water molecules in the vicinity of the enzyme. The free-energy change of the reaction must be relatively small (of the order of RT) since appreciably larger values would lead to a self-poisoning, either by the substrate or by water. It would seem that in those reactions in which the substrate is a charged molecule the value of $-\Delta H$ should be large since there would be large interactions of charged groups of the substrate and the enzyme. On the other hand those reactions involving uncharged substrates should have a large value of ΔS since the substrate should partly destroy the ordered arrangement of water molecules around the polar enzyme.

The enzymatic reaction as usually studied is the resultant of at least three reactions:

$$S + E \cdot (H_2O)_n \rightleftharpoons S \cdot E \cdot (H_2O)_{n-y} + yH_2O \tag{2}$$

$$S \cdot E \cdot (H_2O)_{n-y} \rightleftharpoons P \cdot E \cdot (H_2O)_{n-y-r} + rH_2O \tag{3}$$

$$P \cdot E \cdot (H_2O)_{n-y-r} + (y + r)H_2O \rightleftharpoons E \cdot (H_2O)_n + P \tag{4}$$
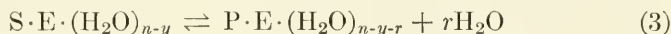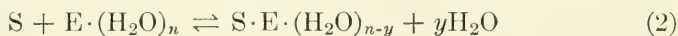
where E, S, and P have their usual meanings and n, m, and r are not necessarily integral. The sum of the three reactions is the reaction usually written

$$S \rightarrow P \tag{5}$$
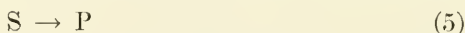
and ΔH and ΔS for this last reaction is the sum of these quantities for reactions 2, 3, and 4. It is not surprising that so much time and effort spent in determining the ΔH and ΔS for reaction 5 has thrown so little light on the individual enzymatic reactions involved. Studies of enzymatic reactions in deuterium and O^{18}-labeled water have furnished methods for the study of these individual steps.[3] A comprehensive study of the elementary reactions involved in the enzymatic catalysis might be illuminating.

The aspect of enzyme action, other than geometrical, involves the energetics of interaction of enzyme and substrate. That these two aspects are not mutually exclusive is illustrated by the previous paragraphs; geometry merges into energetics without a sharply defined boundary. The question of the energetics of enzyme-catalyzed reactions is one about which we know the least. Formally we know that an enzyme speeds up the rate of a reaction by reducing the value of the activation energy. This, however, tells us little new since, in general, fast reactions have low activation energies, and vice versa. The method by which an enzyme reduces the energy of activation of

a chemical reaction is the major problem in our attempt to understand the role of enzymes in the living cell.

The mechanism of catalysis in the non-living world is little more understood than that of the living cell. Here also we recognize the geometric [4] and energetic aspects of the problem.[5] The most obvious division of chemical catalysis is into homogeneous and heterogeneous. Most cases of homogeneous catalysis in water involve hydrogen-ion or hydroxyl-ion catalysis. The mechanism of the catalysis involves intermediates which in most cases have not been isolated.

Most examples of chemical catalysis are heterogeneous. The most common catalysts are metals of the fifth, sixth, seventh, and eighth columns of the periodic table, elements having vacant d orbitals. Considerable evidence has accumulated in the past years which indicates that the properties of heterogeneous catalysts depend not on the interaction of individual atoms of the catalyst with the substrates but on the properties of the bulk metal. The new theory ascribes catalytic activity to the transition metals because of their vacant d orbitals, these orbitals being used for the chemisorption.[6] Couper and Eley [7] have shown that alloying of gold with a palladium hydrogenation catalyst reduces its catalytic efficiency. In bulk palladium approximately 9.4 of the outer ten valence electrons are in the $4d$ shell; 0.6 are in the $5s$. With addition of gold its $6s$ electron fills the $4d$ shell of palladium and reduces the catalytic efficiency.

Catalysis in the biological field is almost exclusively limited to proteins. Even such simple reactions as the hydration of CO_2, or the decomposition of H_2O_2, have enzymes in the cell for their catalysis. The efficiency of the biological catalyst and the importance of the specific protein is strikingly shown by the activity of catalase relative to the iron ion. Haldane [8] has estimated that the catalytic activity for the decomposition of H_2O_2 of hematin is 10^5 and of catalase 10^{10} that of ionic iron. Similarly neither ferrous iron nor heme will combine reversibly with oxygen; the complete iron-porphyrin-globin complex is necessary. Clearly the protein portion of the complete enzyme has a role other than that of a mere carrier of the prosthetic group. The catalytic effect of an enzyme is intimately connected with the structure of the enzyme; the protein does more than supply an active center displayed in proper geometrical relationship to a few positively and negatively charged groups.

The great bulk of enzymatic reactions of the cell can be divided into two classes. Those involving the addition or subtraction of water (or

some substitute such as phosphoric acid) to a molecule or those involving an oxidoreduction.

All the enzymes involved in the latter group of reactions seem to be composed of a metal-containing prosthetic group attached to a protein. Many samples are known composed of an iron porphyrin attached to a protein. Four of the six bonds of the octahedral configuration of the iron atom are firmly bound to the porphyrin; the fifth bond is used to bind the iron porphyrin to the specific protein; the sixth one can be loosely bonded to a water molecule or to the substrate. The role of iron and of the proteins in such enzymes, although unknown in detail, is obviously related to the transfer of electrons from the substrate to the enzyme; the role of the porphyrin is not so obvious. It is possible that the geometry of the iron-porphyrin complex is important chemically. When the iron atom complexes with the planar tetravalent porphyrin molecule, of necessity it leaves two of the six valences free. Since the valences of the iron atom are directed to the six vertices of an octahedron the two free valences are on the opposite sides of the plane containing the iron atom and the porphyrin. It is well known that bifunctional molecules complex more strongly with iron than monofunctional molecules. Were there two adjacent free spaces in the octahedral structure of the iron atom, it would rapidly be filled by the bifunctional amino acids always present in the cells. Because of this particular structure it is not possible for the hematoporphyrin enzymes to be inhibited by an amino acid. For the same reason it is likely that the copper enzymes are so constructed that there is not more than one space in the valence shell of copper for combination with its substrate.

An analogy between metal catalysts of the transition groups with enzymes is not possible, since enzymes have neither vacant d orbitals nor do they show the phenomena resulting from such partially filled shells. Nevertheless it seems unlikely that there is a complete dichotomy in the kind of mechanisms that are employed for catalysis in the living and the non-living world.

All enzymes are characterized by their largeness and their protein nature. Despite a thorough search no example of an enzyme has been found which is not a protein or a large molecule. The backbone of the proteins is a series of repeating peptide bonds separated from each other by the CHR groups. These groups destroy the possibility of conjugation of the double bonds of the peptides. There is evidence that the electrons of the peptide bond can be raised to an excited state by absorption of light of 1850 A. (154,000 cal./mole above the

ground state).[9] This absorption seems to be proportional to the number of peptide bonds per mole indicating little interaction between them. The great energy of excitation makes it unlikely that these states are used in the interaction of the substrate and the enzyme.

In analogy with the crystalline solids, the electrons of the protein molecule could move in a periodic field arising from the regular linear
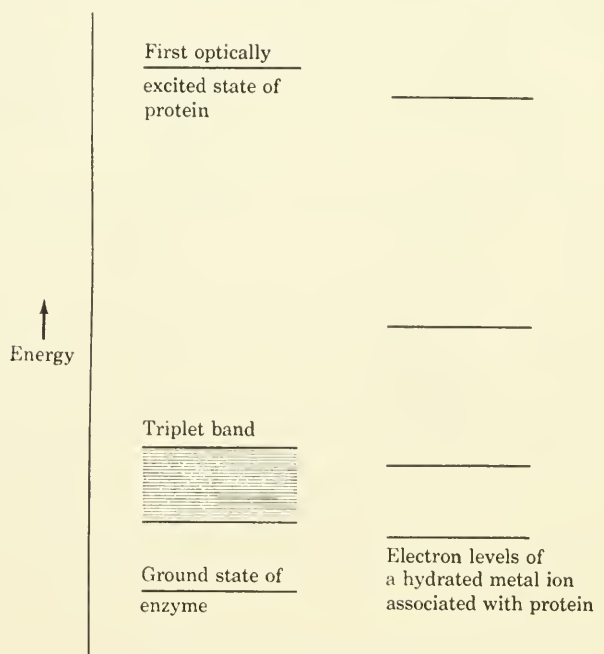
Fig. 1. Hypothetical energy levels of an enzyme.

arrangement of the peptide bonds of the polypeptide chain. The electrons in this excited state would be distributed in a large number of discrete levels which taken together form an energy band (see Fig. 1). The lower edge of this band could approach the ground state rather closely. The width of this band and therefore of the minimum excitation energy of its lower edge would depend on the number of peptide bonds which cooperate, on the size of the protein. This band is similar to that postulated to explain electrical conduction in semiconductors. In these materials, conduction is possible because of the existence of a vacant electron band just above a filled band. It is suggestive in this regard that electrical conduction in semiconductors

is enhanced by addition of impurities (prosthetic groups) which supply unfilled electron levels used to facilitate electron flow. Similar phenomena exist in the photoconduction of MoS_2; a compound known to have catalytic activity.

This excited state must be one which cannot be observed by absorption spectroscopy, since proteins do not absorb light above 1850 A. except for that absorption due to the R groups. If the excited state
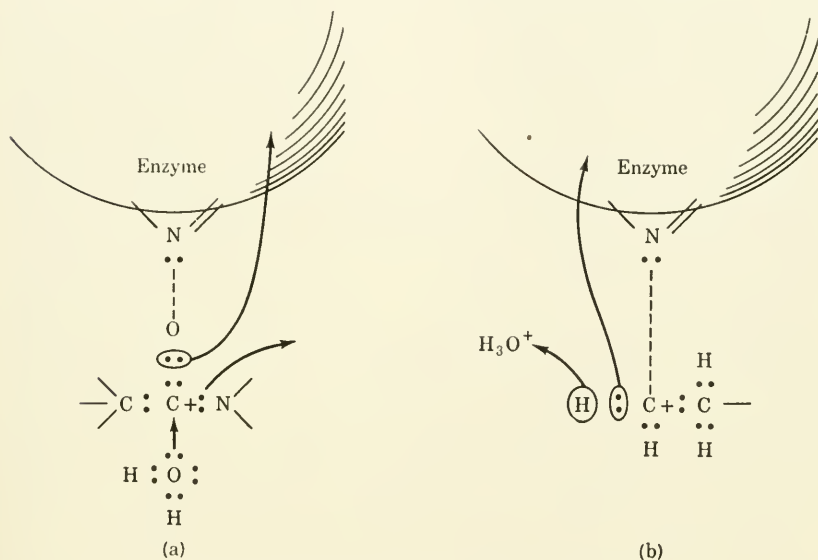
Fig. 2. Interaction of substrate with enzyme.

were a triplet one, it should not give rise to an absorption band, since transitions from the singlet ground state to the excited triplet by light absorption would be forbidden by the usual transition rules.

Electrons in this excited state would not be associated with any particular peptide bond, since they would be distributed over all the peptide bonds. Their average contribution to the electron density of any peptide bond would be very low. On this hypothesis, as a suitable substrate containing a peptide bond approaches a proteolytic enzyme the Coulombic and van der Waals forces would bring the substrate up in a suitable position relative to the enzyme. In this position, as the carbonyl group of the peptide bond approaches the enzyme with the C=O bond pointing toward the enzyme (see Fig. 2), two of the π electrons of the carbonyl group are transferred to the low-lying

triplet state of the enzyme. The energy required for the promotion of the two electrons is in part supplied by the bond which can now form between the electron-deficient oxygen atom and a free electron pair of a nitrogen atom of a peptide bond of the enzyme. This requirement for a lone pair of electrons of a trivalent nitrogen atom may explain why proteins exist in nature rather than the analogous compounds formed from lactic acid derivatives. It would be difficult to form an analogous bond between two oxygen atoms.

The carbon atom of the peptide bond of the substrate has by this transfer of electrons become positively charged (electron deficient). The positively charged carbon atom then attracts by Coulombic forces either a hydroxyl ion or a water molecule (see Fig. 2a). As the hydroxyl (or water molecule) approaches, the C-N bond weakens. Depending on the particular conditions the approaching group may either be reflected, in which case nothing has happened, or the C-N bond may progressively weaken as the OH^- group approaches the positively charged carbon atom until the new OH^- group has completely replaced the original amino group. It will be noted that this mechanism postulates that the essential enzymatic step is the polarization of the carbonyl group. Depolarization and evaporation of the substrate from the enzyme surface restores the system to its original catalytic condition. Such a mechanism explains hydrolysis of peptide bonds, transpeptidation, or O^{18} exchanges in amino acids,[3] and a similar mechanism can obviously explain other hydrolytic reactions.

Some members of this class of enzymes require inorganic ions for their action. In many cases these ions (Ca^{++}, Mg^{++}, etc.) are not very good complexing agents. They may function by supplying a set of energy levels which facilitate transfer of electrons from the substrate to the triplet state of the active enzyme (see Fig. 1). Specificity toward the metal required will be great since the energy levels of the excited protein and the same excited levels of the hydrated metal ion should be approximately equal. An analogous explanation could be offered to explain the concentration of potassium within the living cell. Some enzymes involved in the maintenance of the energy flow in the living cell may have an excited state approximately the same distance above the ground state of the protein as the $4p$ state of potassium is above the filled $3p$ shell. Sodium will not substitute for potassium since the energy difference between the corresponding states of sodium ($3p$ and $2p$) is much greater than between those of potassium ($4p$ and $3p$).

On the above view the mechanism of the enzymatic reactions involving oxidoreductions are closely related to those of the hydrolytic reactions. Here the enzyme must supply a mechanism by means of which electrons can be removed from one substrate and transferred to another. There is no evidence that the transfer is directly from one substrate to another. These enzymes often contain iron or molybdenum, and in some cases the metal is known to be an acceptor for the electrons removed from the substrate. In all cases, it seems reasonable to assume, the metal somehow facilitates the transfer of electrons from substrate to enzyme. The vacant d shells found in these metals may supply a set of electron levels in a manner similar to those of impurities in semiconductors.

The specificity of the protein with regard to catalase activity is in accord with this view. It is not necessary that the substrate react directly with the metal ion. Indeed, in cytochrome c all the six coordination bonds are filled, four by the porphyrin and two by the protein.[10] The substrate could interact with the enzyme at some point distant from the metal atom in a manner similar to that shown in Fig. 2b. As the substrate approaches the enzyme, at an appropriate configuration the two electrons forming the C:H bond enter the triplet state, the hydrogen atom leaves as a hydronium ion, and the remaining positive ion forms a bond with a free electron pair of a tertiary nitrogen of the protein. Further loss of a hydrogen ion from the substrate rearrangement of the electrons and evaporation from the enzyme surface would yield the oxidized substrate. The electrons would be transferred via the triplet state to the iron atom.

Such a mechanism, as outlined above, would permit electrons to enter the enzyme molecule at one point and to leave it at another. Transfer of electrons from one protein molecule to another would not be difficult if both proteins had triplet states of about the same energy. Clearly, the transfer of electrons from one substrate to another could differ not only in time but in space. Szent-Györgyi has briefly described an experiment which suggests that electron flow can take place in excited states of proteins.[11]

An experimental test of the hypothesis presented in this paper is possible. Since molecules in a triplet state are paramagnetic, the active substrate-enzyme complex should be paramagnetic.

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The Biosynthesis of Porphyrins

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There has been tremendous progress in the last 20 years in the elucidation of the biochemical reactions and transformations which occur in living organisms. Some of the general concepts which have emerged, summarized very briefly, are that the basic reactions in the cells are surprisingly simple, that the cell synthesizes its complex molecules from relatively simple and available substances, and that there is a biochemical unity in living matter.

Although these concepts were perhaps not fully appreciated when a study of the biosynthesis of porphyrins was begun 10 years ago, the picture which has emerged is rather a good illustration of these basic concepts. The studies have revealed that the complicated looking molecule protoporphyrin is synthesized from two simple and available compounds, glycine and succinate, by relatively simple reactions. Furthermore it has been established that the synthesis of this esoteric-looking molecule is intimately related to the citric acid cycle, since the "active" succinate utilized in porphyrin synthesis is produced in these cyclic reactions. Although most of the investigations have been concerned with the biosynthesis of protoporphyrin, it appears that all the porphyrins in nature, including chlorophyll, in all different types of cells are synthesized by the same basic pathway. The different porphyrins merely arise by modifications occurring in the side chains in the β positions of the pyrrole units.

In 1945 it was found that the nitrogen atom of glycine is the nitrogenous precursor of protoporphyrin in both man and rat.[1-3] Although protoporphyrin (Fig. 1) consists of two types of pyrrole units, methyl- and vinyl-bearing pyrroles, and methyl- and propionic acid-bearing pyrroles, the finding that N^{15}-labeled glycine was equally utilized for these different pyrrole units [4,5] demonstrated that glycine was the nitrogenous precursor of all four pyrrole units and suggested that a common precursor pyrrole, the source of all four pyrrole rings of the

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porphyrin, is synthesized. This conclusion was well supported by the subsequent experimental results.

It appeared reasonable to expect that, since the nitrogen atom of glycine is specifically utilized for porphyrin synthesis, the carbon atoms of this amino acid might also be involved. It was soon found that, whereas the α-carbon atom of glycine is indeed utilized for porphyrin synthesis,[6–9] the carboxyl group was not.[6,10] This latter negative finding was an important clue in the elucidation of the mechanism by
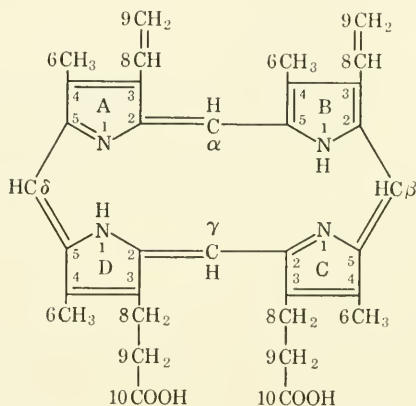
Fig. 1. Protoporphyrin IX. The above numbering system is the same as that previously employed.[8,12]

which glycine and succinate condense. However, on incubation of duck erythrocytes [11] with doubly labeled glycine ($N^{15}H_2$—$C^{14}H_2$—COOH), it was found [7,9] that the dilution for the nitrogen atom was twice that for the α-carbon atom; that is, for every nitrogen atom utilized, two carbon atoms from the α-carbon atom of glycine entered the porphyrin molecule. Therefore, it would appear that eight carbon atoms of the porphyrin molecule arise from the α-carbon atom of glycine, since the four nitrogen atoms of the porphyrin are derived from glycine. In order to establish definitely that eight carbon atoms of the porphyrin are indeed derived from the α-carbon atom of glycine, and, if so, to locate the positions of these carbon atoms in the porphyrin molecule, and to gain some insight into the mechanism of porphyrin synthesis, we developed a chemical degradation procedure of protoporphyrin whereby each carbon atom from a particular position in the porphyrin could be unequivocally isolated [8,12] (Fig. 2). On degrading protoporphyrin synthesized from glycine-2-C^{14} it was indeed found that eight carbon atoms are derived from the α-carbon atom of glycine. The

Fig. 2. Protoporphyrin degradation. The letters and numbers designate positions of the carbon atoms.

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positions located are the four methene bridges [8,9] and one in each pyrrole [8] (Fig. 3). It will be noticed that the carbon atoms in the pyrrole rings, derived from the α-carbon atom of glycine, are in the α-position under the vinyl and propionic acid side chains. This finding supported the suggestion of a common precursor pyrrole first being formed and led to the suggestion that the vinyl side chains arose from propionic acid side chains by decarboxylation and dehydrogenation.

Protoporphyrin IX

$$NH_2 - C^\bullet H_2 - COOH$$

Glycine – 2 – C^{14}

$$NH_2 - C^\bullet H_2 - CO - CH_2 - CH_2 - COOH$$

δ – Aminolevulinic Acid – 5 – C^{14}
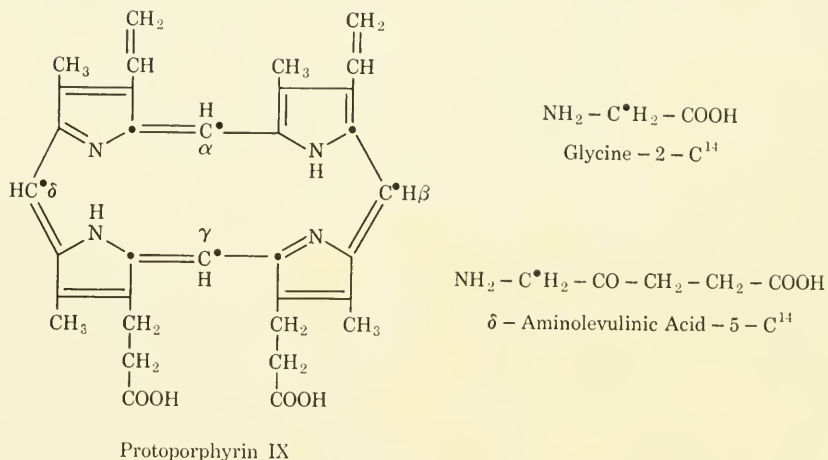
Fig. 3. The carbon atoms of protoporphyrin which arise from the α-carbon atom of glycine and from the δ-carbon atom of δ-aminolevulinic acid.

Having accounted for eight carbon atoms of protoporphyrin, the origin of the remaining twenty-six carbon atoms remained to be determined. It had been found by Bloch and Rittenberg [13] that, on administration of deuterioacetic acid (CD_3COOH) to a rat, the hemin isolated contained deuterium. This indicated that some of the side-chain carbon atoms, at least, were derived from the methyl group of acetate, since these are the only carbon atoms bonded to hydrogen.

In order to determine the extent of utilization of acetate for porphyrin synthesis and to locate all the carbon atoms which may be derived from acetate, duck blood was incubated separately with C^{14}-methyl-labeled acetate and with C^{14}-carboxyl-labeled acetate and the resulting C^{14}-labeled-hemin samples degraded by the method mentioned above. It was found that all the remaining twenty-six carbon atoms were derived from acetate.[12]

The composite C^{14}-distribution pattern among all the labeled twenty-six carbon atoms derived from acetate is given in Fig. 4. Since all four

pyrrole rings had the same C^{14}-distribution pattern, support was obtained for the suggestion that a common precursor pyrrole is first formed. Furthermore, since both the methyl side of the pyrrole units and the vinyl propionic acid sides of the pyrrole units had the same C^{14}-distribution pattern, it was concluded that each side of each pyrrole unit is made from the same compound. It would appear that the compound which condenses with glycine to form the pyrrole unit must be either a 3- or 4-carbon-atom compound. On examination of the structure of protoporphyrin and noting the quantitative distribution

$CH_3 C^{14} OOH$ experiment $C^{14} H_3 COOH$ experiment
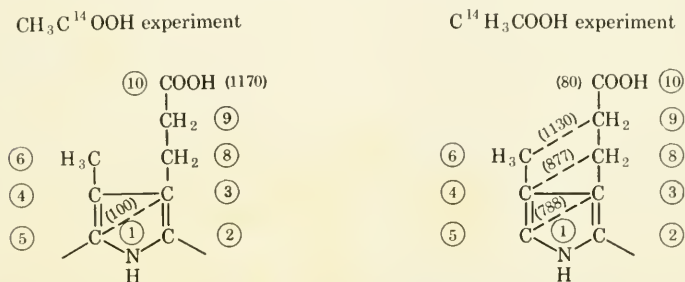
FIG. 4. Average activities of comparable carbon atoms in all pyrrole units. The activities are given in parentheses. The pyrrole unit represented contains a carboxyl group which is found only in rings C and D of protoporphyrin.

of C^{14} among the carbon atoms in the experiments, it can be seen that a 3-carbon-atom compound would satisfy the data as the precursor of the methyl sides of the pyrrole units (carbon atoms 6, 4, and 5) and that the same compound would also be consistent with the data as the precursor of the vinyl sides of the pyrrole units (carbon atoms 9, 8, and 3), excluding carbon atom 2, which is derived from the α-carbon atom of glycine. However, it would appear that a 4-carbon-atom compound would be necessary as the precursor for the propionic acid sides (carbon atoms 10, 9, 8, and 3), again exclusive of carbon atom 2. If a 3-carbon-atom compound were utilized, subsequent carboxylations must have occurred to give rise to the propionic acid side chains in pyrrole rings C and D. On the other hand, if a 4-carbon-atom compound were utilized, decarboxylations must have occurred to give rise to the methyl and vinyl groups. It can be decided which of these two alternative mechanisms operates in the synthesis of protoporphyrin by comparing the data obtained in the experiments using methyl-labeled and carboxyl-labeled acetate. The C^{14} activities of the carboxyl groups (1170 c.p.m.) in protoporphyrin synthesized from carboxyl-labeled ace-

tate are equal to those found in the carbon atoms (1130) adjacent to these groups in the porphyrin synthesized from methyl-labeled acetate (Fig. 4). This equality, i.e., the same degree of dilution, demonstrates that the acetic acid enters as a unit and that the utilization of acetic acid for pyrrole formation is via a 4-carbon-atom unsymmetrical compound. Therefore the common precursor pyrrole originally contained acetic and propionic acid side chains in its β positions; the methyl groups in the porphyrin arose by decarboxylation of the acetic acid side chains, and the vinyl groups arose from decarboxylation and dehydrogenation of propionic acid side chains.

The data obtained in these experiments can readily be explained by assuming the participation of the tricarboxylic acid cycle in porphyrin formation. In the light of the relative distribution of the C^{14} activities among the carbon atoms of the porphyrin derived from acetate, it appeared that the acetate was converted to the 4-carbon unsymmetrical compound via this cycle. The entrance of methyl-labeled acetate in the citric acid cycle can give rise to a 4-carbon-atom compound, derived

Table 1. Relative Distribution of C^{14} Activity in Carbon Atoms of α-Ketoglutaric Acid Resulting from Utilization of C^{14}-Labeled Acetate in Tricarboxylic Acid Cycle *

α-Ketoglutaric Acid	From C^{14}-Methyl-Labeled Acetate (activity of methyl group = 10 c.p.m.)				From C^{14}-Carboxyl-Labeled Acetate (activity of carboxyl group = 10 c.p.m.)			
	Number of Cycles in Tricarboxylic Acid Cycle							
	1st	2nd	3rd	∞	1st	2nd	3rd	∞
COOH	0	0	0	0	10	10	10	10
CH₂	10	10	10	10	0	0	0	0
CH₂	0	5	7.5	10	0	0	0	0
C=O	0	5	7.5	10	0	0	0	0
COOH	0	0	2.5	5	0	5	5	5

* The results are expressed in counts per minute.

from α-ketoglutarate, which would have a relative C^{14}-distribution pattern similar to that found in the porphyrin synthesized from methyl-labeled acetate. For example, if one starts with methyl-labeled acetate with a relative activity of 10 in the methyl group, the α-ketoglutarate formed on the first turn of the cycle would contain C^{14} activity only in the γ-carbon atom and the relative activity would be 10 also (Table 1). On formation of symmetrical succinate, the activities of the methylene carbon atoms would be 5 and 5, and those of the oxaloacetate eventually formed would contain half of the C^{14} activity of the γ-carbon atom of α-ketoglutarate. The recycling of this newly formed oxaloacetate
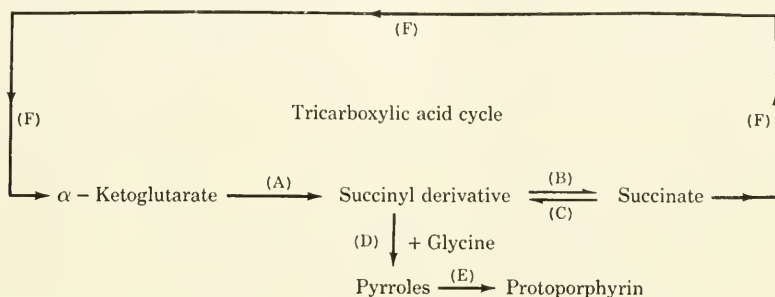
FIG. 5.   The relationship of the citric acid cycle and protoporphyrin formation.

with the labeled acetate would now result in a specimen of α-keto-glutarate having the relative activities shown in Table 1 for the second cycle. In Table 1 the relative activities found in α-ketoglutarate formed from methyl-labeled acetate are given after a number of cycles. It can be seen that a 4-carbon-atom compound arising from α-keto-glutarate after a finite number of cycles would have the same C^{14}-distribution pattern as is found in the 4-carbon-atom unit in the porphyrin synthesized from methyl-labeled acetate; three adjacent carbon atoms are radioactive, and the one arising from the γ-carbon atom has the highest activity. The relationship between the citric acid cycle and porphyrin formation is shown in Fig. 5.[12]

Direct documentation of these conclusions was obtained by studying the utilization of C^{14}-succinate,[14] C^{14}-α-ketoglutarate,[15] and C^{14}-labeled citrate [15] for porphyrin formation. In each case the predicted carbon atom of the porphyrin molecule contained C^{14}. For example, α-keto-glutarate-5-C^{14} and primary carboxyl-labeled citrate produced the same labeling pattern as was found for carboxyl-labeled acetate.

The studies with C^{14}-labeled succinate furnished direct evidence for the participation of a 4-carbon compound in porphyrin formation and

also indicated that the succinyl intermediate is formed from succinate as well as from α-ketoglutarate; that is, reaction C occurs (Fig. 5). In Fig. 6 the labeling pattern which should be found in protoporphyrin synthesized from carboxyl-labeled succinate is given. On degradation of protoporphyrin synthesized from carboxyl-labeled succinate, it was
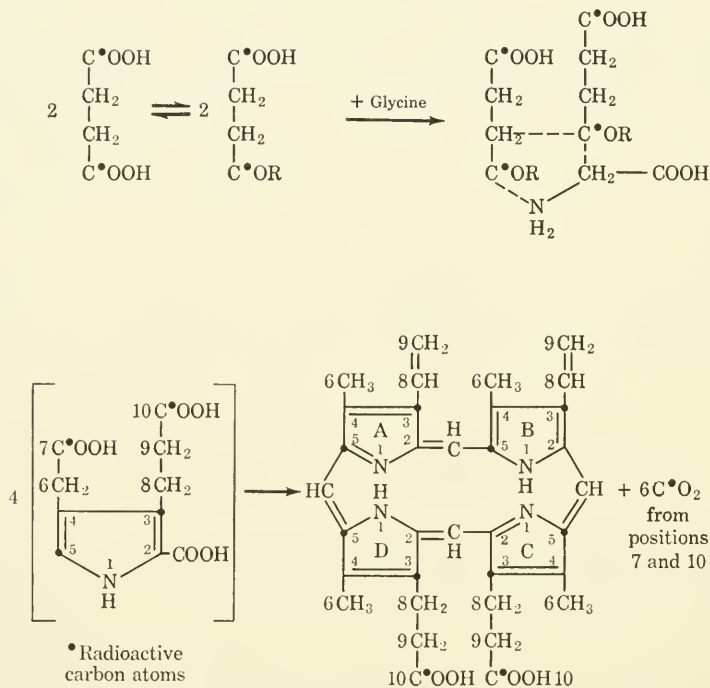
Fig. 6. The position of succinate in protoporphyrin and the labeling pattern obtained in protoporphyrin synthesized from succinate-1,4-C^{14}.

found that the indicated 10-carbon atoms contained the C^{14}.[14] In order to demonstrate that reaction C occurs, a study of the utilization of both methylene-labeled and carboxyl-labeled succinate for porphyrin formation in the absence and presence of malonate was carried out. Theoretically carboxyl-labeled succinate cannot produce labeled porphyrin by entering the oxidative pathway of the citric acid cycle (reaction F, Fig. 5), for in this direction the 4-carbon-atom compound formed from α-ketoglutarate would no longer contain any of the original carboxyl groups of the labeled succinate. Therefore the formation of labeled porphyrin from carboxyl-labeled succinate would be evidence for the occurrence of reaction C. These theoretical considerations and

conclusions can be tested experimentally by blocking reaction F with malonate. If these considerations are valid and if reaction C occurs, the degree of labeling found in protoporphyrin from carboxyl-labeled succinate should not be influenced by the presence of malonate. On the other hand, methylene-labeled succinate can produce labeled porphyrin via reaction F. In this case the degree of labeling from methylene-labeled succinate should be lowered in the presence of malonate. It was found experimentally that the C^{14} activity of the porphyrin synthesized from carboxyl-labeled succinate in the presence or absence of malonate was the same, whereas that of the porphyrin synthesized from methylene-labeled succinate was 50–60% lower in the presence of malonate than in its absence.[14] These experiments demonstrated that the succinate can be converted to "active" succinate via two pathways and that the malonate effect is a reflection of the positions in the succinate which contain C^{14}.

It then became of interest to find the mechanism by which the "active" succinate and glycine combine to form the pyrrole unit of the porphyrin. It was realized that in the initial condensation of glycine and succinate the whole molecule of glycine is involved, since in all experiments in which glycine-2-C^{14} was the substrate the carbon atom in the pyrrole ring and the methene bridge carbon atom (Fig. 3) had the same C^{14} activity and no derivative of the α-carbon atom of glycine (CH_3OH, H_2CO, $HCOOH$, CH_3NH_2) could substitute for glycine. These findings led us to the conclusion that the *same* derivative of glycine was utilized for the pyrrole ring carbon atom and for the bridge carbon atom, even though the bridge carbon atom was no longer attached to the nitrogen atom of glycine as is the ring atom. On consideration of the possible method of condensation of succinate and glycine, which would give rise to a product from which a pyrrole could reasonably be made, the suggested mechanism must also account for a method by which the carboxyl group of glycine is detached from its α-carbon atom, subsequent to the initial condensation, for the carboxyl group of glycine is not utilized for porphyrin synthesis. The condensation of succinate on the α-carbon atom of glycine to form α-amino-β-ketoadipic acid (Fig. 7) would appear to be in agreement with all the experimental findings and conclusions. The compound formed, being a β-keto acid, could then readily decarboxylate and thus provide a mechanism by which the carboxyl group of glycine is detached from its α-carbon atom subsequent to the initial condensation of the whole molecule of glycine with succinate. Further, the product

Tricarboxylic
Acid
Cycle

Succinyl
CoA

Succinate

α–Ketoglutarate

+ Glycine

Ureido group of purines,
formate, etc.

the α–carbon atom to

Succinate-
Glycine
Cycle

$HOOC - CH_2 - CH_2 - C - C - COOH$

$\overset{\text{H}}{\underset{\text{O \quad NH}_2}{\underset{||}{|}}}$

α–Amino–β–ketoadipic acid (I)

$[HOOC - CH_2 - CH_2 - C - CHO]$
$\underset{O}{\overset{||}{}}$

Ketoglutaraldehyde

$- CO_2$

$- NH_3$

$HOOC - CH_2 - CH_2 - C - CH_2NH_2$
$\underset{O}{\overset{||}{}}$

δ–Aminolevulinic acid (II)

[O]

$HOOC - CH_2 - CH_2 - C - COOH$
$\underset{O}{\overset{||}{}}$

α–Ketoglutaric acid

Porphyrin
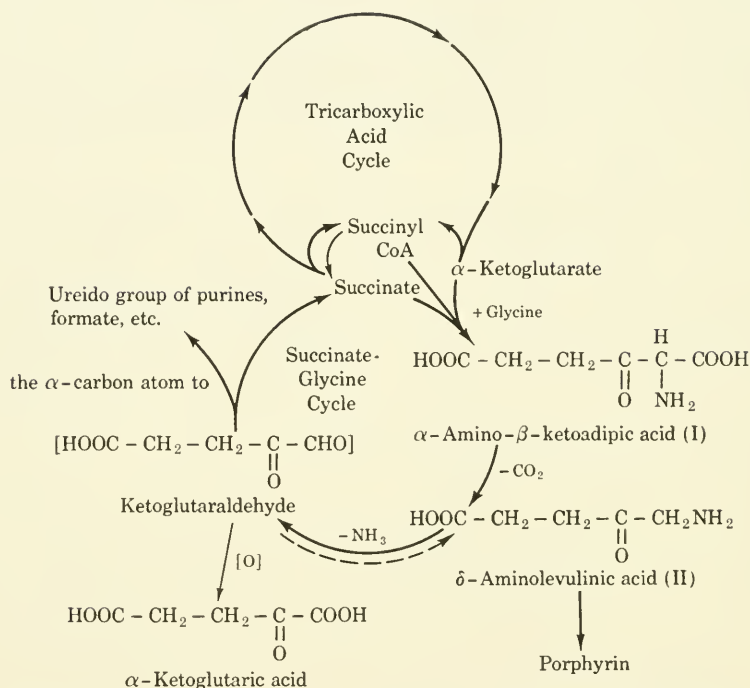
Fig. 7. Succinate-glycine cycle: a pathway for the metabolism of glycine.
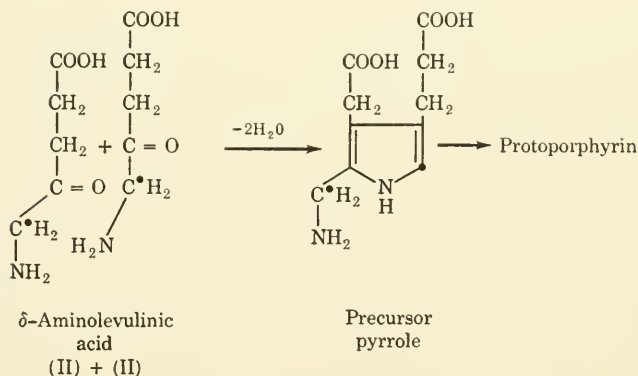
Fig. 8. The mechanism for the formation of the monopyrrole, porphobilinogen, by condensation of two moles of δ-aminolevulinic acid. The carbon atoms bearing the closed circles (•) were originally the α-carbon atom of glycine.

of decarboxylation would be δ-aminolevulinic acid, and condensation of two moles of the latter compound, by a Knorr type of condensation (Fig. 8) would give a reasonable mechanism for formation of a pyrrole in which the α-carbon atom of glycine would be distributed in the positions previously observed. To test this hypothesis, δ-aminolevulinic acid was synthesized and its utilization for porphyrin synthesis studied.[16-18]

In the initial experiments, unlabeled δ-aminolevulinic acid was added to duck red blood cell hemolyzates along with either C^{14}-labeled glycine or C^{14}-labeled succinate. The radioactivities of the hemin samples isolated in these experiments were compared with those obtained from controls in which the unlabeled δ-aminolevulinic acid was omitted. The rationale for these dilution-type experiments is as follows: if δ-aminolevulinic acid is an intermediate formed from the condensation of glycine and succinate, any labeled δ-aminolevulinic acid formed from these labeled substrates will be diluted by the added unlabeled compound, and consequently this should be reflected in the lowered radioactivity of the hemin samples synthesized in the presence of unlabeled δ-aminolevulinic acid. It can be seen from Table 2 that the hemin samples made in the presence of unlabeled δ-aminolevulinic acid

Table 2. Comparison of C^{14} Activities of Hemin Samples Synthesized from Glycine-2-C^{14} (0.05 mc./mM) or Succinic Acid-2-C^{14} (0.05 mc./mM) in the Presence and Absence of Non-radioactive δ-Aminolevulinic Acid

Experiment	Substrates			Isotope Concentration in Hemin	
	C^{14}-Labeled	N^{15}-Labeled	Unlabeled	C^{14}	N^{15}
				C.p.m.	(Atom % excess)
1	Glycine-2-C^{14} (0.05 mM)	—	—	125	
	Glycine-2-C^{14} (0.05 mM)	—	δ-Aminolevulinic acid (0.05 mM)	15	
2	Glycine-2-C^{14} (0.05 mM)	—	—	230	
	Glycine-2-C^{14} (0.05 mM)	δ-Aminolevulinic acid (0.05 mM) *	—	48	0.21
	—	Glycine (0.33 mM) *	—		0.06
3	Succinate-2-C^{14} (0.1 mM)	—	—	660	
	Succinate-2-C^{14} (0.1 mM)	—	δ-Aminolevulinic acid (0.1 mM)	180	

* The isotopic concentrations of these samples were 34 atom % excess N^{15}. In each of the experiments the volume of the hemolyzed preparation was 30 ml. Unlabeled succinate (0.1 mM) was added to the flasks in which labeled glycine was the substrate, and unlabeled glycine (0.33 mM) was added to the flasks in which labeled succinate was the substrate. Each flask contained 1 mg. of iron (ferric).

contained less C^{14} than those of the controls made either from C^{14}-labeled glycine or succinate.[17] These results, which are in full agreement with the hypothesis, can also be explained, however, by the possibility that δ-aminolevulinic acid is acting, not as a diluent, but as an inhibitor of heme synthesis. To rule out the latter possibility, the δ-aminolevulinic acid added in experiment 2 (Table 2) was labeled with N^{15}. It can be seen that, whereas the incorporation of C^{14} from the glycine was lowered, there was a comparatively large incorporation of N^{15} into the porphyrin, thus demonstrating that the lowered C^{14} activity of the hemin sample was due to dilution rather than inhibition. Further proof that δ-aminolevulinic acid is a result of the condensation of glycine and succinate was obtained by incubating red blood cell hemolyzates with glycine-2-C^{14} and unlabeled δ-aminolevulinic acid, and subsequently isolating the δ-carbon atom. In such an experiment it was found that the formaldehyde liberated upon periodate oxidation of a crude fraction containing δ-aminolevulinic acid was highly radioactive.

More rigorous proof that δ-aminolevulinic acid is indeed the precursor for porphyrin synthesis was obtained by degrading a hemin sample synthesized from δ-aminolevulinic acid-5-C^{14} and from δ-aminolevulinic acid-1,4-C^{14}. The δ-carbon atom of the former compound should label the same carbon atoms of protoporphyrin as those which we have previously found to arise from the α-carbon atom of glycine, since according to the hypothesis the latter carbon atom is the biological source of the δ-carbon atom of δ-aminolevulinic acid. Furthermore, the δ-aminolevulinic acid-1,4-C^{14} should label the same carbon atoms of protoporphyrin found to arise from the carboxyl

Table 3. Distribution of C^{14} Activity in Protoporphyrin Synthesized from δ-Aminolevulinic Acid-5-C^{14} and from Glycine-2-C^{14}

Fragments of Porphyrin	Molar Activity (%) in Fragments of Porphyrin Synthesized from	
	δ-Aminolevulinic Acid-5-C^{14}	Glycine-2-C^{14}
Protoporphyrin	100	100
Pyrrole rings A + B (methylethylmaleimide)	24.5	24.6
Pyrrole rings C + D (hematinic acid)	25.2	25.3
Pyrrole rings A + B + C + D	49.7	49.9
Methene bridge carbon atoms	50.3	50.1

groups of succinate since (Fig. 7) these carbon atoms arise from succinate.

It can be seen from Table 3 that the same C^{14}-distribution pattern was found in protoporphyrin synthesized from δ-aminolevulinic acid-5-C^{14} as from glycine-2-C^{14}; 50% of the C^{14} activity resides in the pyrrole rings and 50% in the methene bridge carbon atoms (see Fig. 3).[17,18]

Also it can be seen from Table 4 that the same C^{14}-distribution pattern was found in protoporphyrin synthesized from δ-aminolevulinic

Table 4. Distribution of C^{14} Activity in Protoporphyrin Synthesized from δ-Aminolevulinic Acid-1,4-C^{14} and from Succinate-1,4-C^{14}

	Molar Activity (%) in Fragments of Porphyrin Synthesized from	
Fragments of Porphyrin	δ-Aminolevulinic Acid-5-C^{14}	Succinic Acid-1,4-C^{14}
Protoporphyrin	100	100
Pyrrole rings A + B (methylethylmaleimide)	38.0	39.4
Pyrrole rings C + D (hematinic acid)	61.5	59.5
Pyrrole rings A + B + C + D	99.5	98.5
Carboxyl groups	20.4	20.5

acid-1,4-C^{14} as from succinate-1,4-C^{14}; ten carbon atoms are equally radioactive, 40% of the C^{14}-activity resides in pyrrole rings A and B, 60% of the activity resides in pyrrole rings C and D, and the carboxyl groups contain 20% of the C^{14} activity (see Fig. 6).[19]

Thus all the carbon atoms of protoporphyrin are derived from δ-aminolevulinic acid. The role of δ-aminolevulinic acid in porphyrin synthesis was also actively pursued by Neuberger and Scott,[20] and just subsequent to our initial finding they published a confirmatory paper; further confirmation was published by Dresel and Falk.[21] Furthermore, it may be well to point out that the theoretical formulation of the structure of the precursor pyrrole[16] is the same structure which was determined for porphobilinogen[22] by Cookson and Rimington,[23] a compound excreted in the urine of patients with acute porphyria. These findings make α-amino-β-ketoadiptic acid an obligatory intermediate; we have found experimentally that this β-keto is indeed an intermediate. Injection of δ-aminolevulinic acid or the diethyl ester of α-amino-β-ketoadipic acid gives rise to the urinary excretion of porphobilinogen.[24]

The condensation of "active" succinate and glycine to form δ-amino-levulinic acid subsequently thus far appears to require the partially intact structure of the red blood cell. It has been found that, whereas δ-aminolevulinic acid can be converted to protoporphyrin in either an homogenized preparation or in a cell-free extract, the conversion of succinate and glycine to porphyrin takes place only with intact cells or with those cells which have been hemolyzed with water.[18] Homogenized preparations obtained in a blender are no longer capable of synthesizing protoporphyrin from succinate and glycine. It would appear that on homogenization the functional activity of only those enzymes of the system that are involved in the condensation of succinate and glycine is lost. However, the finding that δ-aminolevulinic acid can be converted to protoporphyrin in a cell-free extract opened up the possibility that soluble enzymes, concerned with each of the steps in this conversion, could be isolated.

Indeed, it was subsequently and independently found in three different laboratories that a highly purified protein fraction from ox liver,[25] duck erythrocytes,[26] and chicken erythrocytes [27] can convert δ-aminolevulinic acid to porphobilinogen. In our laboratory we obtained a highly purified fraction from duck blood which on incubation with δ-aminolevulinic acid-5-C[14] produced labeled porphobilinogen. Since the porphobilinogen is presumably synthesized from two moles of δ-aminolevulinic acid (Fig. 8), its molar radioactivity should be twice that of the δ-aminolevulinic acid used as the substrate. The molar radioactivities of the substrate, δ-aminolevulinic acid, and of the product, porphobilinogen, were found to be 242×10^3 c.p.m. and 487×10^3 c.p.m. respectively. This finding demonstrates experimentally the utilization of two moles of δ-aminolevulinic acid for porphobilinogen formation. Further evidence that porphobilinogen is an intermediate in protoporphyrin synthesis was obtained by incubating equal volumes of the cell-free extract of duck erythrocytes with equimolar amounts of δ-aminolevulinic acid (0.018 mc./mM) and with the enzymatically synthesized radioactive porphobilinogen (0.036 mc./mM) and subsequently isolating the hemin and determining its radioactivity. The radioactivities of the hemin samples synthesized from δ-aminolevulinic acid and from the porphobilinogen were 92 c.p.m. and 85 c.p.m. respectively, after a 2-hour incubation, and 350 and 336 c.p.m. respectively after a 15-hour incubation period.[26] This latter result is in agreement with the findings of Falk, Dresel, and Rimington [28] and of Bogorad and Granick.[29]

Although no evidence has yet been obtained concerning the biological mechanism of conversion of the monopyrrole to the tetrapyrrole structure, several suggestions have been advanced.[29, 30] We would like to suggest still another possibility which may explain the distribution of the α-carbon atom of glycine or the δ-carbon atom of δ-aminolevulinic acid in the porphyrin molecule of the I and III series. This
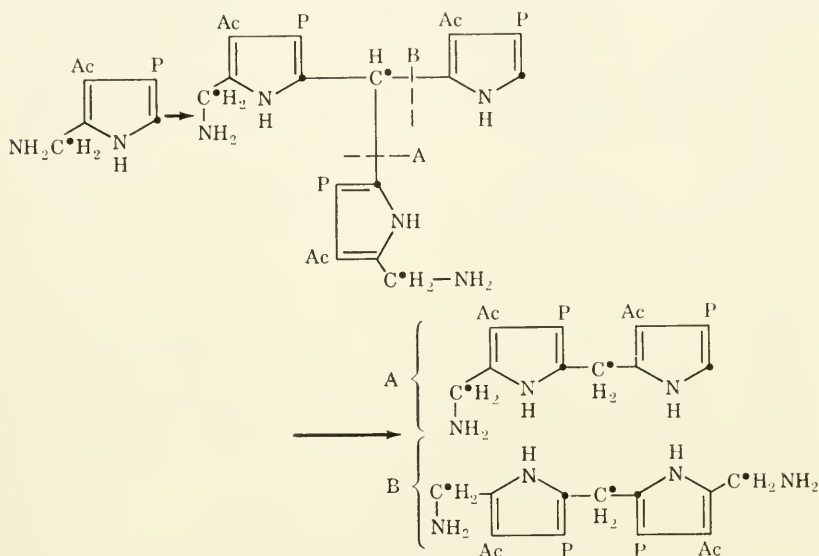
FIG. 9. A mechanism of porphyrin formation from the monopyrrole. Ac = acetic acid side chain. P = propionic acid side chain. • = α-carbon atom of glycine and δ-carbon atom of δ-aminolevulinic acid.

mechanism is based on the synthetic mechanism of dipyrrole and tetrapyrrole formation demonstrated by Corwin and Andrews,[31] and by Andrews, Corwin, and Sharp.[32]

Condensation of three moles of the precursor pyrrole (porphobilinogen), or of a closely related derivative, would lead to a tripyrrylmethane compound, as schematically represented in Fig. 9. The tripyrrylmethane then breaks down into a dipyrrylmethane and a monopyrrole. The structure of the dipyrrylmethane is dependent on the place of splitting. An A split would give rise to dipyrrylmethane A, and a B split would give rise to dipyrrylmethane B. Condensation of two moles of dipyrrylmethane A would give rise to a porphyrin of the I series, and condensation of a mole of A and a mole of B would give rise to a porphyrin of the III series. In the formation of the

porphyrin of the III series it can be seen from Fig. 6 that it is necessary to lose a 1-carbon-atom compound since there are three aminomethyl side chains, and only two are required to condense the two dipyrroles to the porphyrin structure. If the mechanism similar to that outlined in Fig. 6 is concerned with porphyrin synthesis, it would appear that this 1-carbon-atom compound given off could well be formaldehyde. Consistent with this idea is our finding [17] that on the conversion of porphobilinogen to porphyrins either by heating under acid conditions [22] or by enzymatic conversion in cell-free extracts [18] formaldehyde was indeed formed. This was established by heating or incubating porphobilinogen, labeled with C^{14} in the aminomethyl group, and subsequently isolating radioactive formaldehyde as the dimedon derivative.

It would appear that, on conversion of porphobilinogen to porphyrins, formaldehyde from the aminomethyl group is formed and that any postulated mechanism should take this into consideration. It is difficult at present to establish the structure of the intermediate tetrapyrrole compounds which are formed prior to the formation of protoporphyrin. However, we would like to suggest that these intermediate tetrapyrrole compounds may be the more highly reduced state, containing methylene bridge carbon atoms rather than methene bridge carbon atoms, and consequently uroporphyrin and coproporphyrin are oxidized products of the intermediates.

The biosynthetic pathway for porphyrin synthesis, given above, may, from a more general viewpoint, be looked upon as merely one aspect of glycine metabolism. The α-carbon atom of glycine besides being utilized for porphyrin synthesis is also known to participate in the synthesis of several other compounds: the ureido groups of purines, the β-carbon atom of serine, methyl groups, and for formic acid. It would appear that these different compounds and porphyrins may be related via a metabolic pathway of glycine. If indeed these mentioned compounds and porphyrin synthesis are related through a series of reactions occurring with glycine, then an intermediate utilized for porphyrin synthesis may have the same metabolic pattern as is known for glycine. If the succinate-glycine cycle proposed in Fig. 7 [16] were the pathway by which all the compounds are related, then specifically the δ-carbon atom of δ-aminolevulinic acid should have the same metabolic spectrum as the α-carbon atom of glycine. In a study carried out in ducks and rats it was found that the δ-carbon atom of this aminoketone is indeed utilized for the ureido groups of purines, for the β-carbon atom of serine, for the methyl group of methionine, and

is also converted to formic acid. Thus, it has been demonstrated that glycine is metabolized via this pathway.[33]

The condensation of glycine with "active" succinate provides a pathway whereby glycine can be oxidized to carbon dioxide and the intermediates produced in the cycle drawn off for the synthesis of other compounds. This is similar to the citric acid cycle, in which another 2-carbon compound is oxidized to carbon dioxide and intermediates are produced which can be drawn off for synthesis. In the succinate-glycine cycle, succinate is the catalyst instead of oxaloacetate.

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The Role of Carbohydrates
in the Biosynthesis
of Aromatic Compounds

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The diversity and importance of aromatic compounds in biological materials, from simple hydrocarbons to complex alkaloids, have been responsible for several theories of their biogenesis on the basis of structural relationships or known laboratory reactions. Direct experimental attack on this problem began with the discovery by B. D. Davis in 1950 that shikimic acid (SA) is an intermediate in the formation of the aromatic amino acids [1] in certain nutritionally deficient mutants of *Escherichia coli.*

The studies on the formation of tyrosine and phenylalanine from labeled compounds [2-6] do not permit the two sides of the ring to be distinguished from each other, in contrast to those with SA. Accordingly, in collaboration with B. D. Davis,[7] methods were developed for the isolation of SA from filtrates of *E. coli* mutant 83-24, and for its chemical degradation (Fig. 1). It had been observed that, when this organism was grown on a glucose-salts medium with the addition of $NaHC^{14}O_3$, $HC^{14}OONa$, acetate, labeled in either carbon atom, or pyruvate-2-C^{14}, the activity incorporated into SA from these additions was negligible. The participation of the tricarboxylic acid cycle intermediates in the biosynthesis of tyrosine and phenylalanine in yeast had also been excluded by Gilvarg and Bloch,[5] who showed that, although labeled acetate, in the presence of glucose, was incorporated into glutamate, aspartate, and alanine, no activity was observed in the aromatic amino acids.

When glucose labeled in G-1, G-2, G-3, 4, or G-6 * was utilized for

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* The abbreviations S-1, S-2 ⋯ S-7 will be used to denote carbon atoms 1, 2 ⋯ and carboxyl of shikimate; and G-1, G-2 ⋯ to denote carbon atoms 1, 2 ⋯ of glucose.
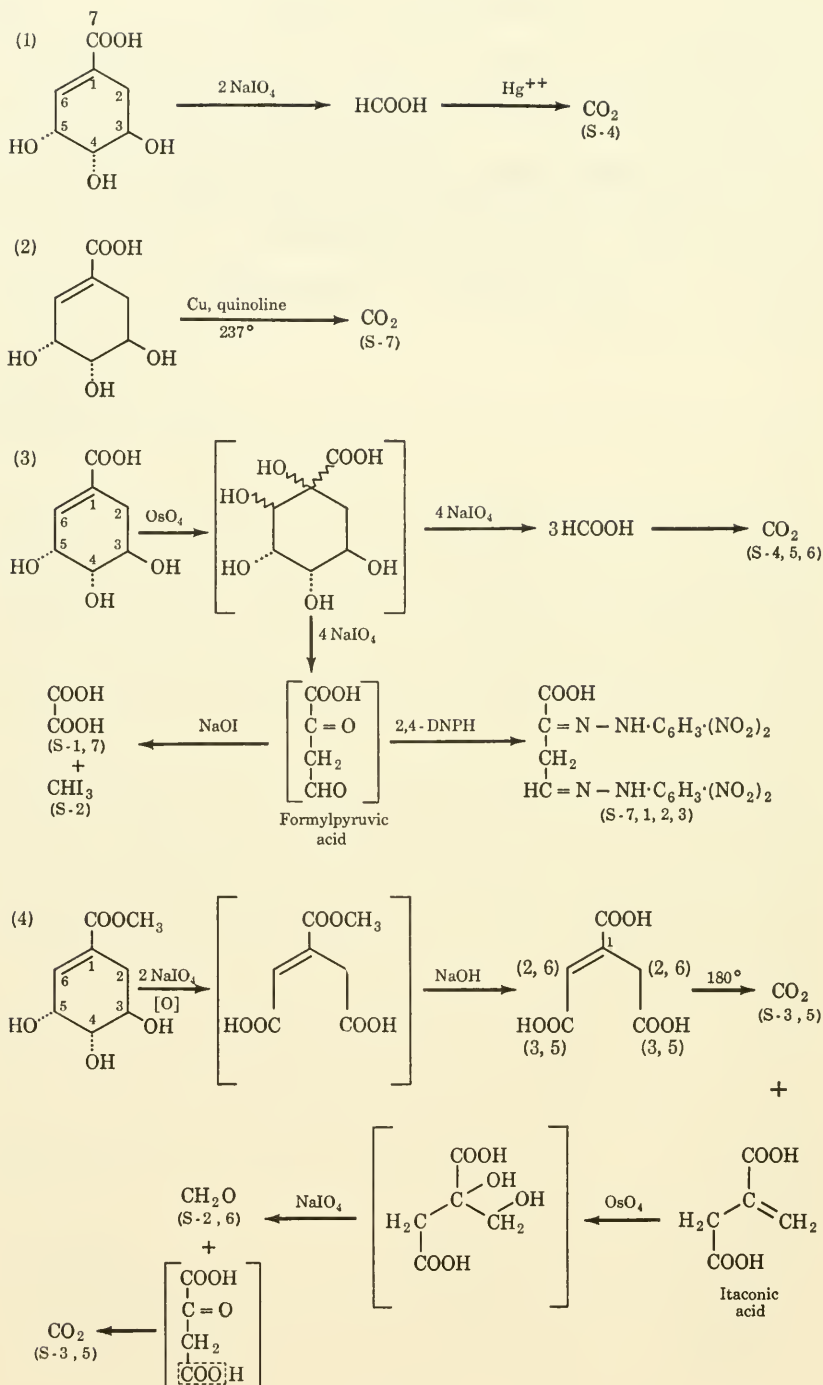
FIG. 1. Reactions used for the degradation of labeled shikimic acid.

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SA synthesis it gave the distribution of activities summarized in Fig. 2. The value for each SA carbon atom is represented as the fraction of activity of the labeled atom of the glucose from which the SA had arisen. Except for S-1 and S-5, the SA atoms were 80–90% accounted for by large contributions from glucose atoms 1, 2, 3, 4, and 6. In addition there were small incorporations which could not be determined accurately. The large "deficiencies" in S-1 and S-5 presumably arise mostly from G-5, the one carbon atom of glucose that could not be tested experimentally.

3 or 4 (0.86)
COOH

2 (0.4)
5 (0.5)

(0.25) 1   1 (0.4)
(0.60) 6   6 (0.5)

(0.22) 2   2 (0.24)
(0.6) 5   3 or 4 (0.59)

3 or 4 (0.9)
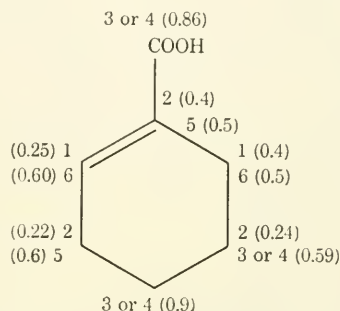
Fig. 2.   Major contributions of glucose carbon atoms to shikimate biosynthesis.

It is clear that S-2 is derived almost entirely from G-1 and G-6, S-1 from G-2 and G-5, and S-7 from G-3, 4. The G-1 and G-6 contributions are about equal, as are those from G-2 and G-5, and so it may be assumed that the G-3 and G-4 contributions are also about equal. From this distribution it may be concluded that S-2, 1, 7 is derived from a three-carbon intermediate of glycolysis, which as pointed out above cannot be pyruvate.

The derivation of the 3, 4, 5, 6 portion of SA from glucose is more complex. S-6, S-5, and S-4 are derived 0.8–0.9 from G-1, 6, G-2, 5, and G-3, 4, respectively. Since the G-6/G-1 and the G-5/G-2 contributions are both present in a ratio of about 2.5/1 it seems reasonable to assume the same ratio for the G-4/G-3 contributions. S-3 arises from G-3, 4 and G-2, also in a 2.5/1 ratio. Such a distribution of label can be explained by assuming that S-3, 4, 5, 6 was derived from tetrose phosphate whose formation had involved, as shown in Figs. 3 and 4, the glycolytic as well as the pentose phosphate pathway.

In reaction 1 (Fig. 3) fructose-6-phosphate (F-6-P) (I), derived directly from the glucose administered, exchanges under the influence of transaldolase with triose phosphate which has been equilibrated by triose isomerase. This exchange incorporates G-1, 2, 3 into the "bot-

tom" three carbon atoms of F-6-P, yielding II. In a steady state the proportion of I and II in the pool of F-6-P (III) will depend on the rates of supply of F-6-P (I) and triose phosphate and on the rate of exchange between them.
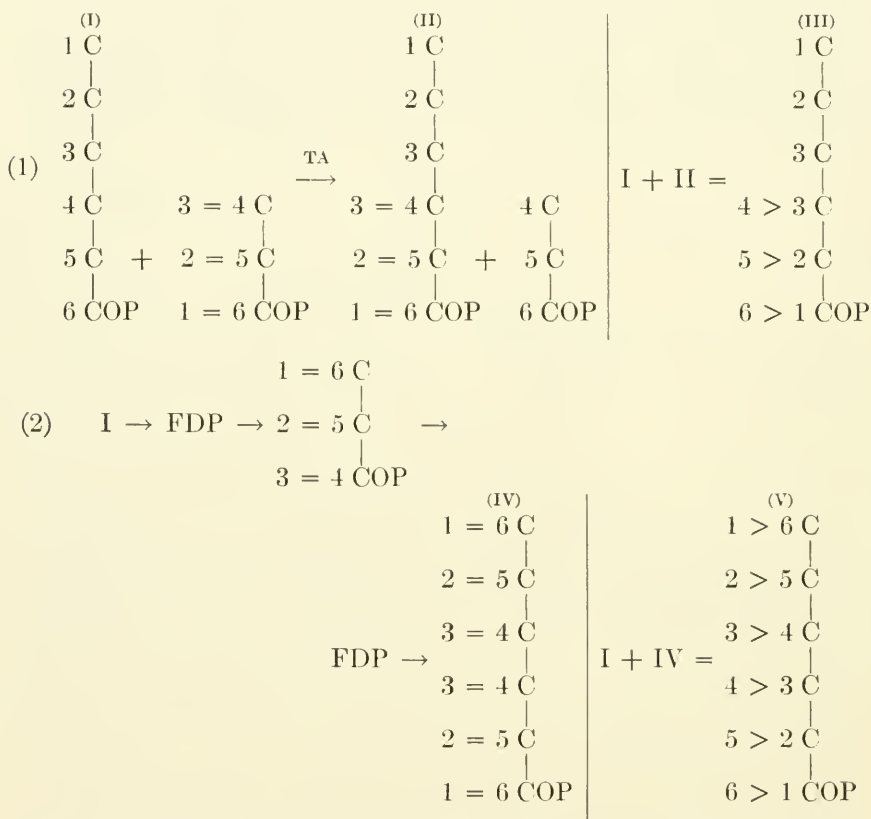
Fig. 3. Possible effects of transaldolase (TA) and fructose diphosphatase (FDP) on the distribution of carbon in the fructose-6-phosphate (F-6-P) pool.

Alternatively, in reaction 2 (Fig. 3), part of the F-6-P may arise by hydrolysis of fructose diphosphate (FDP), in a fraction of which G-1, 2, 3 and G-6, 5, 4 have been equilibrated through the action of aldolase and triose isomerase. The resulting F-6-P is given by IV and the F-6-P pool by V. If both reactions 1 and 2 occur, a different pool will be formed.*

* The enzymes involved in reaction 1 are known to be present in *E. coli*, but the exchange reaction indicated for transaldolase in this sequence, though expected,

From the F-6-P pool tetrose phosphate can then be formed by reactions 3, 4, and 5 (Fig. 4), in which pentose phosphate and sedo-heptulose-7-phosphate are intermediates.[8-12] The net effect of these reactions is to convert one molecule of F-6-P and two molecules of

```
                1 C
                 |
                2 C                                      1 C
                 |                                        |
         (3)    3 C              3 C        3 C          2 C
                4 > 3 C      3 = 4 C        4 > 3 C      3 = 4 C
                 |            |      TK      |            |
                5 > 2 C    + 2 = 5 C   ───→ 5 > 2 C   + 2 = 5 C
                 |            |              |            |
                6 > 1 COP   1 = 6 COP       6 > 1 COP   1 = 6 COP

                                   1 C
                                    |
                 1 C               2 C
                  |                 |
                 2 C               3 C                                3 C
        (4) (a)  3 = 4 C   + 4 > 3 C           1 C   + 4 > 3 C        (a)
                  |          |          TK      |       |
                 2 = 5 C    5 > 2 C     ───→   2 C     5 > 2 C
                  |          |                  |       |
                 1 = 6 COP  6 > 1 COP          1 C     6 > 1 COP
                                               2 C

                 1 C        1 C        TK       3 = 4 C     3 = 4 C
                  |          |          ───→    |           |
                 2 C        2 C                2 = 5 C   + 2 = 5 C   (b)
                  |          |                  |           |
            (b)  3 = 4 C  + 3 = 4 C            1 = 6 COP   1 = 6 COP
                  |          |
                 2 = 5 C    2 = 5 C
                  |          |
                 1 = 6 COP  1 = 6 COP

                 1 C
                  |
                 2 C
                  |
                 1 C                                      1 C
                  |                                        |
         (5)     2 C                         2 C          2 C
                  |                           |            |
                 3 = 4 C     3 = 4 C         3 = 4 C      3 = 4 C
                  |          |      TA        |            |
                 2 = 5 C   + 2 = 5 C   ───→  2 = 5 C   + 2 = 5 C
                  |          |                |            |
                 1 = 6 COP  1 = 6 COP        1 = 6 COP    1 = 6 COP
```
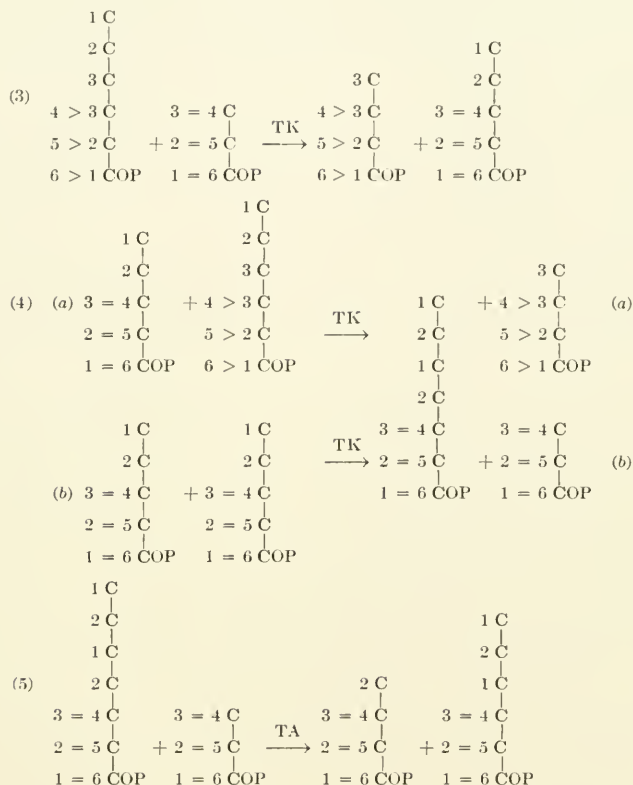
Fig. 4.   Synthesis of tetrose phosphate via the pentose phosphate pathway from the pooled F-6-P of reaction 1, Fig. 3.

triose phosphate to three molecules of tetrose phosphate. In two of these the "bottom" three carbon atoms are derived from the corresponding atoms of F-6-P, and in the third tetrose molecule they are derived from the triose phosphate.

has not been directly explored. The phosphatase required for reaction 2 has been demonstrated in preliminary experiments on extracts of this strain of *E. coli*. (P. R. Srinivasan and D. B. Sprinson, unpublished results. We are indebted to Dr. E. Racker for generous supplies of purified FDP and glucose-6-phosphate dehydrogenase required in this assay).

With the F-6-P pool (III) resulting from reaction 1, reactions 3 and 4a each yield a molecule of tetrose of the composition G-3, 4 > 3, 5 > 2, 6 > 1. Reaction 5 would then give rise to a molecule of the composition G-2, 3 = 4, 2 = 5, 1 = 6. Alternatively, the production of two molecules of the first kind of tetrose in reaction 3 can be followed by reactions 4b and 5, again yielding a molecule of the second kind of tetrose. In either case the ratio of G-3 to G-2 in carbon 1 of the pooled tetrose would be 2.

The F-6-P pool (V) from reaction 2 would yield through the same process a pair of tetrose phosphates identical with the above in carbon atoms 2 to 4. In carbon atom 1, however, G-3 > 4 would replace G-3 and G-2 > 5 would replace G-2.

It is clear that the isotope distribution observed in atoms 4, 5, 6 of SA is consistent with their origin from carbon atoms 2, 3, 4 respectively of tetrose phosphate formed as described above. In one-third of the tetrose molecules these atoms are derived from triose phosphate, which would account for a G-1, 2, 3/G-6, 5, 4 ratio of 1/6 in S-6, 5, 4. From the higher ratio observed (1/2.5), it can be calculated that about two-thirds of the pooled F-6-P was derived from the intact chain of glucose and one-third had incorporated triose phosphate, possibly by direct exchange (reaction 1) or by hydrolysis of equilibrated FDP (reaction 2).*

Similarly, the isotope distribution observed in S-3 (G-3, 4/G-2 in a ratio of 2.5/1) is consistent with the origin of S-3 from carbon atom 1 of tetrose phosphate. In the latter atom a ratio of 2/1 would be expected, as noted above, for the G-3/G-2 contribution derived from "unexchanged" F-6-P (I). The effect of reaction 1 on the F-6-P pool would not influence the isotopic composition of this atom of tetrose, whereas reaction 2 would cause small incorporations of G-4 and G-5. Theoretically the importance of reaction 2 could be evaluated from a precise determination of these incorporations, but such data are not available.†

* Let a = fraction of G-1 in position 6 of pooled F-6-P (III or V). Assume fraction of G-1 in position 3 of pooled triose phosphate = 0.5. Assume also that S-3, 4, 5, 6 is derived from tetrose molecules of which two-thirds obtain their bottom carbon atoms from F-6-P and one-third from triose. Then:

$$\text{Fraction of G-1 in S-6} = \frac{(G\text{-}1)}{(G\text{-}1) + (G\text{-}6)} = \frac{0.25}{0.85} = 0.29 = \frac{(0.5 \times 1) + 2a}{3}$$

$$a = 0.185$$

Fraction of F-6-P pool derived by exchange (reactions 1 or 2, Fig. 3) is $2a$, or 0.37.

† As a further consequence of reactions 3 to 5 (Fig. 4), the F-6-P regenerated in reaction 5 (which would amount to one-third of the glucose entering these

A further consequence of these considerations is that attachment between the two intermediates utilized in SA formation involves carbon 1 of the tetrose and carbon 3 of the triose. As will be pointed out later this does not correspond to the known ways of forming heptoses.

A complete series of experiments on the biosynthesis of tyrosine, phenylalanine, the tryptophan from variously labeled glucose is unavailable for comparison with the present studies. However, several relevant investigations are known.

The incorporation of approximately 0.5 of an atom of G-1 into carbons 2, 6 of tyrosine and phenylalanine in yeast [5] agrees with the presence of 0.65 G-1 in S-2, 6. In agreement also are the incorporations, in *E. coli*, of 1.0 to 1.1 atoms of G-6 into carbons 2, 6 of both tyrosine [13] and SA. The earlier results of Ehrensvärd and collaborators [2] on tyrosine formation from acetate-1-C^{14} (showing a high incorporation of label into carbons 4 and 3/5 and essentially none elsewhere in the ring) are in agreement too, if it is assumed that this compound is incorporated via glucose-3,4-C^{14}.

In contrast, subsequent results of Ehrensvärd et al. have indicated that carbon 1 of acetate is extensively incorporated into at least three atoms of the ring of tyrosine, [3,4] and into three consecutive atoms of the benzene ring of tryptophan. [14] A similar incorporation of G-3, 4 into tryptophan has been observed by Rafelson. [15] These results appear to be in conflict with the finding (Fig. 2) that G-3, 4 enters significantly into only two atoms of the ring of SA. The tryptophan data have led to the suggestion [15,16] that glucose-3,4-C^{14} is converted to heptose-3,4,5-C^{14},[8,12] and that the intact carbon chain of the latter is cyclized to SA-4,5,6-C^{14}. However, utilization of the intact carbon chain of such a heptose or of heptose formed by any known mechanism seems excluded by the results presented above, as well as by enzymatic studies to be discussed later.

In order to test various intermediates of the glycolytic as well as the pentose phosphate pathways as precursors of SA, a cell-free test system was developed. In most cases the formation of 5-dehydro-shikimate (DHS), the precursor of SA,[17] rather than SA was studied,

reactions) should yield the triose G-1, 2, 1 (or G-1 > 6, 2 > 5, 1 > 6) and the tetrose G-1, 3 = 4, 2 = 5, 1 = 6 (or G-1 > 6, 3 = 4, 2 = 5, 1 = 6). From the observed insignificant incorporation of these fragments into SA, it appears that under these experimental conditions the contribution of reactions 3 to 5 to the F-6-P pool is small.

It has been assumed throughout that in triose phosphate G-6, 5, 4 = G-1, 2, 3. This is a simplifying assumption, since S-2, 1, 7, which are derived from a three-carbon intermediate of glycolysis, appear to show a small excess of G-6, 5, 4 over 1, 2, 3.

since the reduction [18] of DHS to SA required an additional substrate, isocitrate, and TPN for generating the required TPNH.

Extracts of strain S3-24 of *E. coli* were able to convert hexose phosphates, ribose-5-phosphate, or sedoheptulose-7-phosphate (S-7-P) to DHS in a yield of about 5%.[19] S-7-P plus FDP did not yield significantly higher values. However, sedoheptulose-1,7-diphosphate (SDP) [20] was almost quantitatively converted to DHS.[21] When SDP-4,5,6,7-C[14] was incubated under these conditions, the SA obtained had the same activity, and only in S-3, 4, 5, 6, showing that a four-carbon fragment from SDP is utilized directly in shikimate formation.[21]

Table 1. Synthesis of DHS from E-4-P + PEP and from SDP * †

	Per Cent Conversion	
Substrates and Additions	1 Hour	2 Hours
E-4-P + 0.3 µM PEP	88	86
+ fluoride + 0.3 µM PEP	88	88
+ fluoride + 0.5 µM 3-PGA	0	0
+ iodoacetate + 0.3 µM PEP	90	90
+ iodoacetate + 0.5 µM 3-PGA	90	90
SDP	39	83
+ fluoride	0	0
+ fluoride + 0.5 µM FDP	0	0
+ fluoride + 0.5 µM 3-PGA	0	0
+ fluoride + 0.5 µM pyruvate	0	0
+ fluoride + 0.3 µM PEP	37	80
+ iodoacetate	0	0
+ iodoacetate + 0.5 µM FDP	0	0
+ iodoacetate + 0.5 µM 3-PGA	46	83
+ iodoacetate + 0.5 µM pyruvate	0	0
+ iodoacetate + 0.3 µM PEP	46	83

* Cell-free extracts were prepared by subjecting cells of freshly harvested *E. coli* mutant S3-24 [B. D. Davis, *J. Biol. Chem.*, *191*, 315 (1951)] to sonic vibration. The incubation mixtures contained 0.1 ml. of extract (2 mg. of protein), 5 µM of $MgCl_2$, 50 µM of $PO_4^{=}$ buffer pH 7.4, 0.25 µM of E-4-P + 0.3 µM of PEP, or 0.25 µM of SDP, + additions (10 µM of KF, or 0.5 µM of iodoacetate) in a final volume of 1 ml. When iodoacetate was added, the solution, 0.95 ml., was preincubated at 37° for 15 minutes prior to the addition of substrate. Following incubation at 37° for the indicated length of time aliquots were removed for the bioassay of DHS with *Aerobacter aerogenes* mutant A170-143S1 [B. D. Davis and U. Weiss, *Arch. exp. Pathol. and Pharmakol.*, *220*, 1 (1953)].

† Abbreviations: E-4-P, D-erythrose-4-phosphate; PEP, phosphoenolpyruvate; SDP, sedoheptulose-1,7-diphosphate; FDP, fructose diphosphate; 3-PGA, D-3-phosphoglyceric acid.

The high conversion of SDP to DHS cannot be due to direct cycliza-
tion of the seven-carbon chain of SDP. Carbon atoms 7, 1, and 2 of
SA were shown to arise from G-(3, 4)-(2, 5)-(1, 6), respectively. Such
a sequence of glucose carbon atoms in carbons 1, 2, and 3 of heptose
has never been observed. The reverse order, i.e., G-(1, 6)-(2, 5)-(3, 4),
would be expected in carbon atoms 1, 2, and 3 of heptose.[12, 20]

Since S-2, 1, 7 are derived from a three-carbon product of glycolysis,
but not pyruvate, it was surmised that carbons 1, 2, and 3 of SDP
are converted to phosphoenolpyruvate and then condensed with tetrose
phosphate to a seven-carbon precursor of DHS. In support of this
scheme, it was found (Table 1) that the conversion of SDP to DHS
was completely inhibited by fluoride and iodoacetate but that it was
completely restored by phosphoenolpyruvate.[22] Moreover, synthetic
D-erythrose-4-phosphate [23] and phosphoenolpyruvate were almost quan-
titatively converted to DHS (Table 1). The efficient conversion of
SDP to DHS may therefore be explained on the basis of the following
reactions:

SDP $\rightleftharpoons$ D-Erythrose-4-phosphate + Dihydroxyacetone phosphate [20]

Dihydroxyacetone phosphate $\rightleftharpoons$ Phosphoenolpyruvate

D-Erythrose-4-phosphate + Phosphoenolpyruvate $\rightarrow$ $\rightarrow$ DHS
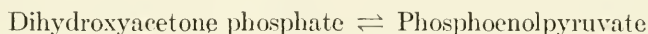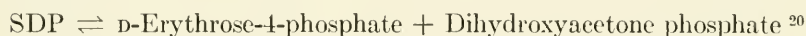
Although the details of the reactions are still unknown, it may be
postulated at present that the pathway from glucose to DHS is in part
as shown in the diagram below.
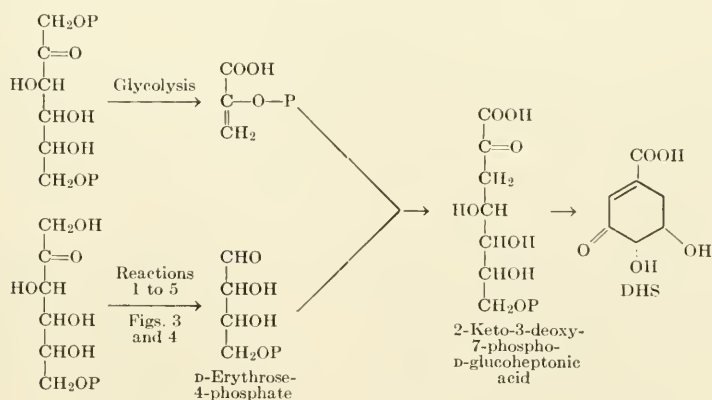
It is of interest that several classes of alicyclic compounds, e.g., the
carotenoids and steroids, in the biosynthesis of which an isoprene unit
appears to be involved, are derived from acetate. On the other hand,

the biosynthesis of the aromatic amino acids, and presumably the alkaloids and flavonoids derived from them,[24, 25] is dependent on several reactions in the metabolism of carbohydrates which either precede, or are unrelated to, the production of acetate. "Intuitively" and on structural grounds this division was foreseen many years ago.[24]

It is a pleasure to acknowledge the contributions, intellectual as well as experimental, of the investigators who have participated in this investigation in our laboratories, Drs. H. T. Shigeura, P. R. Srinivasan, M. Sprecher, and M. Katagiri, as well as the stimulating and pleasant collaboration with Dr. B. D. Davis. I am grateful to Dr. D. Rittenberg for his support and encouragement during the early period of this investigation. Without his generosity it could not have been undertaken.

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On Determining the Chemical Structure of Proteins

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A decade ago an essay under this title would have seemed hopelessly visionary. That it is not so today testifies to the precipitate progress made in the intervening years by biochemistry in general and protein chemistry in particular. Commencing with the introduction of chromatographic methods into amino acid chemistry by Martin and Synge, this progress has culminated in the brilliant studies of Sanger (cf. ref. 1 for references) that have revealed completely the arrangement of the amino acid residues in the insulin molecule. Despite this striking success, determining the chemical structure of a protein is still far from the routine procedure that will be necessary if proteins as a group are to become susceptible to searching structural analysis. On the basis of current knowledge, however, it is possible to formulate in some detail the steps that will be required to determine the chemical structure of a protein. It is also possible to apprehend the nature of some of the difficulties remaining, and, in a few cases, to suggest possible methods by which they may be overcome. This essay will attempt to discuss the problem from this point of view, using ribonuclease as a specific example.*

For the purposes of the present discussion, the determination of the chemical structure of a protein will be limited to finding the sequence of amino acids in the peptide chain (or chains), ascertaining the nature and position of any cross linkages in the molecule, and determining the manner of attachment to the peptide chains of any non-amino acid

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* The author owes much to the many fruitful and stimulating discussions he has enjoyed with Dr. Stanford Moore and Dr. C. H. W. Hirs. It is particularly fitting that the studies on the structure of ribonuclease carried out by Dr. Hirs form an important part of this essay, for he is a recent graduate of the Department of Biochemistry at Columbia University. Permission to include this work, some of it unpublished at the time of writing, is gratefully acknowledged.

moieties that may be present, such as carbohydrates, nucleic acids, or porphyrins. On the basis of this definition, determining the chemical structure of a protein can be broken down into the following sequence of operations: (*a*) Purification and isolation of a sample of a protein sufficiently pure to warrant detailed structural work. (*b*) Quantitative amino acid analysis of the purified protein. (*c*) End-group analysis to determine the number of peptide chains in the molecule. (*d*) Rupture of the disulfide bridges or other cross linkages in the molecule. (*e*) Separation of the peptide chains from one another, if more than one is present. (*f*) Partial hydrolysis of a peptide chain. (*g*) Fractionation of the mixture of peptide fragments formed on partial hydrolysis. (*h*) Determination of the structure of the isolated fragments.

It will be apparent that formulation of the problem in this way implicitly assumes that proteins have a definite and determinable chemical structure.* It assumes that when a given bovine pancreas synthesizes ribonuclease, for example, the amino acid residues in the chains of the various protein molecules will be laid down in the same order from one molecule to the next. It does not preclude the possibility that more than one kind of molecule possessing ribonuclease activity may be synthesized by the pancreas, but it does assume that the number of such kinds of molecules will be small and that the individual species of proteins from a single organism are not populations of molecules the organic chemical structure of which varies more or less continuously around a mean.

In short, this conception of the proteins ascribes to them the attributes of well-defined chemical compounds similar in fundamental character to, though more complicated in structure than, the other types of substances synthesized by living organisms. There is considerable evidence in favor of this conception, the most compelling of which is the fact that Sanger has been able to establish an unambiguous chemical structure for insulin. Were beef insulin to consist of a family of closely related molecular species, it is difficult to see how a unique structure could have been derived. Recent results with ribonuclease point in the same direction. This conception also has the pragmatic advantage that it encourages investigators to attack the problem of protein structure from the point of view of the organic chemist. Organic chemistry has no techniques for handling complex structural problems involving heterogeneous populations of molecules. No matter

* For a summary, with references, of the contrary point of view, cf. Colvin, Smith, and Cook.[2]

how complex the structure, however, where there is homogeneity there is hope. If the hope is ill-founded and proteins really are families of molecules, detailed investigations of their structure are bound to reveal this fact.

In finally deciding whether or not the biochemical events directing the synthesis of protein molecules are so ordered as to permit exact structural duplication from one molecule to the next, it will be necessary to decide not only whether a given protein preparation is heterogeneous, but also whether heterogeneity, if found, has been imposed in the process of isolating the material from natural sources. Opportunities abound for introducing heterogeneity where none originally existed. At the outset, the choice of source material may be crucial in this connection. For years, insulins derived from the ox, the pig, and the sheep were tacitly assumed to be identical by many investigators simply because each had identical effects on blood sugar. The recent amino acid analyses of Harfenist [3] and the structural studies of Sanger [4] have finally proved, however, that the insulins of these species are slightly different. This finding should not be too surprising in view of the abundant immunological evidence demonstrating the species specificity of proteins. Most investigators would agree that structural work on a protein preparation derived from several animal species would probably be a waste of time, but how many would agree on just what constitutes a species? What degree of genetic homogeneity must one demand before molecular homogeneity can be expected? Does individuality extend to the molecular level? It does not seem inconceivable that in the case of species, such as man, possessing an unknown and uncontrollable genetic constitution, true homogeneity of some types of proteins may only be attainable in a preparation derived from a single individual.

Even granted a suitable starting material containing a mixture of initially homogeneous proteins, however, the problems involved in isolating a single molecular species without introducing alterations in the molecule remain among the most intractable facing the protein chemist. For a long time, virtually the only preparative procedures available made use of fractional precipitation in some form. Fortunately, recent years have seen the introduction of multistage techniques of high resolving power such as countercurrent distribution, zone electrophoresis, and chromatography. Chromatography in particular would appear to possess several features that, in principle, should make it ideally adapted to work with proteins. The method is gentle, flexible, has high resolving power, and can be used for both analytical and preparative

purposes. Nevertheless, despite intensive efforts in many laboratories (cf. Zittle,[5] Moore and Stein,[6] and Porter[7] for references), only a handful of proteins have been chromatographed successfully. Usually, however, successful chromatography has led to purer products and new information, indicating that if the method could be more widely employed protein chemistry would benefit greatly.

The difficulties in chromatographing proteins doubtless arise from the fact that proteins are large, fragile, polyvalent molecules. Because of their size, they cannot penetrate into the particles of most column packings the way small molecules do but must be bound largely at the surface. Unless the column packing has a large surface, therefore, the capacity may be so small that effective chromatography is impossible.* A limited capacity will also render the behavior of the column very sensitive to variations in the composition of the mixture being chromatographed. Displacement of one protein by another, and competition between proteins for a limited number of binding sites, may complicate the interpretation of the effluent curves. Under such circumstances, rechromatography, determinations of enzymatic or other activities in the effluent, electrophoresis, or amino acid analyses of the individual peaks will all prove helpful as ancillary means of following the fractionation and will serve to minimize errors of interpretation.

The fact that proteins are polyvalent molecules undoubtedly makes effective chromatography more difficult. Most proteins contain several residues of each of the amino acids; hence, no matter what types of linkage bind the protein to the column packing, these linkages are almost sure to be multiple. As has been pointed out before, notably by Tiselius,[9] polyvalent molecules are likely to be all adsorbed or all eluted. The R_f is likely to be either one or zero and to change abruptly from one extreme to the other over a rather narrow range of experimental conditions. Satisfactory elution analysis, however, requires a reversible distribution of solute between stationary and mobile phases leading to R_f values intermediate between zero and one. It has seldom been possible to find such conditions with proteins, and for this reason an eluent of constantly changing pH or ionic strength or both (so-called "gradient elution") has of necessity been employed in several

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* The high capacity for proteins exhibited by XE-64, a ground form of IRC-50, is probably related to its large surface, inasmuch as a bead form of the same resin does not yield satisfactory chromatograms. It is not clear whether the high capacity possessed by the ion-exchange materials made by Sober and Peterson[8] from cellulose depends upon the surface of the cellulose or a loose gel structure that permits penetration of protein into the column packing.

laboratories, including our own. In this procedure, the chromatogram is begun with an eluent incapable of eluting the protein in question ($R_f = 0$), whereupon the composition of the eluent is gradually changed to one in which the protein has an R_f of 1. The exact point of emergence of the protein will thus be a function more of the rate of change of the eluent than of the length of the column. Although worth-while purifications can be effected in this manner,[9,10] elution analysis of proteins has, in our experience, been found to be superior when it can be made to work properly.

Satisfactory elution analysis has been possible with several proteins on both partition and ion-exchange columns. Despite the fact that these proteins are polyvalent molecules, test-tube experiments showed unquestionably that, in several instances, reversible distribution between resin (IRC-50, in each case) and buffer existed. Moreover, the magnitude of this distribution coefficient could be changed in a predictable fashion by altering the pH or the ionic strength of the buffer phase. In short, several proteins, among them ribonuclease,[11] have been found to behave much like simple substances. It is pertinent to inquire why this should be so. From a logical point of view, the problem really is not why most proteins chromatograph unsatisfactorily but why some behave well. Ribonuclease, for example, contains a minimum of fifteen possible cationic sites (cf. Table 1) which could exchange with Na^+ on a buffered IRC-50 column. A clue may be furnished by the electrophoretic studies of Crestfield and Allen[12] that show that the isoelectric point of ribonuclease drops from pH 7.48 in dilute acetate buffers to pH 5.49 in 0.2 N phosphate buffers of the type used in chromatography. Apparently phosphate ions form a feebly dissociable complex with ribonuclease, and it may be that the existence of this complex facilitates chromatography on the ion exchange resin. Complexing agents have been utilized with success in other fields, notably in the case of the rare-earth elements by Spedding and his associates[13] and in the case of the sugars by Khym and Zill.[14] Perhaps a systematic search for agents that form complexes with proteins might widen the scope of elution analysis. Whether or not chromatography is the best way to procure them, it is unquestionably true that a larger number of rigorously purified proteins remains one of the critical needs of the protein chemist.

In considering which proteins were likely to be good subjects for detailed structural study, the fact that ribonuclease could be purified chromatographically was one of the most important considerations leading to its selection. Its small size (mol. wt., 14,000) and the

absence of tryptophan from the molecule were also strongly in its favor. As a first step in the investigation, detailed amino acid analyses were performed so that an accurate balance sheet in terms of amino acids could be kept as the structural studies progressed. Although the quantitative amino acid analysis of a protein hydrolyzate no longer presents serious problems, it has become apparent that the number of amino acids that may decompose on hydrolysis and the extent of the decomposition may vary from laboratory to laboratory and from protein to protein. Possibly this variation could be minimized if the temperature at which the hydrolysis is conducted were precisely controlled. Some decomposition during acid hydrolysis has been noted at one time or another for serine, threonine, cystine, tyrosine, aspartic acid, glutamic acid, proline, methionine, histidine, lysine, and arginine.[2, 3, 15–17] (Tryptophan, of course, decomposes nearly completely.) The variable nature of this decomposition requires that individual corrections must be worked out for each protein studied. Analysis after two times of hydrolysis, say 20 and 70 hours, permits extrapolation to zero time and seems to yield the most accurate results obtainable at present. The longer time of hydrolysis also allows some estimate to be made of those amino acids, such as valine or isoleucine, originally bound in peptide linkages resistant to acid hydrolysis. The amino acid composition of ribonuclease given in Table 1 was determined in this manner.[17] Corrections for decomposition had to be made for serine, threonine, cystine, tyrosine, aspartic acid, glutamic acid, proline, and arginine. Isoleucine was liberated slowly. Despite these corrections, however, the number of residues of each amino acid present to the extent of ten residues per mole or less is known with considerable assurance. For those amino acids present in larger quantities, a reasonable experimental error of $\pm 4\%$, coupled with the uncertainties introduced by corrections, may cause an error of $\pm$ one residue in the final value. With proteins larger than ribonuclease, this limitation might extend to most of the amino acids.

End-group analyses of ribonuclease were performed by Anfinsen, Redfield, Choate, Page, and Carroll.[18] When the DNP technique of Sanger was employed, it was established that ribonuclease consisted of a single peptide chain bearing lysine as the amino-terminal amino acid, followed in turn by glutamic acid, threonine, and alanine. By the use of carboxypeptidase, the carboxyl-terminal amino acid was found to be valine, followed back along the peptide chain by phenylalanine, isoleucine, or leucine, alanine, tyrosine, and methionine in undetermined order. On the basis of this information and the analytical

Table 1. Amino Acid Composition of Ribonuclease

(The values for the stable amino acids are the average of six determinations)

	Residues per Mole	
Amino Acid	Found	To Nearest Integer
Aspartic acid *	15.8	16
Glutamic acid *	11.8	12
Glycine	3.05	3
Alanine	12.0	12
Valine	8.95	9
Leucine	2.15	2
Isoleucine	2.85	3
Serine *	15.0	15
Threonine *	10.45	10
Half cystine †	8.15	8
Methionine	3.75	4
Proline *	4.79	5
Phenylalanine	2.97	3
Tyrosine *	5.87	6
Histidine	3.80	4
Lysine	10.0	10
Arginine *	3.97	4
Amide NH_3 ‡	17.0	(17) §
	Total	126

Calculated molecular weight	13,895
Nitrogen recovery	97%
Weight	99%

* Extrapolated values for amino acids that show decomposition on hydrolysis.
† The average of triplicate determination of cystine as cysteic acid.
‡ Average of two determinations of amide NH_3.
§ Amide groups not included in the summation of the total number of residues.

data in Table 1, Anfinsen et al. proposed the structure for ribonuclease shown in Fig. 1.* The existence of four disulfide bridges in the molecule was assumed when attempts to detect sulfhydryl groups failed. Ledoux [19] has claimed, on the basis of experiments with oxidizing and reducing agents such as H_2O_2, HCN, and reduced glutathione, that sulfhydryl groups are essential for enzymatic activity. Davis and Allen [20] and Dickman and Aroskar,[21] on the other hand, could find no evidence for the presence of sulfhydryl groups in the molecule. This

* At the time this structure was proposed, it was not appreciated that there are probably five and not four proline residues in the molecule.

apparent contradiction may be resolved by the observation [20, 21] that traces of copper inhibit the enzyme strongly. It seems entirely possible that HCN and sulfhydryl compounds could enhance enzymatic activity by sequestering inhibitory traces of copper that might be present in either the enzyme, the substrate, or some of the reagents used in the assay procedure. According to this view, copper could not inhibit enzymatic activity by reacting with sulfhydryl groups of the enzyme, which is the usual assumption advanced to explain inhibition by copper, but must be bound at some other site in the molecule. The
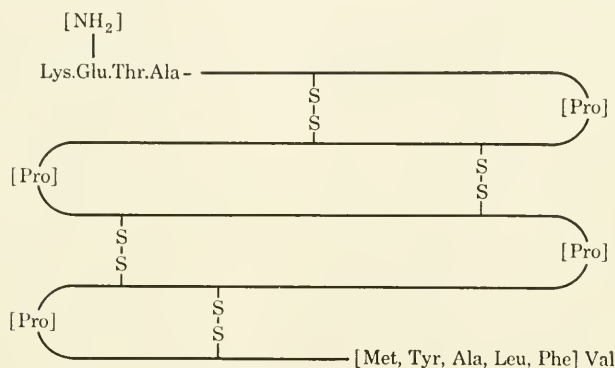
Fig. 1.   Generalized gross structure of ribonuclease (from Anfinsen, Redfield, Choate, Page, and Carroll [18]).

imidazole ring of one or more of the four histidine residues in the enzyme seems a likely binding site, particularly in view of the findings of Weil and Seibles [22] that photooxidation of only one of the histidine residues in ribonuclease leads to a 74% decrease in enzymatic activity. If, in fact, a histidine residue is associated with the active center of the enzyme, it seems possible that the studies now in progress might reveal something of the structure associated with this active center.

If disulfide bridges exist in a protein molecule, it is desirable that they be ruptured before partial degradation is attempted. The peptide chains, if there is more than one, should then be separable, as they were in the case of insulin.* If, as in ribonuclease, only one chain exists, hydrolysis of peptide bonds will not necessarily lead to a reduc-

* The peptide chains of insulin were separable on the basis of solubility. To separate the chains obtained from other proteins, techniques that have been found useful for the separation of large peptides, such as countercurrent distribution, or chromatography on IRC-50, or low-cross-linked polystyrene sulfonic acid resins, may prove advantageous.

tion in molecular weight unless the disulfide cross links have first been cleaved.[18] Moreover, if hydrolysis by proteolytic enzymes is contemplated, rupture of disulfide bonds is frequently necessary before enzymatic attack can occur. Finally, Ryle and Sanger [23] have shown that a mixture of peptides of the structure R—S—S—R′ and R″—S—S—R‴ can rearrange easily to give R—S—S—R″ + R′—S—S—R‴. It is obviously desirable to rupture disulfide bonds and eliminate this possibility, inasmuch as the determination of the sequence of amino acids in a long peptide chain already presents sufficient problems without introducing rearrangements to complicate the picture still further!

Two general methods for cleaving disulfide bonds exist, the oxidative and the reductive. Sanger employed the performic acid oxidation procedure of Toennies and Homiller [24] in his work with insulin, by which means each mole of cystine yields two moles of cysteic acid. Schram, Moore, and Bigwood [25] and Mueller, Pierce, and du Vigneaud [26] have described conditions under which the conversion is virtually quantitative, and Hirs [27] has found that in ribonuclease methionine is also quantitatively transformed to the sulfone. With the definition by Mueller et al.,[26] Hirs,[27] and Thompson,[28] of conditions under which tyrosine is fully stable, the performic acid oxidation procedure appears admirably suited for use with proteins, such as insulin and ribonuclease, that contain no tryptophan. With the large majority of proteins that contain tryptophan, however, difficulties will be encountered, for Witkop and his co-workers [29] have shown that tryptophan is unstable to oxidation. If a single product, such as formylkynurenine, could be obtained in good yield after oxidation, the instability of tryptophan would make little difference. Formylkynurenine containing peptides would then be formed on partial hydrolysis, and total acid hydrolysis of these peptides would yield kynurenine which should readily be determined chromatographically. It seems likely, however, that oxidation of a tryptophan-containing protein with performic acid, followed by partial hydrolysis, would result in the formation of a number of different fragments derived from each tryptophan residue, thus greatly complicating subsequent structural work.

The difficulty with tryptophan might be circumvented if the disulfide bridges of a protein were cleaved by reduction instead of by oxidation. Sodium in liquid ammonia or lithium borohydride (on the unesterified protein) might be capable of reducing all the disulfide bonds in the molecule without attacking other vulnerable groups (cf. Roberts,[30] for example, and Bailey [31]). It would then, of course, be necessary to cover the newly formed sulfhydryl groups by some reagent

that would not react elsewhere in the molecule. One of the most specific reactants for an —SH group is another —SH group in the presence of oxygen. Whether protein—SH groups formed by reduction could be induced to react completely in the presence of oxygen with an excess of another SH compound such as cysteine or thioglycolic acid remains to be determined. Under any circumstances, the question of how to deal with disulfide bridges * in proteins containing trypto- phan may prove to be one of the crucial bottlenecks in future work with protein structure.

Once a single peptide chain free from cross bridges is obtained, three general approaches to the determination of its structure are avail- able. In one, the sequence of amino acids is derived by hydrolyzing the chain into relatively small fragments employing more or less random hydrolysis with strong acid. The large number of di-, tri-, and tetrapeptides formed must then be separated and the structure of each determined. This is essentially the approach utilized by Sanger, but, despite his notable success, this method has serious draw- backs which he well appreciated.[32] An enormous number of peptides are formed after such treatment, most of them in relatively poor yield. The problems of fractionating and isolating pure peptides from such a mixture are great. Although each individual peptide is of relatively simple structure, this advantage is offset by the large number of peptides requiring study. It is doubtful whether such an investigation could be completed successfully for peptide chains much longer than the thirty amino acids contained in the B chain of insulin. It does not appear to be a generally feasible approach for determining the structure of a large number of complex proteins.

A second possible method of determining the sequence of amino acids in a peptide chain is by stepwise degradation starting at either the carboxyl or amino end. Promising techniques for accomplishing this are available, or on the horizon, and it may well be the approach of choice for small peptides. It seems doubtful, however, whether any stepwise procedure will be suitable for the degradation of long chains of amino acids such as occur in intact proteins. The losses involved in each step seem insupportable. If, for example, 1 gm. of a protein were degraded stepwise and a yield of 75% obtained at each

* The existence of diester phosphate bridges in proteins, and methods for cleav- ing them, have been suggested by the work of Perlmann.[33] Diester sulfate bridges are also a possibility after the finding of Bettelheim [34] that tyrosine-O-sulfate is a constituent of fibrinogen.

step, only 15 mg. of material would remain after fifteen steps. Clearly, more information can be derived from 1 gm. of protein by other means.

The most appealing approach to the determination of the sequence of amino acids in a peptide chain consists of specific hydrolysis of the chain at selected linkages, separation of the relatively few fragments formed, followed by further specific cleavage of the larger fragments when necessary, and direct structural analysis of the smaller fragments. Although specific chemical methods would be ideal, at the present time proteolytic enzymes appear to offer the greatest promise as analytical reagents for the selective hydrolysis of peptide chains.* Some, such as trypsin, have an extremely sharp specificity. All operate under mild conditions of pH and temperature, so that after hydrolysis, amide linkages are still intact and non-amino acid moieties such as carbohydrates or porphyrins are likely to remain attached to that portion of the peptide chain to which they were originally joined. Sanger employed trypsin, pepsin, chymotrypsin, and papain in his work with insulin. The enzymes were used as ancillary tools, however, to establish sequences of amino acids in the neighborhood of peptide bonds that were particularly labile to acid hydrolysis. More recently, Bell and his associates [35] used trypsin, pepsin, and chymotrypsin as primary hydrolytic agents in their studies with ACTH. Trypsin has been employed by Tuppy and Bodo [36] in work with cytochrome c, by Gorini, Felix, and Fromageot who studied lysozyme,[37] and by Monier and Jutisz [38] who isolated some basic peptides from protamines. When enzymes are employed as primary hydrolytic agents, it is, of course, essential to eliminate the possibility that the products isolated might have been formed as a result of rearrangements catalyzed by the enzymes. Unless this can be done with some assurance relatively early in the investigation, an enormous amount of labor could be wasted proving the structure of artifacts.

In beginning partial degradation studies with ribonuclease, a tryptic hydrolyzate of the oxidized protein was chosen for the first investigations.[27, 39] From the classic specificity studies of Bergmann, Fruton, and Hofmann, taken in conjunction with the analytical results shown in Table 1 and the data of Anfinsen et al. on the N- and C-terminal amino acids, it would be anticipated that fourteen peptides should be found in a tryptic hydrolyzate. Nine of them should terminate in lysine, four in arginine, and one, corresponding to the C-terminal fragment, should be devoid of basic amino acids. Free lysine, from the

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* The possibility of using proteolytic enzymes in this way was clearly envisaged by Max Bergmann and stated in a Harvey Lecture delivered by him in 1935.

N-terminal position, might or might not be present, because little is known as to whether trypsin can hydrolyze bonds of this type.

The rate at which oxidized ribonuclease is split by trypsin is shown in Fig. 2. Amino acid analyses of the oxidized preparation employed showed that cystine had been converted quantitatively to cysteic acid and methionine to the sulfone, but that all the other amino acids were untouched. Moreover, end-group analysis by the DNP technique
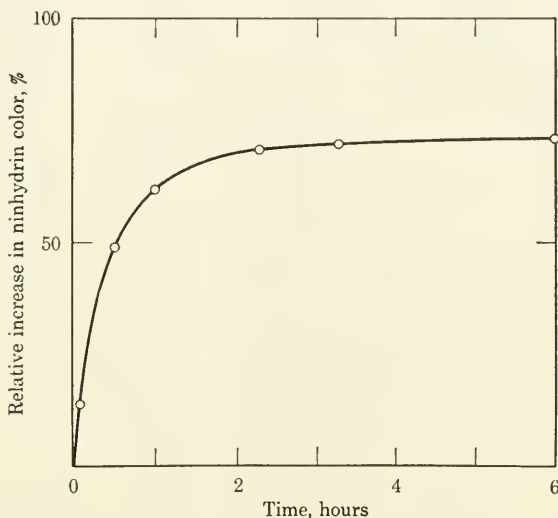
Fig. 2. Hydrolysis of oxidized ribonuclease by trypsin at pH 7.0 and 25°; ribonuclease concentration, 0.5%; trypsin concentration, 0.0025%.

revealed that the peptide chain had not been cleaved to any significant degree during the oxidation. On the basis of the curve shown in Fig. 2, it was decided to investigate the peptides present after both 3 and 20 hours of tryptic action. For this purpose, the hydrolyzate was fractionated on a column (150 × 2 cm.) of Dowex 50-X2 with the results shown in Fig. 3. The experiment was performed with about 200 mg. of ribonuclease so as to yield a sufficient quantity of each peptide for further structural investigation. As a first step, the quantitative amino acid composition of each peptide was determined after acid hydrolysis by chromatography on columns of Dowex 50-X4.[40] The buffer salts from the eluent used in the Dowex 50-X2 column usually did not interfere in this process and were not removed. The amino acid composition of each peptide obtained in pure form in the initial fractionation is shown on the curve in Fig. 3. The analyses
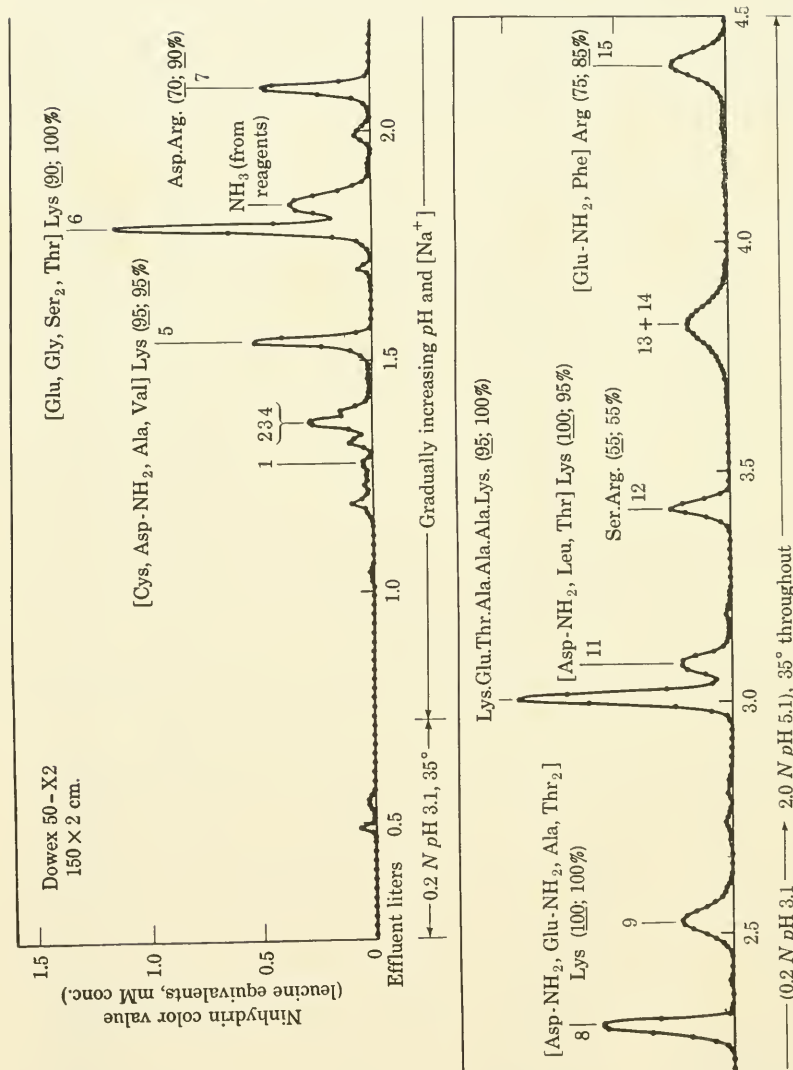
Fig. 3. Chromatography of 20-hour tryptic hydrolyzate of 200 mg. of oxidized ribonuclease (for conditions of hydrolysis, cf. Fig. 1). The figures in parentheses represent the yield of each peptide after 3 and 20 hours of hydrolysis.[7]

gave whole numbers for each of the amino acids in the peptides with an accuracy of ±5 to 10%. The numbers in parenthesis give the yield of each peptide obtained after 3 and 20 hours of hydrolysis, on the assumption that each one of these fragments occurs only once in the molecule.* On the basis of the specificity of trypsin, the basic amino acid, lysine or arginine, is assigned to the C-terminal position. Eventually it would be well to check this point experimentally, but for the present this assignment seems reasonable. It will be noted that peptide 10 contains two lysine residues. Inasmuch as DNP end-group analyses placed one lysine at the amino-terminal position, it seems extremely probable that this peptide represents the amino-terminal sequence. The first four amino acids of this sequence were determined by Anfinsen et al. to be Lys.Glu.Thr.Ala-, so that the finding of two more alanine residues and a lysine makes it possible to extend this sequence to seven amino acids without further structural work.

The mixture of peptides labeled 2, 3, 4, occurring at the beginning of the chromatogram, and those at 9 and at 13 + 14 were rechromatographed on Dowex 50-X2 under slightly different conditions to give the results shown in Fig. 4. Three peptides were separated from the front part of the original chromatogram (a 20-hour tryptic hydrolyzate), two containing lysine and one arginine. Both lysine peptides had the same 21 amino acid residues and probably differed only in their amide content. The fact that the faster moving of the two lysine peptides, the center peak in the top curve of Fig. 4, was present to only a very small extent after 3 hours of tryptic digestion is compatible with this supposition. The arginine peptide is also very large, containing nineteen amino acid residues. It is unstable, however, the yield decreasing from 55% in the 3-hour hydrolyzate to 15% in the 20-hour hydrolyzate. The reason for this instability is not certain at the moment, but possibly this fragment contains some linkage that is extremely susceptible to the action of chymotrypsin, a trace of which cannot be ruled out as a contaminant of the trypsin.

Peptide 9 is very large and contains two lysine residues. The presence of proline may explain why one of the lysyl bonds is not split by trypsin to yield two peptides. If the sequence -Lys.Pro- existed, the lysylprolyl bond might be very resistant to tryptic hydrolysis. In

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* The figures with rules under them were computed from the actual amino acid analyses of the peptides. The other yields for different times of hydrolysis were computed by comparing the area of the peak with the area of the corresponding peak for which the amino acid composition was known.
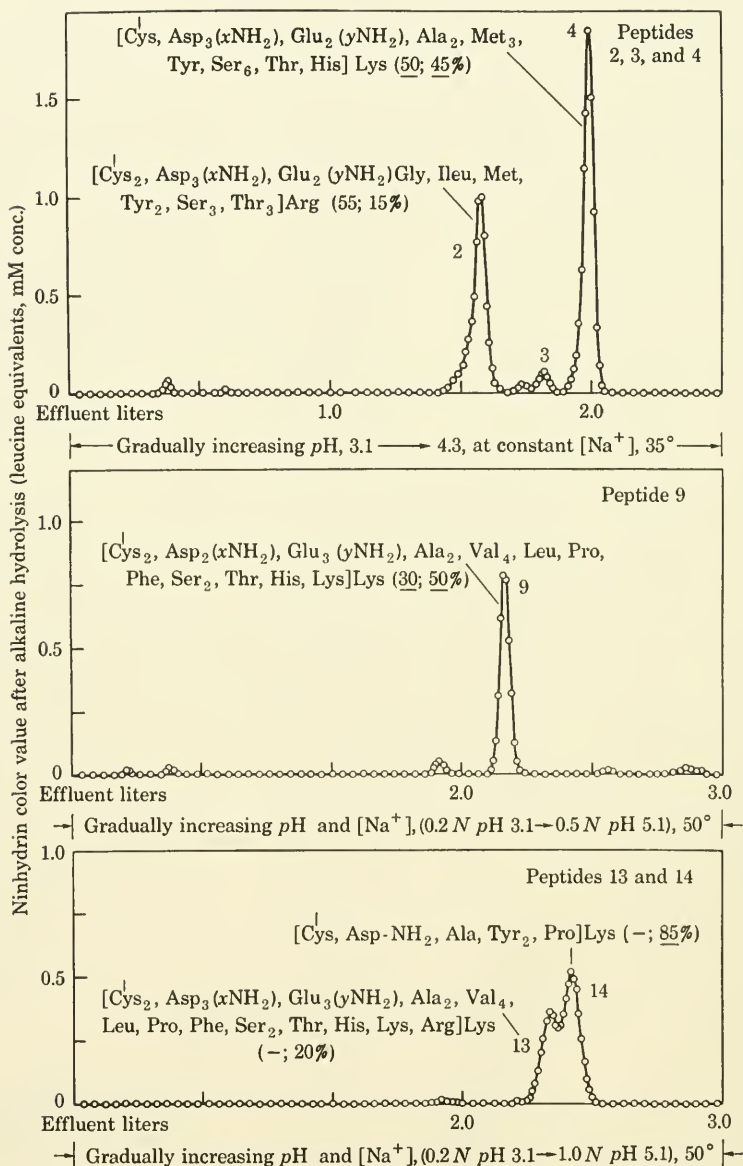
FIG. 4. Rechromatography of peptides from a tryptic hydrolyzate of oxidized ribonuclease on a 150 × 2 cm. column of Dowex 50-X2. The peptides fractionated are taken from the overlapping peaks shown in Fig. 3, except that peptides 2, 3, and 4 were obtained from a 3-hour tryptic hydrolyzate.

ACTH, for example, a similar bond was found by Bell [35] not to be hydrolyzed by trypsin.

Rechromatography of the next to last peak in Fig. 3 gave the two peptides shown at the bottom of Fig. 4. One is a heptapeptide containing lysine found in good yield. The other, however, contains twenty-four amino acid residues including two lysine and one arginine residue and was obtained in low yield. Since all the lysine and arginine residues in the molecule have already been accounted for, this peptide must represent a combination of two other peptides. From its composition, it could be formulated as aspartylarginine (peptide 7) added to peptide 9. Whether this peptide is an intermediate degradative product or a rearrangement product cannot be determined for certain at present, but, since the yield of aspartylarginine increases with time, the former possibility seems more likely.

The peptides characterized thus far account for 105 of the 126 amino acid residues in ribonuclease. Missing are four residues of valine, three of proline and aspartic acid, two of alanine, isoleucine and histidine, and one of glutamic acid, glycine, half-cystine, phenyl-alanine, and tyrosine. Also missing is a peptide devoid of basic amino acids that should arise from the carboxyl-terminal end of ribonuclease. According to Anfinsen et al.,[18] the last six residues in the molecule are -met, tyr, ala, leu or ileu, phe, and val. It has not been possible thus far to isolate such a carboxyl-terminal fragment from the Dowex 50-X2 columns. It may be that the "end piece" of ribonuclease is particularly susceptible to the action of traces of chymotrypsin, which, as has been noted above, may contaminate the trypsin. It is tempting to assume that all the amino acids listed above as not accounted for in the basic peptides should be assigned to the carboxyl-terminal end piece, but only future work can decide the validity of this assumption.* The balance sheet does prove, however, that isoleucine and not leucine is present in the end fragment, because both leucine residues have been found in peptides shown in Figs. 3 and 4. The existence of a residue of methionine near the carboxyl end cannot be reconciled with the analyses of the peptides reported above, because all four of the methio-nines supposed to exist in the molecule have been accounted for. This discrepancy will also have to be resolved by future work.

Although the work on tryptic digests of ribonuclease is still far from elucidating the complete structure of the molecule, it has demonstrated

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*A large peptide devoid of basic amino acids and believed to represent the carboxyl-terminal segment has recently been isolated. Its composition is discussed in ref. 39.

several things in quite a clear-cut fashion. In the first place, it seems justifiable to conclude that under the proper circumstances, proteolytic enzymes will prove to be remarkably general and useful tools for determining the structure of proteins. The principal theoretical draw-back to their use, namely, that they catalyze rearrangements, seems not to be operative, at least in the case of trypsin.* It is difficult to conceive that the group of peptides shown in Figs. 3 and 4 could be artifacts. Practically all of them were obtained in yields of from 50 to 90% of theory after only 3 hours of tryptic digestion. It hardly seems credible that rearrangements could proceed so rapidly and so completely in homogeneous solution. The absence in most of the peptides of more than one residue of a basic amino acid also argues against transpeptidation. Of course it cannot be assumed that, because transpeptidation does not occur to a significant extent when trypsin acts on ribonuclease, it will never occur. By the use of quantitative procedures, however, it should be possible to insure against undetected rearrangements. When the peptides can be separated quantitatively and their amino acid composition determined quantitatively, there is far less likelihood of being misled. In the final analysis, proof for the validity of the results obtained with trypsin can only emerge from an examination of the products formed by the action of other enzymes. Such studies with ribonuclease are now under way. It can be said already that chymotrypsin splits oxidized ribonuclease into twenty to thirty fragments that can be separated on the Dowex 50-X2 columns. About fifteen of these fragments are found in major quantities. Pepsin also attacks oxidized ribonuclease but with the formation of more peptides in poorer yield.†

The work with tryptic digests has also demonstrated quite clearly that columns of Dowex 50-X2 provide extremely effective means for separating mixtures of peptides. The resolving power is great, and even peptides containing twenty or more amino acid residues can be handled without difficulty. Moreover, such columns possess the great advantage that they can be scaled up readily to a size sufficient to permit the isolation of enough of a peptide for subsequent structural studies. In the present work, the columns have been operated in the sodium form. Doubtless quite similar separations could be achieved with the ammonium form of the resin and NH_4OAc or formate as

* The subject of the synthesis of peptide bonds by proteolytic enzymes has been elegantly summarized by J. S. Fruton in a Harvey Lecture given November 17, 1955.
† J. L. Bailey.

eluents, both of which can be removed by volatilization. Alternatively, eluents containing sodium can be desalted by passage over a small bed of NH_4-Dowex 50, whereby Na is exchanged for the volatile ammonium.

Once the amino acid composition of the various peptides obtained from peptic and chymotryptic digests of ribonuclease has been determined and correlated with the data obtained by the use of trypsin, it should be possible to learn a great deal about the way the molecule is constructed even before sequence studies on the individual peptides have been performed.* Moreover, from the array of data thus presented, it should be possible with some assurance to choose for detailed investigation those and only those peptides the amino acid sequence of which will contribute the most to an understanding of the final structure of the protein. It should also be possible to choose those peptides the size and composition of which are most suitable for study by existing techniques. In this manner the number of sequence determinations required will be reduced to a minimum. There is not space to go into the question of the actual determination of the sequence of amino acids in peptides. If the peptides chosen for such an investigation contain from four to ten amino acid residues, there is little doubt that a sequence for each can be determined by one or a combination of several existing methods.

Structural analysis would be simplified greatly if there were available more proteolytic enzymes possessing a specificity as sharp as that of trypsin, only directed towards other linkages, such as those involving proline, or histidine, or the aromatic amino acids. Possibly the enzymologist will be able to come to the aid of the analyst and provide the necessary tools. If, however, enzymes of the requisite specificity cannot be found in nature, possibly they can be created artificially. Microorganisms seem to be able to do almost anything, given sufficient provocation. Perhaps with the aid of synthetic substrates they can be induced to synthesize enzymes capable of hydrolyzing the particular types of peptide linkages in which the protein chemist is interested.

It should be emphasized that there is nothing extremely novel in

* The separation and analysis of the peptides formed by the action of chymotrypsin has recently been completed by Dr. Hirs. On the basis of his data, it has been possible to construct, as a working hypothesis, a tentative, partial structural formula showing how the peptides present in the tryptic and chymotryptic hydrolyzates might have originally been joined together in the molecule of oxidized ribonuclease [Hirs, Stein, and Moore, *J. Biol. Chem.*, in press]. The expectations expressed in this paragraph have, therefore, been realized.

the approach to determining the structure of a protein summarized in this essay. Enzymatic hydrolysis with several different enzymes, followed by fractionation of the peptides thus produced and amino acid analysis of each before ultimate sequence determinations on a selected few, is the procedure used in essence by Bell and his associates in their studies on ACTH. It is also a simple and logical development of the approach the organic chemist has employed for years in the structural analysis of simpler compounds. The general use of quantitative techniques will, it is felt, simplify the task with proteins and make for less uncertainty in the final result. Obviously, a large number of routine quantitative amino acid analyses are required, partly as a substitute for, and always as a prelude to, detailed structural work. To render this approach truly feasible, therefore, these amino acid analyses must be made extremely simple and quick. It seems safe to say that none of the existing procedures completely fills this need. There are two obvious ways of improving this situation. One is to render paper chromatography truly quantitative and more fully mechanized. The other is to render current column methods faster and more automatic, a subject under continued study in our laboratory.

The amount of information now available on the structure of proteins is so limited that it is too early to expect any ironclad conclusions to emerge. Nevertheless, a few suggestive facts are worth noting. There seems to be some tendency for like types of amino acids to cluster together along the peptide chain. For example, in insulin we find the three sequences, -Glu.Glu-NH$_2$-, -Glu-NH$_2$.Leu.Glu.Asp-NH$_2$-, and -Asp-NH$_2$.Glu-NH$_2$-, thus placing acidic amino acids and their amides near one another. In another part of the insulin molecule we find, in the A chain, three out of six half-cystine residues in the protein in the single sequence -Cys.Cys.Ala.Ser.Val.Cys-. There is also a cluster of three aromatic amino acids together in the sequence -Phe.Phe.Tyr-, and finally, the only two strongly basic amino acid residues in the molecule are tucked off at one end of the B chain separated by only seven amino acids. This clustering of like R groups is even more obvious in the ACTH molecule. There is a sequence of fourteen amino acid residues near the N-terminal sequence, of which seven are either lysine or arginine. At one point the sequence -Lys.Lys.Arg.Arg- occurs. At the opposite end of the chain there is a cluster of five acidic amino acid residues out of a sequence of eight. Although the information on ribonuclease is still meager, it is already apparent that the basic amino acids also tend to cluster in this molecule. Of the four arginine residues, two are separated from another basic

amino acid (either lysine or arginine) by two amino acid residues and one arginine is separated by three. Of the ten lysine residues, seven are separated from another basic amino acid by six amino acid residues or less. In another part of the molecule we find three alanine residues in a row, and in another peptide seven out of twenty-one amino acid residues are serine. Recently, Davie and Neurath [41] have isolated the hexapeptide that is split off when trypsinogen is transformed to trypsin and proved it to have four aspartic acid residues joined together in the structure Val.Asp$_4$.Lys. If, in fact, this apparent tendency of amino acids of like structure to group together turns out to be a general occurrence, it means that in the peptide chain there will be found areas in which the positive charge density is high, areas in which the negative charge density is high, and areas in which van der Waals forces predominate. There is suggestive evidence based on studies of enzyme specificity and of the manner in which protein molecules interact with one another that is compatible with this idea. How these areas are disposed on the surface of the protein molecule will, of course, be a function of the manner in which the peptide chain is folded. At this stage of development, speculation is tempting, but the real need is many more facts. It appears that at last the protein chemist is about to possess tools adequate to the difficult task of accumulating these facts.

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Glycogen Turnover

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Since the time of Claude Bernard it has been appreciated by bio-chemists that glycogen represents an animal storage form of glucose. Glycogen is well suited to this storage function for several reasons. Its high molecular weight and low intrinsic viscosity permit its accumu-lation in tissue fluids with but slight effect upon the osmotic pressure or the fluidity. The readily reversible phosphorylase reaction permits both the synthesis of glycogen from hexose phosphate and glycogen breakdown to glucose-1-phosphate to proceed with very small energy transfers. The breakdown of liver glycogen, under hormonal regulation by epinephrine and probably also by glucagon, serves as an elegant homeostatic device to sustain the blood glucose concentration under a wide variety of circumstances.

The storage of glycogen in liver or in muscle is not a dead storage. It is, on the contrary, very much alive in that the constituent atoms of the glycogen molecule, even in the animal maintained in a balanced nutritional state, are continuously being regenerated de novo at the expense of a variety of precursors. Since, under such controlled experi-mental conditions, the quantity of glycogen in a given tissue remains approximately constant, coincident with new glycogen synthesis the occurrence of continuous glycogen breakdown must be postulated.

The fact that, in the animal in nutritional balance, glycogen of liver and muscle is in a dynamic steady state, undergoing continuous "turn-over," was first observed in the study of the appearance of carbon-bound deuterium in glycogen isolated from animals whose body fluids were enriched with deuterium oxide.[1] In these experiments glycogen was isolated from tissues of rats maintained for varying periods of time with their body water enriched with D_2O, and a progressive enrichment of glycogen with stably bound deuterium was noted. When glucose

was abundantly present in the diet, the deuterium concentration in glycogen approached asymptotically a maximal value of about 30% of that in the body water. However, when lactate served as the source of glycogen carbon, glycogen containing about 60% as high a concentration of deuterium as body water was recovered,[2] and this value approximated the fraction of all hydrogen in glycogen which is carbon-bound.

From these results it could be concluded that glycogen was undergoing turnover at the expense of available glucose. This glucose was isotopically labeled insofar as it was synthesized in the D_2O-enriched medium, but was subject to dilution prior to glycogenesis by such glucose as might be derived from the diet.

In order to translate the results of these experiments into meaningful quantities, certain assumptions were made and equations were derived designed to relate the change in isotope abundance as a function of time to the turnover rate of glycogen. It could be shown that, if:

i = isotope concentration in glycogen at time t

i_{max} = maximal isotope concentration, achieved at $t = \infty$

then:

$$k = \frac{1}{t} \ln \frac{i_{max}}{i_{max} - i}$$

where k is the turnover rate constant, the fraction of the total pool replaced per unit time. The turnover rate, as weight of glycogen replaced per unit time, could then be evaluated as the product of k and the total weight of glycogen in the tissue. By this method, estimates of a half-life of about 1 day for liver glycogen and 4 days for muscle glycogen of rats were made.

The several assumptions upon which this treatment rests include the following:

1. That the pool size remains constant over the period of observation; that the rates of entry into and departure from the pool be equal.

2. That these rates in turn be constant.

3. That each new molecule of glycogen entering the pool be enriched with deuterium at the same concentration, namely, i_{max}.

4. That perfect mixing take place; that the deuterium concentration of each molecule of glycogen leaving the pool be the same as the average deuterium concentration of the pool at that time, namely, i.

The first three of these assumptions appear to us to be entirely plausible. The last assumption, however, is much more troublesome.

It implies perfect mixing of newly introduced with preformed molecules; it requires complete randomization of isotopic and non-isotopic molecules and random selection of material from this pool for destruction. For want of a method of approach, this fourth assumption was not explored at that time. Our more recent studies have led to a realization that this assumption is incorrect.

Since our more recent exploration of this assumption has depended upon the newer knowledge of glycogen structure and of the several enzymes related to glycogen breakdown, it may be well to digress sufficiently to review the pertinent aspects of these developments. It is generally agreed that glycogen is a branched polysaccharide in which the major glucosidic linkage is α-1,4′ and the minor linkage is α-1,6′. The ratio of 1,4′ to 1,6′ linkages in typical glycogen samples range from 10 to 18.[3] Of the three possible branching patterns that have come into serious consideration the arboreal pattern first advocated for amylopectin by Meyer [4] is almost certainly the dominant one. It is in the nature of an arboreally branched polyglucoside containing n glucose residues that there must be, per molecule, one free reducing end and $n - 1$ glucosidic bonds. In glycogen the number of branch points should equal the number of α-1,6′ glucosidic bonds and will be one less than the number of non-reducing ends. Discrepancies between experimentally secured values and the above expectations have resulted in the suggestion that the structure of glycogen may not be of a simple arboreal nature,[5] but, for the purposes of the present discussion, the arboreal structure will be taken for granted.

The experimental basis for the above assignment of structure rests largely upon the examination of products secured when glycogen is subjected to various enzymic degradations. When exhaustively treated with β-amylase, glycogen was found by Meyer to yield maltose and a limit dextrin which represented approximately 50% of the glucose residues initially present. This dextrin was totally refractory to further attack by β-amylase.[6] In Cori's laboratory this product was further studied, and, in addition, a limit dextrin was secured from glycogen by the action of purified phosphorylase and inorganic orthophosphate.[7] This latter limit dextrin, LD (glycogen, phosphorylase), insensitive to further attack by phosphorylase, could be rendered phosphorylase-sensitive again by the action of a specific amylo-1,6-glucosidase, which yielded glucose by the selective hydrolytic cleavage of α-1,6′ glucoside bonds of exposed glucose residues.[8] The exposure of glycogen to *simultaneous* attack by phosphorylase and by amylo-1,6-glucosidase yielded

a mixture of glucose-1-phosphate and glucose as the sole products, free glucose presumably arising uniquely from the hydrolysis of α-1,6′ bonds. The ratio of these products was therefore taken as equal to the ratio of 1,4′ to 1,6′ links and the abundance of free glucose as a measure of the number of branch points.[9] By the *alternating* attack
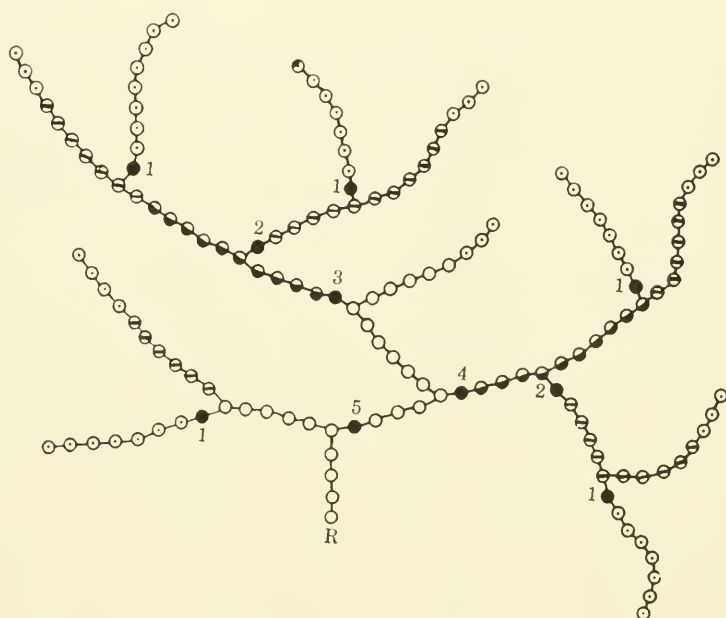
R, reducing end of molecule.
⊙, ⊖, and ◓, glucose residues removed by first, second, and third degradation with phosphorylase, respectively.
●, glucose residues split off as free glucose by amylo-1,6-glucosidase.

FIG. 1. The structure of glycogen (from J. Larner, B. Illingworth, G. T. Cori, and C. F. Cori, *J. Biol. Chem., 199*, 641, 1952).

upon glycogen by phosphorylase and amyloglucosidase, a series of limit dextrins could be secured differing one from another by the successive elimination of tiers of glucosidic residues. Regardless of whether glycogen or a susceptible limit detrin thereof served as precursor, the product secured after each treatment with phosphorylase represented a loss of 35–45% of the glucosidic residues initially present.[10] A schematic representation of what was happening is shown in Fig. 1, from which it will be seen that the alternating exposure to phosphorylase and amyloglucosidase has resulted in the peeling of the molecule, removal of successive peripheral layers, much as an onion might be peeled.

In view of the lack of information concerning the metabolic homo-
geneity of glycogen in tissues of the intact animal, it was considered
of interest to re-explore the intimate nature of glycogen regeneration.
Is glycogen turnover a process in which a molecule is broken down
completely to be replaced by a newly formed one, or does it involve
removal and replacement of a fraction of the glucosyl residues within
a given glycogen molecule? The experiments designed to this end have
taken advantage both of the newer knowledge of glycogen structure
and of the specific enzyme activities discussed above. Glycogen ex-
hibits a certain structural homogeneity, not shared by many of the
mammalian macromolecular substances, in that on total hydrolysis
all of its carbon is recoverable exclusively in glucose. The question
of whether glycogen constituted, metabolically, a perfectly mixed pool
of glucose residues wherein, after isotopic enrichment by one or an-
other device, glucose residues, regardless of their location in the mole-
cule, had an equal probability of being labeled could now be studied.

The basic experimental design has been simple. Into normal rats
and rabbits glucose uniformly labeled with C^{14} has been injected. After
various intervals of time, animals were sacrificed and glycogen was
isolated from livers and carcasses. After purification of these products,
their radioactivities were determined. Samples were then subjected to
one or more enzyme digestions, and the radioactivity of the products
of such degradations were examined.[11]

Enzyme digestions, in the earlier experiments, were limited to treat-
ment with barley malt β-amylase. The commercially available prep-
aration of this enzyme, although far from pure, is demonstrably free
of α-amylase, amylo-1,6-glucosidase, and maltase activity. Glycogen
subjected to exhaustive treatment with β-amylase gives, in excellent
yields, maltose and a limit dextrin, LD (glycogen, β-amylase), which
resists all further attack by this enzyme. In effect this enzyme bisects
the glycogen molecule into two approximately equal portions, the
peripheral cortex of unbranched termini, recoverable as maltose, and
the central medullary portion, recoverable as the limit dextrin.

When the concentrations of C^{14} in these two portions of a given
sample of glycogen were compared, they were found, in general, to be
unequal. Samples of glycogen were studied, which were obtained from
rat livers and rat carcasses, from 3 to 48 hours after intraperitoneal
injection of glucose-C^{14}. Results are given in Table 1. In the earlier
time intervals, the specific activity was in all cases higher in the maltose
(periphery) than in the LD (glycogen, β-amylase). Indeed, despite
a relative rise in the specific activity of maltose and a relative fall in

Table 1. Distribution of Radioactivity in Rat Liver and Carcass Glycogen after Intraperitoneal Injection of Glucose-C^{14}

Relative Specific Activity *

Time, hours	In Liver Glycogen		In Carcass Glycogen	
	Periphery	Limit Dextrin †	Periphery	Limit Dextrin †
3	178	36	127	56
6	146	51	129	56
6	—	—	156	56
6	—	—	148	59
6	129	74	157	56
6	134	71	150	61
12	92	101	137	65
24	84	105	133	72
24	79	122	124	81
48	64	107	117	89

* Expressed as per cent of specific activity of glycogen.
† The limit dextrin here referred to is LD (glycogen, β-amylase).

that of the limit dextrin, even 48 hours after the injection of glucose-C^{14} the periphery of the carcass glycogen molecule was more radioactive than was the center. Liver glycogen was somewhat different in this regard in that by about the twelfth hour the radioactivity was found to be approximately uniformly distributed between maltose and LD (glycogen, β-amylase), whereas in the experiments of longer duration the central core of the molecule (limit dextrin) was consistently more radioactive than the peripheral (maltose) tier. The distribution of isotope between the periphery and limit dextrin is plotted in Fig. 2 as a function of time after glucose-C^{14} injection.

In the interpretation of these results it must be borne in mind that the glucose-C^{14} was all injected at the outset of the experiment and that therefore the specific activity of circulating glucose was constantly declining throughout the period of observation. On the basis of results of Feller et al.[12] it may be estimated that the rate of this decline was such as to give a half-time of about 1 hour. Undoubtedly by the twelfth hour the specific radioactivity of circulating glucose available for glycogen synthesis was lower than the mean specific activity of the glycogen into which it was being incorporated. Whereas the earlier data reflect the incorporation of glucose-C^{14} into glycogen, the later data are a consequence of introduction of glucose-C^{12} into glycogen-C^{14}. The data strongly suggest that, whereas circulating glucose serves as a fairly immediate precursor of the glucosidic residues situated periph-
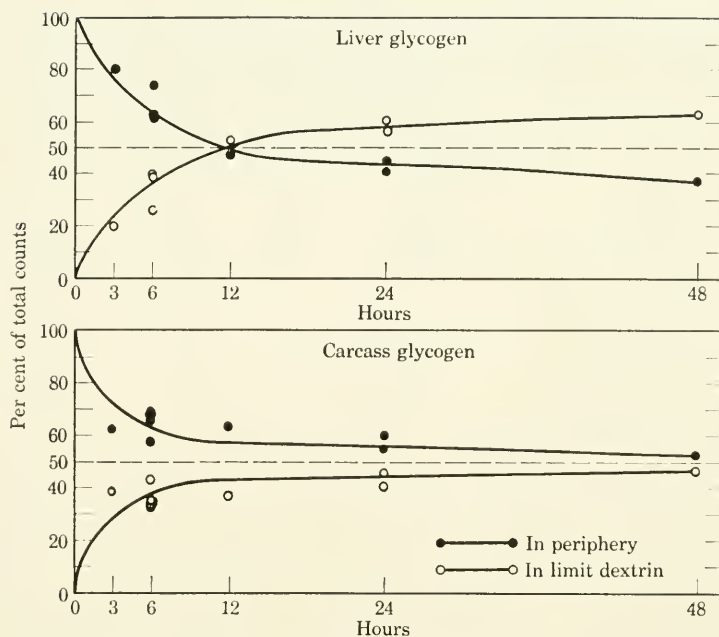
Fig. 2. Distribution of radioactivity in liver and carcass glycogen between periphery and limit dextrin. The total counts per minute in the maltose and in the limit dextrin (glycogen, β-amylase) have been determined in each sample of glycogen after exhaustive treatment with β-amylase.

erally in the glycogen molecule, it is these glucosidic residues rather than circulating glucose which give rise to the glucosidic residues situated in the central core of the molecule. This relationship between precursors and products may be expressed by the sequence:

Circulating glucose $\rightarrow$ Glucosidic residues at periphery of glycogen $\rightarrow$ Glucosidic residues at center of glycogen

It is interesting to compare the distribution of radioactivity within the molecule of liver glycogen derived from the fasted and from the fed rat. The experiments recorded in Table 2 represent such a comparison. One rat was fasted for 24 hours prior to glucose-C^{14} injection; another had continuous access to diet. Three hours after injection each rat was killed, and the distribution of isotope within glycogen was studied. The striking finding was that, whereas in the liver from the fed rat the major portion of the label was in the periphery (maltose) of the glycogen, the glycogen recovered from the liver of the fasted

**Table 2.   Distribution of Radioactivity in Glycogen from Fasted and
Fed Rats Killed 3 Hours after Administration of Glucose-C^{14}**

	Liver Glycogen		Carcass Glycogen	
	Fasted Rat	Fed Rat	Fasted Rat	Fed Rat
Specific activity: *				
Glycogen	33,500	366	3870	369
Periphery	36,600	651	5190	469
Limit Dextrin	31,600	131	2580	206
Per cent of total c.p.m. in:				
Periphery	49.2	80.2	58.8	61.8
Limit dextrin	50.8	19.8	41.2	38.2

* Specific activity reported as c.p.m. per milliatom C.

rat appeared to be labeled nearly uniformly.   This equality of labeling
of the maltose and limit dextrin shows that, in the liver glycogen
recovered when glucose is given after a suitable period of fasting,
virtually the entire molecule, center as well as periphery, has arisen
from blood glucose.   This is clearly a consequence of the depletion
of hepatic glycogen which results from fasting.   The specific activity
of carcass glycogen was far higher when derived from the fasted than
from the fed rat, probably because of less dilution of injected glu-
cose-C^{14} by non-isotopic glucose in the fasted rat.   Nevertheless, it is
noteworthy that the distribution of isotope within this glycogen be-
tween maltose and LD (glycogen, β-amylase) was not greatly different
from that in the corresponding sample from the fed animal.   This latter
finding reflects the well-established observation that muscle glycogen,
in contrast to liver glycogen, is not depleted by fasting.

It would appear, from these results that glycogen regeneration in
tissues involves primarily the addition of glucoside residues derived
from blood glucose to non-reducing ends of pre-existing polysaccharide
molecules.   The reactions whereby this occurs probably include those
catalyzed by hexokinase, phosphoglucomutase, and phosphorylase.
These reactions alone would account for the appearance, after glu-
cose-C^{14} administration, of radioactivity in the maltose liberated from
glycogen by the action of β-amylase.   The introduction of isotope
into the limit dextrin (glycogen, β-amylase), although a slower process,
takes place both in liver and in muscle and indicates the occurrence
of another distinct process involving the establishment of new points
of branching in the glycogen molecule.

To explore this process more fully, it was deemed advisable not merely to bisect the glycogen molecule by treatment with β-amylase but to dissect it, layer by layer, by the alternating exposure of glycogen to phosphorylase and amylo-1,6-glucosidase (debranching enzyme). To this end the methods devised in Cori's laboratory [3, 8, 10] were reproduced and applied to glycogen samples which were obtained from animals after injection of glucose-C^{14}.[13]

A preliminary experiment was conducted upon a sample of glycogen secured from rabbit muscle. This animal was killed 6 hours after intravenous injection of glucose-C^{14}, and glycogen was isolated from muscle and analyzed for C^{14}. Treatment of this polysaccharide with crystalline rabbit-muscle phosphorylase in a phosphate buffer yielded glucose-1-phosphate and a limit dextrin, LD-1-(glycogen, phosphorylase). This product was assayed for radioactivity and was then treated with a crude rabbit-muscle amylo-1,6-glucosidase solution in the absence of inorganic phosphate. The enzyme was destroyed, and phosphorylase and inorganic phosphate were added, to yield, ultimately, a second limit dextrin, LD-2. Repetition of this procedure yielded a third product, LD-3. The extent of each digestion was measured by the sum of the glucose-6-phosphate plus glucose liberated at each step. Radioactivity measurements were performed upon each polysaccharide and, where feasible, also upon phenylglucosazone samples prepared from the products of each successive degradation step. Excellent recoveries of isotope were secured from these combined analyses, so that in later studies only the polysaccharides were analyzed.

The results secured are given in Table 3. Included also are the results of a single-step β-amylase degradation performed upon the

Table 3.   Distribution of Radioactivity in Rabbit-Muscle Glycogen 6 Hours after Intravenous Injection of Glucose-C^{14}

Sample	Relative Specific Activity	Per Cent of Glycogen Digested, Cumulative
Glycogen	100	0
LD-1 (phosphorylase)	69.2	37.9
LD-2	47.3	63.3
LD-3	29.9	77.7
LD (β-amylase)	61.3	44.0

same glycogen sample. From the values in this table, the specific activities of the glucose residues eliminated by each degradation step

have been evaluated and the results have been plotted in Fig. 3. The ordinates, in this and succeeding figures, represent counts per minute per milliatom carbon, corrected to 100 c.p.m. per milliatom C in the initial glycogen sample. The abscissas represent the percentages of glucosyl residues, initially present in glycogen, which have been eliminated by the successive digestions. With reference to the structure of glycogen (Fig. 1), R is the sole reducing end of the molecule which is approached from the non-reducing ends by the repeated enzyme
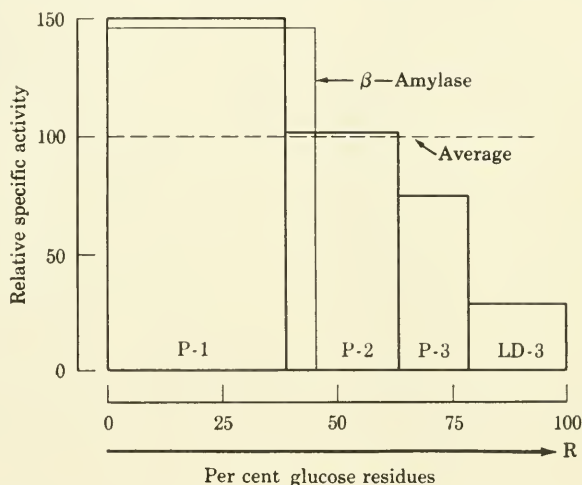
Fig. 3. Distribution of radioactivity in rabbit-muscle glycogen 6 hours after injection of glucose-C^{14}.

treatments. The area in each block represents the total c.p.m. contained in that segment of the molecule.

From the data in Table 3 it will be seen that each successive limit dextrin obtained was less radioactive than the parent substance from which it was secured. This finding confirms the metabolic inhomogeneity of glycogen noted previously. From the schematic representation (Fig. 4) of the tierwise degradation of a glycogen molecule, together with the data plotted in Fig. 3, a clearer picture of the distribution of isotope in this sample of glycogen is obtained. Here it will at once be seen that the outer layer of the glycogen molecule (P-1) is more radioactive and the central core (LD-3) is less radioactive than the average of all the glucoside residues in the molecule. As one progresses from the non-reducing ends of this 6-hour glycogen sample centrally toward the reducing end, R, the enrichment of isotope diminishes in a fairly smooth fashion.
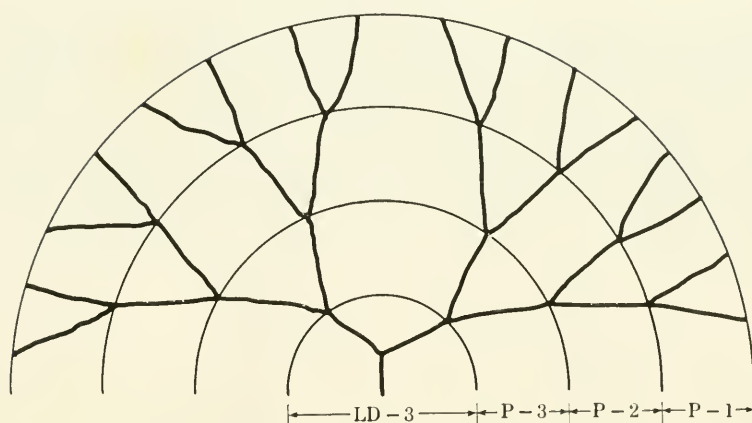
FIG. 4. Schematic representation of the tierwise degradation of a glycogen molecule.

In order to gain insight into the time course of the distribution of isotope in glycogen, studied by this technique, samples of glycogen from rat carcass and liver, which had previously been studied by the β-amylase method, were reinvestigated. These samples were derived from rats which were killed 6, 12, 24, and 48 hours after intraperitoneal injection of glucose-C^{14}. The results of these studies are summarized in Tables 4 and 5.

Table 4. Relative Specific Activity in LD (Phosphorylase) from Rat-Carcass Glycogen

	6 Hours	12 Hours	24 Hours	48 Hours
Glycogen	100	100	100	100
LD-1	74	75	92	91
LD-2	56	54	80	87
LD-3	45	52	73	70

Table 5. Relative Specific Activity in LD (Phosphorylase) from Rat-Liver Glycogen

	6 Hours	12 Hours	24 Hours	48 Hours
Glycogen	100	100	100	100
LD-1	74	104	101	115
LD-2	44	108	103	139
LD-3	23	98	117	149

At each point in time studied, rat-carcass glycogen bore certain similarities to rabbit-muscle glycogen described above. The relative

specific activity of each derivative was lower than that of the parent
substance from which it was derived. In each sample of carcass glyco-
gen (Fig. 5), the outermost tier of glucose residues (P-1) had a higher
specific activity than did the central core (LD-3) and, in each case,
LD-3 was the least radioactive portion of the molecule. With the
passage of time, the difference in specific activity between the periph-
eral and the central portions of the molecule diminished slowly.
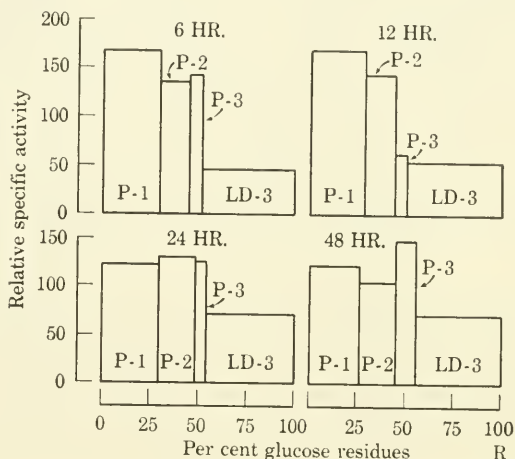
FIG. 5.   Distribution of radioactivity in rat-carcass glycogen.

Rat-liver glycogen, isolated 6 hours after injection of glucose-C^{14},
resembled, in regard to isotope distribution (Table 5), the samples
secured from rat carcass. Again each successive limit dextrin was less
radioactive than its precursor, and the specific activity decreased reg-
ularly as the molecule was entered from the non-reducing termini (Fig.
4). However, with the passage of time, a striking change occurs which
is clearly manifested in the 48-hour sample. Here we find a reversal
of the sequence of specific activities of the serial polysaccharides,
LD-3 > LD-2 > LD-1 > glycogen. This is reflected in the recon-
struction of the glycogen molecule in Fig. 6, where it will be seen that
the outermost tier (P-1) is now the least radioactive and that the
radioactivity systematically increases as the reducing end of the mole-
cule is approached.

From these results it is clear that, in addition to a process which
adds glucose residues to the non-reducing ends of preformed glycogen
molecules, glycogen regeneration entails a second process which results
in the introduction of glucosyl residues into inner tiers of the molecule.

The presumed source of a glucosyl residue in inner tiers is a glucosyl residue in an outer tier, which hypothesis requires that new branch points be continuously established. A mechanism whereby this may happen has recently been elucidated by Larner,[14] who has given the name amylo-(1,4 → 1,6)-transglucosidase to a branching enzyme which generates α-1,6′ glucosidic bonds at the expense of α-1,4′ bonds. He has demonstrated an effect in vitro which places a labeled glucosyl residue, initially in the outermost tier, into an inner tier of the glycogen
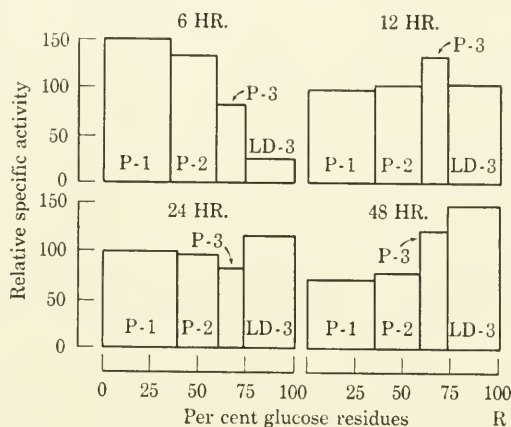
FIG. 6. Distribution of radioactivity in rat-liver glycogen.

molecule. It must be supposed that it is this or some similar mechanism which is responsible for the effect which we have observed, namely, the gradual transfer of labeled glucose residues, initially most abundant at the periphery of glycogen, into more and more centrally situated laminae.

From these studies a picture, or rather a motion picture, is presented of at least a part of the process of glycogen regeneration. It is quite clear that this is not a process wherein a newly synthesized glycogen molecule is created in place of another one which is destroyed. Rather, new glucose residues are introduced into the glycogen reservoir by a process of continuous accretion at the periphery of pre-existing glycogen molecules. By a second process, involving establishment of new branch points, glucose residues in the glycogen molecule become progressively more remote from the outermost tier of glucosyl residues. Since, under most circumstances, including those of the present experi-

ments, the animal is in approximate nutritional balance and analytically in an approximately steady state, the process of glycogen growth must be offset by a process of glycogen decay. The steps involved in this process are presumed to be the phosphorolytic uncoupling of peripherally situated glucosyl residues operating in conjunction with the hydrolytic cleavage of exposed glucosyl residues in α-1,6′ linkage due to the action of the debranching enzyme, amylo-1,6-glucosidase.

It should be noted that the description just given of glycogen turnover may also serve to explain glycogen accrual and depletion. If this proves to be the case, it represents an unusual mode of storage in that no change in the number of glycogen molecules is postulated. If glycogen accrual results merely from the action of phosphorylase and branching enzyme, if depletion is the result simply of phosphorylase and debranching enzyme activity, then changes in glycogen content, in contrast to alterations in fat or protein content, are reflections of changes in mean molecular weight of glycogen, the number of molecules remaining constant. This would be a happy arrangement in that it would permit large fluctuations in the magnitude of the glycogen reserve to occur with no accompanying changes in the colligative properties, notably osmotic pressure, of tissue fluids.

Regardless of whether the count of glycogen molecules in a given adult tissue is or is not constant, it is clear that during growth the number of glycogen molecules must at some stage increase. Within the limits of our present understanding of the actions of phosphorylase, amylo-1,6-glucosidase and amylo-(1,4 → 1,6)-transglucosidase, no explanation for replication of glycogen molecules exists. Replication, when and if it does occur, may result from the α-amylolytic activity of tissues, generating a plurality of seeds for glycogen synthesis from a single glycogen molecule.

It appears likely that much of what has been said about the nature of glycogen synthesis in the living animal may also apply, with very minor modifications, to the synthesis of the less highly branched plant polysaccharides, the starches. Certain differences exist, however, which may raise the question of whether polysaccharide synthesis occupies the same crucial position in animal as it does in vegetable economy. If attention is momentarily focused upon glycogen of liver, it will be recalled that this is considered to be a mobile reservoir of glucose and of energy. It is, however, a small reservoir, amounting calorically in the rat to some 3% of the total daily caloric requirement. Furthermore, after it has been virtually eliminated, as by fasting or by injec-

tion of epinephrine, the animal is not demonstrably sick and indeed under most circumstances its chance of survival has been but little diminished when compared with that of a litter mate whose liver is rich in glycogen. Lastly, the major process of glycogenesis, the phosphorylase reaction, is the precise reversal of the major process of glycogenolysis. In other words the route *in* is the reversal of the route *out;* hence glycogen is a sort of biochemical blind alley. In summary, the reserve of liver glycogen is small, the animal does quite well without it, and it is a cul-de-sac. It will be recalled that on these very grounds the vermiform appendix in man has been designated as a vestigial organ.

Is liver glycogen then to be considered a biochemical vestige? The probable answer is in the negative, in view of the fact that under certain stresses of short duration, liver glycogen does serve as a useful source of extra blood glucose. The fact remains, however, that, whereas the well-nourished potato accumulates polysaccharide, the well-nourished rat accumulates characteristically not glycogen but lipid. The major elastic compartment for energy storage in adult mammals is the depot fat, not the carbohydrate compartment. This fact was drawn to our attention some years ago when we had occasion to compare rates of glycogenesis and lipogenesis in adult and fetal rats.[15] Although the fetus is, until shortly before term, very poor in depot fat, its glycogenic capacity, per gram of tissue, is far greater than that of the adult. The possibility that this difference in fetal and adult habits might represent a sort of recapitulation of phylogeny suggests itself.

In quest of a more satisfactory explanation for the lack of fat storage in fetal tisues, one is forced to consider the generally stated functions of subcutaneous depot fat. This adipose tissue serves as an energy reservoir, it functions as mechanical upholstery against physical trauma, and it acts as thermal insulation in homothermic species. For none of these functions does the mammalian fetus have any need. It is nourished not discontinuously but continuously by the maternal circulation. It is well protected against physical trauma from without by maternal tissues and amniotic fluid. It resides in a precisely thermoregulated environment. It is therefore not surprising that the fetus receives its investiture of subcutaneous adipose tissue only shortly before term, as if in anticipation of its ejection from its highly protected environment.

A further inspection of the difference in energy-storage habits of

plants and animals reveals certain interesting exceptions. Whereas plants in general accumulate polysaccharides and are poor in lipids, many important vegetable oils do occur. With few exceptions, however, major lipid storage in plants is restricted to seed parts. Again, whereas it is the habit of animals to accumulate lipid rather than polysaccharide, in the tissues of molluscs one finds large amounts of glycogen and but little fat. These exceptions suggest the possibility that the habit of preferential lipid storage is an adaptation to motility. An obvious advantage of lipid storage over polysaccharide storage is that, per calorie stored, lipid weighs far less than carbohydrate. This is of little survival benefit to sessile forms of life such as the higher plants, but it may be of real importance to the mouse, who must evade his natural enemies, or the cat, who must capture the mouse. Of all the tissues of higher plants, it is uniquely in the seed parts that light weight may be considered to be of survival value. It is necessary both for the survival of the individual and of the species that seeds be disseminated, and, regardless of the vector, the less a seed weighs the higher the probability that it will be carried away from its source. Molluscs are undoubtedly motile, but of all animal species few are better protected against natural enemies and few have less need for motility in order to survive. What has been said of molluscs may also be said of the mammalian fetus.

From the foregoing it will be seen that in the starch-glycogen group of polysaccharides nature has produced a system peculiarly and elegantly adapted to the function of energy storage. Each glycogen molecule acts as a minute and elastic glucose reservoir at a molecular level and is continuously acquiring and losing glycosyl residues at its periphery. As an energy reservoir it suffers, however, in comparison with fat in that, per calorie stored, it adds far more to organism weight. Although this is unimportant to the survival of such forms as do not depend upon motility, such as the tuber, the tree, the mollusc, or the fetus, it may be of importance to the plant seed and the adult mammal. In these latter forms the habit of lipid storage has largely supplanted the habit of polysaccharide accumulation.

Reverting to the question of whether mammalian-liver glycogen is a biochemical vestige, we are still inclined to the opinion that it is not. It is, however, regarded as entirely possible that a transfer from a predominantly glycogenic habit to a predominantly lipogenic habit has real survival value to many motile forms of life and that this change has, in the long course of evolution, actually occurred.

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The Veratrum Alkamines

OSKAR WINTERSTEINER

This essay attempts to give a brief account of the advances made in the structure elucidation of the veratrum alkamines, and in particular to bring out how the peculiar dichotomy of skeletal structure which sets these alkaloids, as a group, apart from other families of steroidal bases came to be recognized. Naturally only the facts most directly relevant to the elaboration of the presently accepted structures can be presented here. The literature cited covers most of the more recent work, except that on cevine, for which the reader is referred to the bibliography in the comprehensive 1954 paper on the constitution of this alkamine by Barton et al.[1] More detailed if slightly outdated résumés of the chemical and other aspects of the subject can be found in review articles.[2]

Of the more than two dozen veratrum alkaloids now known the majority is of the conjugated type (esters or glucosides); the much smaller group of unconjugated bases (alkamines) from which these are derived comprises only eight well-characterized members. They all contain twenty-seven carbon atoms and one nitrogen atom, which in all but two instances (jervine, $C_{27}H_{39}O_3N$; veratramine, $C_{27}H_{39}O_2N$) is tertiary. The tertiary bases include rubijervine and isorubijervine $(C_{27}H_{43}O_2N)$, and a group of highly oxygenated compounds (zygadenine, $C_{27}H_{43}O_7N$; * cevine, germine, $C_{27}H_{43}O_8N$; and protoverine, $C_{27}H_{43}O_9N$), which occur in nature predominantly in combination with acids, i.e., as the alkamine moieties of the hypotensically active and hence medicinally important ester alkaloids.

Much of our present knowledge of the chemistry of the alkamines we owe to the fundamental studies of W. A. Jacobs, who, together with L. C. Craig, began exploring this subject in 1937. In the initial phase

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* This alkamine, together with certain ester alkaloids derived from germine, occurs in *Zygadenus venenosus*, which is not a member of the genus *Veratrum*.

of this work the prime objective was to gain insight into the nature of the underlying carbon-nitrogen skeleton by means of soda-lime distillation and selenium dehydrogenation. This approach proved to be immediately fruitful at least as far as the nitrogenous portion of the molecule was concerned. Particularly informative in this respect was the finding that 3-methyl-6-ethylpyridine (I) was formed on selenium dehydrogenation from all the alkamines then so investigated, and that the corresponding piperidine base was formed from cevine on soda-lime distillation. There could be no doubt then that the nitrogenous moiety was a substituted piperidine ring, joined, as it turned out later, to the rest of the molecule through the α-carbon of the ethyl group and, in the tertiary bases, also through the nitrogen atom. The structural significance of three other basic dehydrogenation products, 3-methyl-5-hydroxypyridine (II), from veratramine, a base which in all probability is 3-methyl-5-hydroxy-6-ethylpyridine (III), from jervine, and an isomer of jervine recently [1] assigned structure IV, from cevine, will become evident later.

It will be noted that the carbon skeleton of I is that of the cholesterol side chain. It was then not unreasonable to adopt the working hypothesis that the remainder of the molecule was made up of the androstane skeleton. Strength was added to this supposition when it was found (Prelog and Szpilvogel, 1942; Craig and Jacobs, 1943) that solanidine, $C_{27}H_{43}O$, the main alkaloid of the potato plant (Solanum tuberosum), then already known to be a steroid, likewise contained its nitrogen atom in a 3-methyl-6-ethylpiperidine moiety. Against it, however, stood the fact that painstaking fractionation of the neutral dehydrogenation products from jervine and cevine (up till then the only alkamine so investigated), had failed to disclose the presence of any phenanthrene derivatives. In fact, with the exception of a tricyclic compound from cevine, 1,2-cyclopentenonapthalene (V), none of the numerous physically and analytically well-characterized hydrocarbons obtained from these alkamines could be identified structurally, and some of the tetra- and pentacyclic members of the series exhibited ultraviolet characteristics which indicated a relationship to fluorene (VI) and 1,2-benzofluorene (VII) rather than to phenanthrene. For this reason Jacobs and Craig for a time gave consideration to a modified steroidal nucleus with a five-membered ring B and a methyl group at C_5. However, when in 1943 they shifted their attention to rubijervine, and later to its newly isolated isomer isorubijervine, it became increasingly evident that these two alkamines, at any event, must be normal steroids. Thus, rubijervine yielded on selenium dehydrogena-

tion an isomer of Diels' hydrocarbon, in all probability 1′-methyl-1,2-cyclopentanophenanthrene (VIIIa), and isorubijervine, 1,2-cyclopentanophenanthrene (VIIIb) itself. Furthermore, the presence of the usual 3-hydroxy-5,6- double-bond grouping in both compounds could be readily demonstrated by oxidation to the corresponding Δ^4-unsaturated ketones and reduction of the ketones to the Rosenheim-positive allylic alcohols corresponding to allocholesterol.
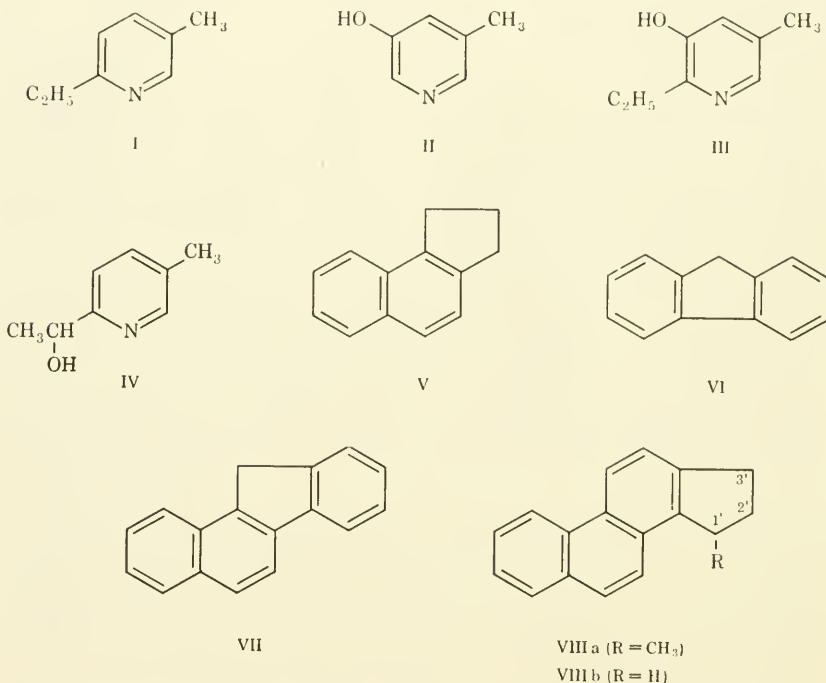
The last vestige of doubt regarding the significance of these results vanished when it could be shown that both these alkamines were indeed derivatives of solanidine (IX), which differed from this base merely by the presence of a second hydroxyl group. With rubijervine (X), the conversion to solanidine was effected by selective oxidation of this group, for sound reasons assumed to occupy the 12 position and to be α oriented, and Wolff-Kishner reduction of the resulting 12-monoketone.[3] In isorubijervine (XI) the additional hydroxyl group is primary (formation of a monoketomonocarboxylic acid $C_{27}H_{41}O_3N$ on oxidation of the 5,6-dihydro derivative[4] and resides on the methyl carbon atom 18. This site was first deduced[4] from the absence of

methyl substitution in the cyclopentene ring of the dehydrogenation product VIII*b*, but a more cogent argument evolved from the quaternary base nature of the intermediates XII*a* and XII*b* (originally regarded as the normal 18-O-tosylate and 18-iodide[5] in the sequence by which isorubijervine was converted to solanidine;[5-7] clearly, of the methyl carbons in the vicinity of the basic group only C-18 fulfills the steric requirements for facile formation of a bond to the nitrogen atom.

IX (R = H) Solanidine
X (R = ···OH) Rubijervine

XI Isorubijervine

XIIa (X = p−CH₃−C₆H₄−SO₃)
XIIb (X = I)
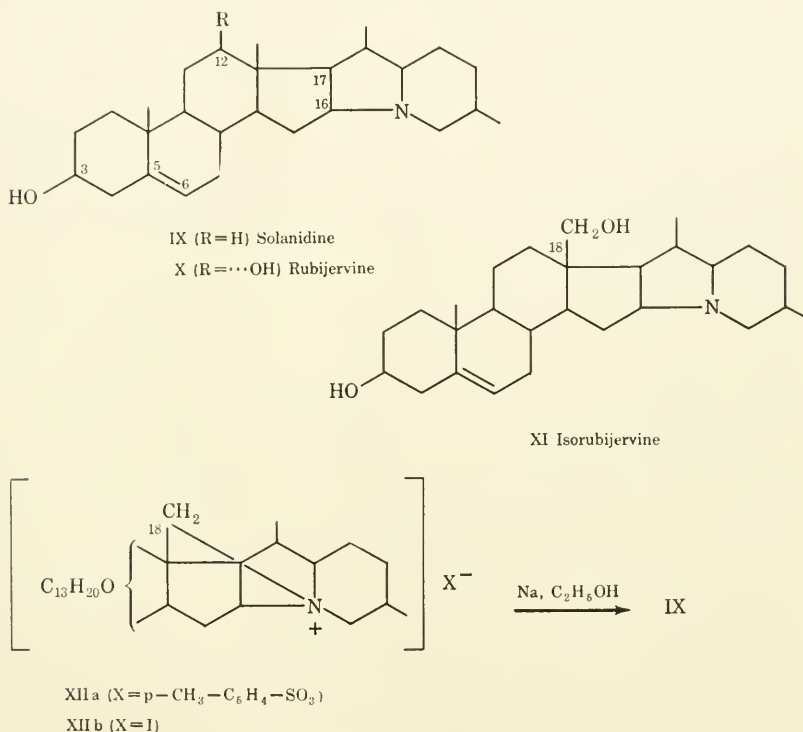
With the presence of a normal steroidal nucleus in these two bases assured, it was only natural that in the tentative expressions which had meanwhile been proposed for jervine[8] and cevine[9] these alkamines were also accorded the normal structure. Implicit in these formulations was the assumption that the "abnormal" fluorene-like hydrocarbons formed from these bases in the selenium dehydrogenation were probably artifacts resulting from skeletal rearrangements such as sometimes occur in this high temperature reaction. That, to the contrary, these products accurately mirror the original ring system was first

brought out in a reinvestigation of jervine, begun in 1950 in the laboratory of the writer, which definitely established the perhydrobenzfluorene structure XIII for this alkaloid.

Much valuable information on jervine was already on hand from the work of Jacobs with Craig, and with Sato.[8] Thus the evidence adduced by these authors for a normal A/B ring system with a hydroxyl group at C-3 and a 5,6 double bond was incontestable. The function of the other two oxygen atoms present could not be ascertained by simple chemical means. One of these was assumed to be oxidic and to link C-23 in the piperidine ring (corresponding to the position of the hydroxyl group in the dehydrogenation product III) to C-16 in a normal ring D. The second inert oxygen atom was revealed as part of an α,β-unsaturated ketone grouping by the absorption spectrum (λ_{max}, 250 mμ). In view of the complete lack of reactivity towards ketone reagents the ketonic carbonyl was placed in position 11 of a normal six-membered ring C, and this in turn necessitated accommodating the conjugated double bond in 8, 9.

That the latter proposition was incorrect became apparent from a study of the ultraviolet characteristics of diacetyl-7-ketojervine and its 5,6-dihydro derivative,[10] which showed that the new α,β-unsaturated ketone system created in the former compound by the introduction of the 7-keto group could not be contiguous with that pre-existing in jervine, and hence that ring C, if containing the unreactive keto group, could not be normally constituted. In the further pursuit of the problem the use of acetolyzing agents proved to be decisive. Thus, treatment of jervine with boiling acetic anhydride and zinc chloride yielded a nitrogen-free compound, $C_{23}H_{30}O_3$, which contained the unreactive keto group as part of a dienone system. Its structure XIV followed in essence from its oxidative degradation to acetaldehyde and the en-1,4-dione XV and the alkali-induced aromatization of the latter to the phenol XVIa, the absorption spectrum of which left no doubt as to the presence of an α-indanone (or α-tetralone) system carrying a phenolic hydroxyl group in the position indicated. The alternative structures XVIb and XVIc could be excluded on the grounds that the keto group which had survived the aromatization was completely unreactive to ketone reagents.[11,12]

When milder acetolytic conditions were employed, ring E was opened without loss of the side chain and the reaction led through the intermediate XVII to the indanone XVIII, the structure of which was readily deducible from its spectrum and from the fact that on reduction it formed a product showing benzenoid absorption.[11,13] The presence
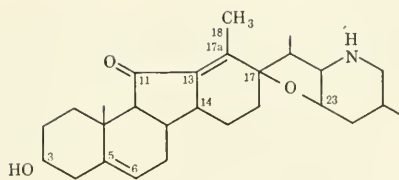
XIII Jervine
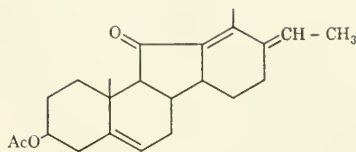
XIV
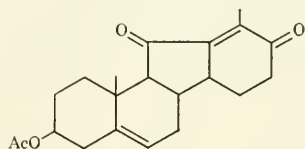
XV
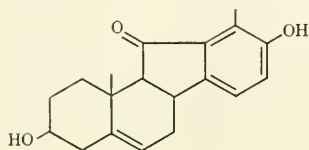
XVIa
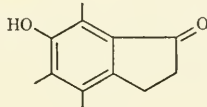
XVIb
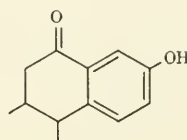
XVIc
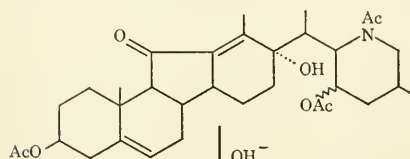
XVII
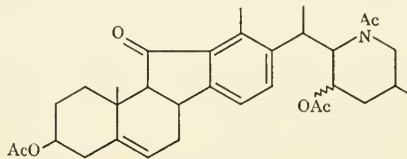
XVIII
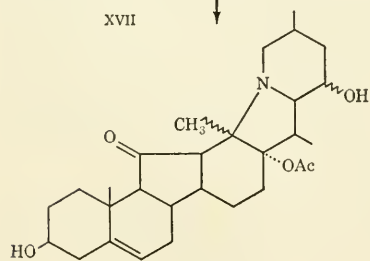
XIX
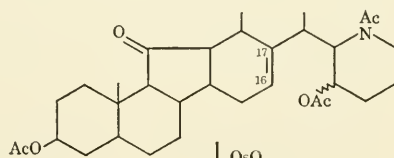
XX
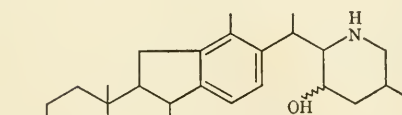
XXII Veratramine
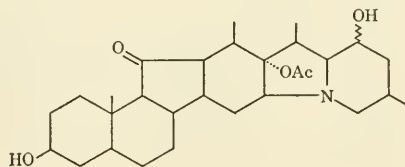
XXI

of a new, non-acetylatable hydroxy group in the precursor XVII of the indanone provided the information that the oxidic bridge of jervine was attached in ring D to the tertiary carbon atom 17. It is interesting, and perhaps of biogenetic significance, that this feature renders XVII, and generally "open" derivative of this type, prone to undergo rearrangements catalyzed by alkali in which the nitrogen atom, after losing its acetyl group by N → O migration to that hydroxyl group, adds to an activated and sterically favored site in ring D. Thus XVII on O-deacetylation with cold alkali rearranges to the sterically hindered and hence very weak tertiary base XIX,[13] whereas the olefin XX, an acetolysis product of tetrahydrojervine, on hydroxylation with osmium tetroxide and treatment of the adduct with sodium sulfite yields, besides the normal 16,17-glycol, a tertiary amine of type XXI,[14] reminiscent of rubijervine.

A concurrent study of the secondary base veratramine [15] showed that this alkamine was closely related to the acetolysis product XVIII from jervine; in fact it differed from it structurally only by the absence of the 11-keto group. The presence of a preformed benzene aromatic ring, first deduced from the absorption spectrum (Jacobs and Craig, 1945), rests on solid chemical evidence,[15] as does the allocation of the two secondary hydroxyl groups to positions 3 and 23, and of the double bond to the 5,6 position.[15, 16] Structural correlation with XVIII and hence with jervine was achieved through the 5,6-dihydro derivative of XVIII,[17] which proved to be identical with an indanone-like compound found among the chromic acid oxidation products of triacetyl-dihydroveratramine.[15] Veratramine is therefore XXII. The finding that on permanganate oxidation it afforded benzene-1,2,3,4-tetracarboxylic acid,[18] aside from disposing of an earlier expression advanced by Jacobs and Sato [16] which differed from XXII only by inclusion of C-18 in a six-membered ring C, made secure the allocation to position 17a of the methyl group representing that carbon atom.

In the sequel Jacobs and Pelletier,[19] through a careful re-evaluation of the absorption spectra of the "abnormal" dehydrogenation products and some of their partly hydrogenated derivatives, came to the conclusion that the perhydrobenzfluorene skeleton should be allowed not only to the two secondary bases but also to cevine and the other tertiary ester alkamines. Particularly indicative in this respect were two large basic fragments from cevine not previously mentioned here, namely, cevanthridine, $C_{25}H_{27}N$, and veranthridine, $C_{26}H_{25}N$, now formulated, respectively, as XXIII and XXIV. The characteristic ease with which these compounds formed the corresponding 11-ketones
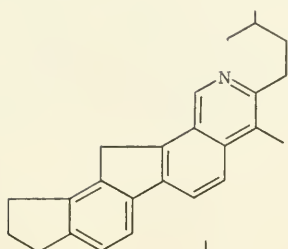
XXIII Cevanthridine
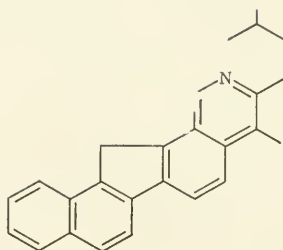
XXIV Veranthridine
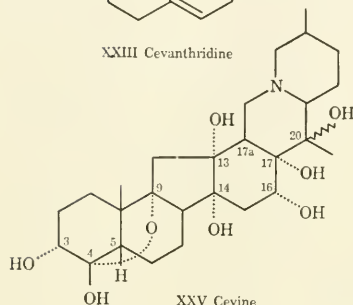
XXV Cevine
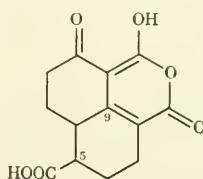
XXVI Decevinic acid
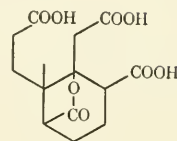
XXVII
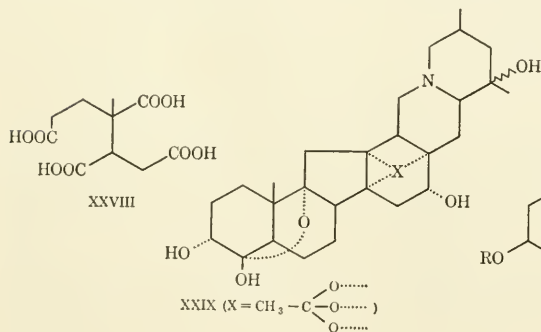
XXVIII
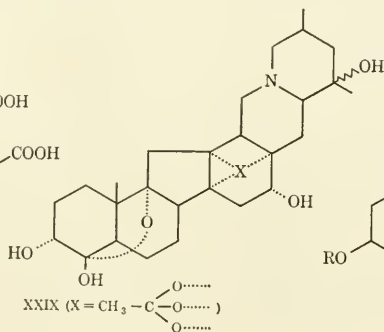
XXIX (X = CH₃—C—O······)
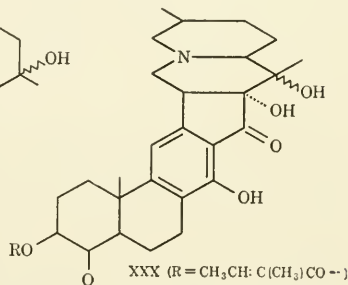
XXX (R = CH₃CH: C(CH₃)CO ‑‑)
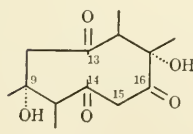
XXXI
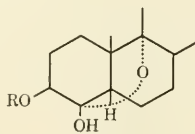
XXXIII Cevagenine

XXXII   (R = H) Veracevine
XXXIIa (R = CH₃CH: C(CH₃)CO —) Cevadine
XXXIIb (R = 3,4 − (MeO)₂C₆H₃CO —) Veratridine
XXXIIc (R = CH₃CO —) Cevacine

XXXIV b

XXXIV a

1 tert. OH
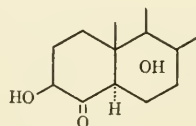
XXXV Germine
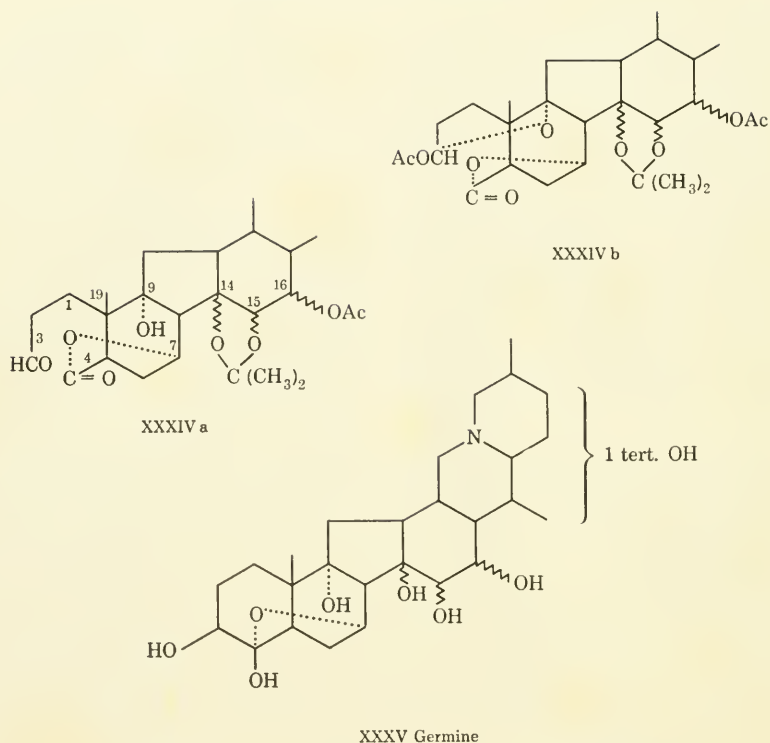
(9-fluorenones) on oxidation is quite in line with the assigned struc-
tures.[20]   The contraction of ring A in XXIII may have its cause in
the multiple substitution of this ring, in cevine, with oxygen (cf. below).
On the other hand, this explanation is not applicable to some of the
dehydrogenation products of jervine which likewise show this feature.

With the skeletal structure of cevine thus in essence defined by
XXIV, there remained still the formidable task of determining the
location of its eight oxygen atoms, all but one of which could be
assumed to be hydroxylic.   Studies towards this end had been in prog-
ress since about 1951 in several laboratories, including those of Barton
(London), Jeger and Prelog (Zürich), and Woodward (Harvard).
Before long these investigators, by pooling their information, were able
to advance in a joint communication [1] structure XXV for cevine.   It
is not possible to do justice here to the intricate and ingenious argument
essential to the elaboration of this formula, nor to survey the host of
experimental facts, some already on record from the work of Jacobs,
and many others of more recent vintage, which could be marshalled

in its support. A very brief and cursory exposition of the main lines of evidence will have to suffice. One of these developed out of a reconsideration of the properties and mode of formation of decevinic acid, $C_{14}H_{14}O_6$, a degradation product already extensively explored by Jacobs and Craig and now shown to be XXVI. Decevinic acid is formed by pyrolytic dehydration of one of the chromic acid oxidation products of cevine, a lactone tricarboxylic acid of the composition $C_{14}H_{18}O_8$. The recognition of this lactone as XXVII, partly implicit in XXVI, and of another closely related oxidation product, a hexane-tetracarboxylic acid $C_{10}H_{14}O_8$, as XXVIII showed these fragments to be derived from a normal A/B ring system substituted with oxygen at C-3, C-4, and C-9, and beyond that provided confirmatory evidence (preservation in XXVII of carbon atom 13 * in the oxidative scission) for ring C being five-membered (Jeger, Prelog, Woodward).

At this point it was possible to accommodate in the positions mentioned a masked secondary α-ketol system which had been disclosed by the work of Barton, and further to place tentatively an independent ditertiary glycol grouping (Stoll and Seebeck, Barton) at the C/D bridge atoms 13 and 14. More decisive evidence on this latter point, as well as on the location of one other hydroxyl group, derives from the properties and reactions of the "anhydrocevine" formed from cevine on vigorous acetylation followed by hydrolysis (Stoll and Seebeck). This compound is now shown to be a tritertiary orthoacetate (Barton) which must be formulated as XXIX, since other possible sites for the hydroxyl groups linked together in the orthoacetate grouping may be excluded on the strength of periodate titration data, as well as for other reasons (for instance, XXX below). The presence of a secondary hydroxyl group at C-16 is deduced, inter alia, from the structure (XXX) assigned to a product which is formed from the ester alkaloid cevadine (XXXIIa) on chromic acid oxidation. This compound is undoubtedly a phenolic indanone and is thought to arise from the intermediary seco-13,14-diketone XXXI by transannular Claisen condensation (C-13 → C-15), followed by dehydration at C-9, and aromatization of ring C (Jeger, Prelog). The allocation of the remaining hydroxyl group, which must be tertiary, to C-20 rests in essence on the (as yet unproved) structure of the dehydrogenation product IV.

It is now well established that an isomer of cevine, veracevine, and not cevine itself is the true native alkamine occurring in the ester

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* Numbering according to proposals for steroid nomenclature now before Union internationale de chimie pure et appliquée (cf. ref. 12). Barton et al.[1] designate the carbon atoms here numbered 13 and 17a as 12 and 13, respectively.

alkaloids (Pelletier and Jacobs, Kupchan et al.). Veracevine, which can be obtained from the latter by methanolysis, is transformed into cevine under the conditions (vigorous alkaline hydrolysis) generally used for liberating the alkamine moiety. Yet another isomer, cevagenine (Stoll and Seebeck), is formed from veracevine on mild treatment with alkali. Cevagenine differs from cevine and veracevine in that it contains a ketonic carbonyl. These isomers are formulated by Barton et al. as shown in XXXII, XXXIII, and XXV, and accordingly the three known ester alkaloids, cevacine, veratridine, and cevadine, have to be expressed as XXXIIa, b, and c, respectively. It would lead too far afield to review here the evidence for the configurational assignments for C-3, C-5, and C-9 in these isomers and for the asymmetric centers in the remainder of the molecule.

Germine, a native alkamine which occurs in nine of the ester alkaloids presently on record, is under the influence of alkali analogously isomerized to the ketonic isogermine [21] and further to the non-ketonic pseudogermine,[22] and before long evidence was at hand showing that this behavior is referable to the presence of an isomerizable α-ketol system similar to that of veracevine [23a] but differing from it in that the terminus of the hemiacetalic ether bridge is C-7 and not C-9.[23b] This was deduced from: (1) The finding that all three germine isomers form tetraacetates (which is possible only if the hydroxyl function at that terminus is primary or secondary). (2) The fact that the hemiacetalic ring is five-membered (formation of an aldehydo-γ-lactone on periodate oxidation of germine (or pseudogermine) acetonide.[23a] (3) The exclusion of the alternative non-tertiary γ-positions 1 and 19 for that hydroxyl function on the grounds that germine, like cevine, can be degraded to the tetracarboxylic acid XXVIII which carries no oxygen at these carbon atoms. Finally Kupchan and Narayanan,[24] on the basis of additional but perhaps somewhat less compelling evidence which cannot be given here in detail, came to ascribe structures XXXIVa and b, respectively, to the aforementioned germine acetonide aldehydo-γ-lactone and its diacetate and thus to formulate germine itself as XXXV.

Although structural information on the other two tertiary alkamines of this group, zygadenine and protoverine, is still scant, there can be little doubt that they, too, conform with cevine in regard to the nature of the skeleton and the presence of an isomerizable ketol system.

Thus, except for what remains to be done on these two alkamines, the task of the organic chemist in this field has been largely accomplished. With the coexistence in several species of two principal groups

of alkaloids differing in their skeletal structure clearly established, it is legitimate to speculate on mechanisms by which the perhydrobenz-fluorene system, observed here in nature for the first time, might arise biogenetically from the normal steroid nucleus. The structures now before us, as such, are not informative in this respect, except perhaps insofar as it can be inferred from the relative prevalence of functional groups, or of equivalent unsaturation, in the C/D ring moiety that this portion of the molecule has lived through an eventful biogenetic past. However, a significant clue (though it is one derived from purely organic-chemical facts and hence, in the eyes of biogeneticists, perhaps not too trustworthy) is available from the work of R. Hirschmann, N. L. Wendler, and their colleagues on ring C/D rearrangements in certain sapogenins appropriately substituted with oxygen in ring C.[25]

XXXVI Rockogenin

XXXVII

XXXVIII

XXXIX
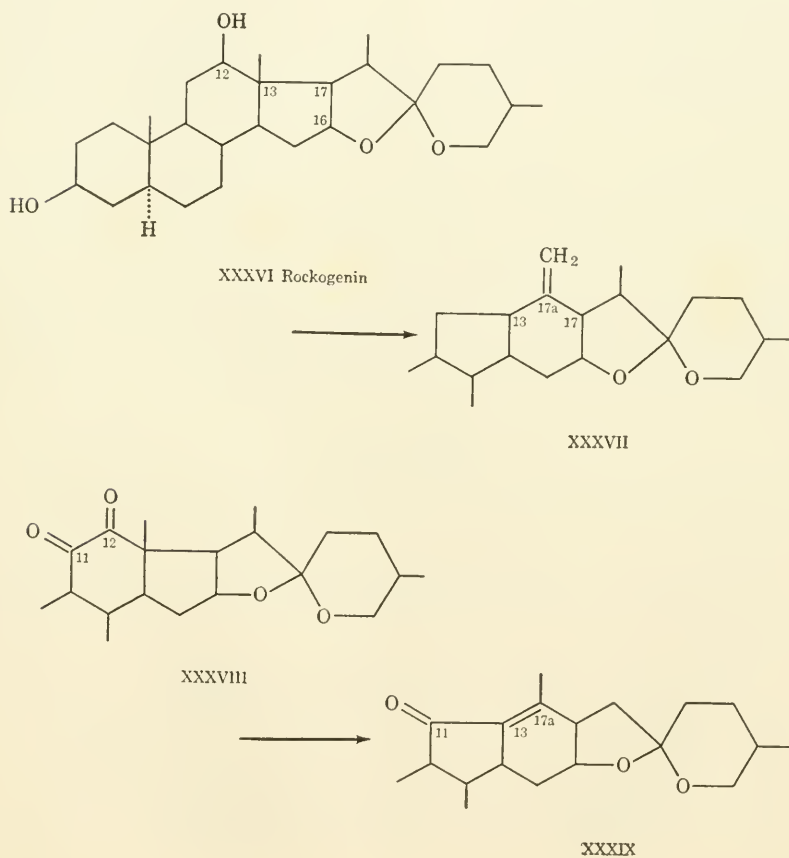
It was shown by these investigators that rockogenin (12β-hydroxy-tigogenin, XXXVI), when treated in the form of its 3-methylsuccinate-12-mesylate with potassium t-butoxide in t-butanol, or with the alcohol alone, was transformed in fairly facile reaction into a mixture of the C-nor/D-homospirostene XXXVII and its 17,17a double-bond isomer. Similarly, the 12-toluene-p-sulfonylhydrazone derivative of 11-keto-hecogenin (XXXVIII) with alkali yielded the rearrangement product XXXIX, reminiscent of jervine. Since an 11β-hydroxy-12-keto bile acid also undergoes this rearrangement, it is clear that the constitutional requirements are rather simple: a 12-keto or 12β-hydroxyl group in an activated state such as supplied here by the tosyl group in the derivatives used. The authors suggest that the veratrum alkaloids possessing the modified nucleus may arise in the plant by rearrangements of this kind from similarly constituted precursors. Against this hypothesis can be held the fact that there is no evidence for the occurrence in the plant sources of rockogenin and hecogenin of products of the type obtained by Hirschmann et al. The question could be presumably settled by growing veratrum plants in the presence of 1-C^{14}- and 2-C^{14}-labeled acetate and determining the isotope distribution in suitable degradation products of the more abundant "abnormal" alkaloids, but, aside from the great practical difficulties inherent in a project of this kind, it would obviously have to await the demonstration of a constant distribution pattern of acetate carbons in several classes of plant steroids including normally constituted steroid alkaloids, since so far it is only a presumption that the pattern ascertained for the cholesterol nucleus holds true for all steroids.

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The Chemical Basis of
Heredity Determinants

STEPHEN ZAMENHOF

Students of the history of science cannot fail to notice how often the issues most essential for our species are continuously avoided. Thus, the science of *heredity* which affects us more than astronomy was practically non-existent until the second half of the nineteenth century although Mendel's conclusions (1866) were actually much easier to arrive at and to accept than those of Copernicus (1530) or of Harvey (1628). As late as 1872 Spencer writes: "We are obliged to confess that Life in its essence cannot be conceived in physico-chemical terms."

By the end of the first half of this century the foundations for chemical explanation of several biological phenomena had already been laid. However, the phenomenon of heredity was not attractive to the chemist, perhaps because of a fear of the multitude of substances involved and their insurmountable complexity; indeed, in contrast to simpler biochemical functions, nothing less than the whole cell or at least the chromosomal apparatus seemed indispensable as heredity determinant. The remains of this Spencerian attitude still hamper modern research on the chemistry of the transmission of heredity.

Heredity Determinants

Although the hypothetical "working gene" may be, indeed, a very complex system, not all the elements of such a system need be essential for the determination of heredity: a few may be actual *heredity determinants,* and all the others merely auxiliary elements. This essay deals with the search for the decisive factors.

If one approaches the problem with an unbiased mind one also has to test the possibility that the heredity determinants are not chemical

substances. A set of "genes" could be, for instance, a set of specific reactions going on, or a specific distribution of independent molecules; such suggestions were, indeed, made in the past. To dispose of these notions one has only to turn to the simplest living entities, the crystalline viruses. Each of these consists of a single molecule of nucleoprotein; there are no reactions going on, no distribution of independent molecules because only one is present. Since it is a matter of common sense (or definition) that each living entity carries its own heredity, one has to conclude that the only heredity determinants of these viruses are indeed chemical substances: either nucleoproteins as such or their components, nucleic acids * and/or proteins. One could argue that perhaps a different principle is involved in higher organisms; however, as will be shown below, the evidence there points in the same direction.

The next more complex living entities studied in this respect are the bacterial viruses. The viruses of *Escherichia coli* consist mainly, but not exclusively, of nucleoprotein, about 40% of which may be nucleic acid (here deoxyribonucleic acid or DNA). As shown by Hershey and Chase,[1] upon infection practically only the DNA of the virus reaches the inside of the host and is allowed to reproduce (determine) the new virus particle; the DNA, then, must be the only heredity determinant of these species.

In bacteria, the discovery of the transforming phenomenon (Griffith [2]) and of the nature of the transforming principle (Avery, MacLeod, and McCarty [3]) made it clear that there, too, the DNA alone is capable of acting as a heredity determinant; however, the question as to whether the DNA is the *sole* or only one of many heredity determinants still remains debatable. The subject of transforming phenomena will be discussed in detail below.

The situation in higher organisms is, naturally, more complex, and evidence of the above-mentioned degree of validity has not yet been presented; however, there, too, all the existing evidence, though indirect, points to DNA as a heredity determinant.

The spermatozoa, which carry the entire heredity of the male, may contain over 90% of deoxyribonucleoprotein (referred to dry weight); the high deoxyribonucleoprotein content of somatic chromosomes, undoubtedly concerned with the orderly transmission of heredity, is well realized. But, although the composition and gross structure of DNA seem to remain unchanged when the spermatozoon (and ovum)

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* The nucleic acid involved here is the ribonucleic acid (RNA); this RNA, however, seems to consist of giant molecules resembling more, in this respect, the cellular DNA than RNA.

changes into somatic cells of the same heredity (for a review see ref. 4), the composition and structure of nuclear protein may undergo drastic changes: the change of protamines into histones is but one example. In addition, the study of the quantity of DNA and of protein (per single set of chromosomes) revealed that, although the quantity of DNA remains the same in all cells of a species,[5] the quantity of protein varies a great deal. All these findings can be taken as an indication (though not as an absolute proof) that in higher species also the DNA serves as a determinant of heredity.

This concept encountered difficulties, however. Workers in the field were still influenced by the authority of "old masters" (especially P. A. Levene [6]) who, on the basis of erroneous chemical analysis, concluded that the DNA from all sources contains equimolar amounts of individual purines and pyrimidines; that these form subunits ("tetranucleotides"); and that, in fact, all deoxyribonucleic acids are identical, being merely composed of identical "tetranucleotides." Lehmann-Echternacht even reported that he had actually isolated this (purely fictitious) substance. Of course, the identical and simple DNA molecules could not serve as highly specific heredity determinants.

These erroneous views about the structure of DNA were slowly corrected, mainly through the work of Chargaff and his collaborators (for a review see ref. 4). It was found that the DNA has a highly complex asymmetrical structure;[7] that the individual purines and pyrimidines, as a rule, are not present in equimolar amounts; that the "tetranucleotide" unit simply does not exist;[8] that the composition of the whole DNA is different for each species but similar for different organs of the same species;[9] and that the DNA of one type of cell is actually a mixture of different molecules.*[10,11] All these features are necessary if the DNA molecules are to serve as heredity determinants; but the finding of these features does not, of course, furnish a proof that the DNA molecules actually *are* heredity determinants.

The Transforming Phenomenon

The study of the nature of the transforming principle furnished the most acceptable proof that *one* heredity determinant is DNA. The transforming phenomenon will be discussed at greater length because it offers unique possibilities for studying the correlation between the

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* Assuming the molecular weight of DNA to be of the order of 5×10^6, the number of possible combinations of sequence of different nucleotides for the DNA molecules of just one composition is of the order of 10^{9000}.

structure and the function of heredity determinants. The reasons for this will become clear from the description of the phenomenon and of the agent involved.

In 1928, Griffith [2] reported that, from mice that had been injected with living non-encapsulated (R) pneumococci mixed with heat-killed encapsulated (S) ones, living S pneumococci were recovered. Of particular interest was the fact that the specific type of S cells was the same as the heat-killed cells rather than the type from which the living R cells were derived. Once the new feature (production of the capsule of a specific type) was established, it was retained and reproduced in subsequent generations as if a new gene had been added to the genetic make-up of the receptor cells; indeed, such transformed cells, when heat-killed, could in turn induce the transformation of R cells exactly in the same way as did the original S cells.

Alloway was the first to demonstrate that the presence of the whole "donor" (S) cells is not necessary for the phenomenon. This investigator prepared cell-free aqueous extracts of the heat-killed S cells, passed the extract through a bacterial filter and demonstrated that the filtrate is still capable of transforming the R cells into S cells.

In 1944, Avery, MacLeod, and McCarty [3] purified the extract further and found that the responsible agent (the "transforming principle") had all the properties of a highly polymerized deoxypentose nucleic acid. This single finding laid the foundation for the chemical study of the transforming principle.

Species Susceptible to Transformation

To date, reproducible transformation phenomena are known only in bacteria. A transformationlike phenomenon in viruses has been reported [12] and confirmed; however, it is difficult to judge whether the nature of this phenomenon is similar to that of bacterial transformation. The transformation of organisms higher than bacteria has not yet been reported.

Of bacteria, the organism used originally by Griffith, the pneumococcus, is still most widely experimented upon, because of the large body of knowledge accumulated, the comparatively low pathogenicity of this organism, and, above all, the reproducibility of results. The disadvantage of this species is the presence in the bacterial cultures of an enzyme, deoxyribonuclease (DNAase), which tends to destroy the transforming principle.

The transformation in several other bacteria has been reported. These are *Escherichia coli, Shigella paradysenteriae, Proteus, Salmo-*

nella, Staphylococcus, tubercle bacillus, *Alcaligenes radiobacter, Phyto-monas tumefaciens,* and *Brucella.* However, these reports have not as yet been confirmed by others and thus far cannot be used for a routine study of the transforming principle.

Besides pneumococcus, the only two species in which the transformation phenomenon can be repeated day after day with the same reliability are *Hemophilus influenzae* [13] and *Neisseria meningitidis.* [14] *Hemophilus influenzae* lends itself particularly well to quantitative studies on the transforming principle because of the absence of DNAase from bacterial cultures.

Features Transferable in the Transformation Phenomenon

The feature transferred (induced) in the transformation phenomenon, as originally discovered by Griffith, was the production of capsules of type I, II, or III of pneumococcus; as is well known, each capsule contains polysaccharide specific for its type. Further studies of McCarty and Avery demonstrated that the production of capsules of types VI or XIV can also be induced by using the transforming principles from the cells of these types. It is probable that transformation could be demonstrated for every one of the 70-odd known types of pneumococcus. The feature transferred need not be limited to polysaccharide however. Austrian and MacLeod demonstrated acquisition of a specific M protein through transformation in pneumococci. The six capsular substances *a, b, c, d, e,* and *f* produced after the transformation of *Hemophilus influenzae* [13] at first appeared to be polysaccharides, but closer chemical study [15, 16] revealed that most of them form a new class of immunologically active compounds, the polysugar phosphates. The substance of type *b* is of particular interest: it appears to consist of a polyribophosphate chain, as it exists in pentose-nucleic acids, in which the place of the purines and pyrimidines is occupied by a second similar chain, linked to the first in $1:1'$ glycosidic linkages. The substance of type *a* appears to be a polyglucophosphate and that of type *c* a polygalactophosphate. The transferable feature in *Neisseria meningitidis* is the production of capsular substances of type I or IIa which appear to be more complex than polysaccharides. Other transferable features include induction of fermentation of salicin, change in metabolism of glucose and lactic acid, production of mannitol phosphate dehydrogenase, and change in *quantity* of polysaccharide produced; in this last case it was suggested that the genes involved form indeed a series analogous to what is known in higher organisms as an *allelic* series. Still other transferable features include change

from sensitivity to resistance to penicillin, streptomycin, and sulfanilamide, and a change from resistance to sensitivity to streptomycin (Hotchkiss et al.). This class of transformation phenomena is of particular interest because the gradations involved (one-step or multistep acquisition of resistance) resemble closely the gradations acquired by a natural process, i.e., spontaneous mutation.

In summary, then, one can say that the phenomenon of transformation involves a great variety of genetic characters, and, were it not for the nature of the phenomenon, it would closely resemble spontaneous mutation.

The Nature of the Transforming Phenomenon

Although a considerable amount of work has been done in the field of bacterial transformations, the nature of the phenomenon itself remains far from clear. At present, the following conclusions appear logical:

The molecules of the transforming principle are heredity determinants because their presence determines the presence of hereditary characters. However, it is not known whether all hereditary characters can be accounted for by the (joint) action of all the molecules of the transforming principle in the cell; it is still conceivable that the determination of the most fundamental features proceeds through an entirely different mechanism.

The transforming principle seems to consist of DNA molecules. On successful transformation these molecules must reproduce (or be reproduced) because more of them are obtained. The reproduction must take place inside the cell because the DNA is never found outside, and because a thorough destruction of the cell is necessary to isolate the transforming principle. Furthermore, within 3 minutes after the transforming principle is added to the cells, it becomes completely protected against the action of added strong DNAase.[13] Thus, it appears that on transformation the molecule of DNA must penetrate the cell. However, the mechanism of this hypothetical penetration remains unknown; the DNA is composed of giant molecules and ought to be stopped by a normal bacterial membrane; at neutral pH the DNA molecules are highly negatively charged and ought to be repelled by the majority of bacterial cells which are also known to be negatively charged, especially when coated with polysugar phosphates.[15, 16] Only a small proportion of receptor cells (about one per hundred in pneumococcus, 10 to 10^3 times less in *H. influenzae*) is actually sus-

ceptible to transformation; such cells must therefore be in a special physiological state.[17]

Upon entering the susceptible cell the DNA may become fixed to the genetical "locus," presumably on bacterial chromosomes. That some sort of "fixation" or precipitation is necessary has been postulated on the demonstration that the molecule of DNA of *E. coli* in solution is actually longer than the cell itself.[18] The fact that the transforming principle as extracted from the cell seems to be in the form of a nucleoprotein [3, 19] also indicates some binding of DNA. In such an hypothetical fixation or acceptability inside the cell, two or more transforming principles may compete for the substrate. A phenomenon of competition (*a* vs. *b*, or *b* vs. *c*) has indeed been demonstrated in *H. influenzae;* [17] such competing transforming principles can be demonstrated to obey the law of mass action.

The problem of hypothetical competition for the "locus" may be closely connected with the problem of possible DNA exchange. When an R (non-encapsulated) cell is transformed into an S (encapsulated) cell, one may suppose that a missing transforming-principle molecule has been added (to a bare locus?); however, one may also postulate that an inactive (or less active) molecule has been replaced by an active one. This latter explanation appears more probable for the following reasons: (1) Many gradations of "roughness" are known, corresponding to various amounts of polysaccharide produced, and in some cases introduction of a transforming principle may abolish the action of an already existing gene (compare "allelism" in higher organisms). (2) S cells of one type can be transformed directly into S cells of another type.[20]

The Transforming Principle

From the foregoing discussion one can easily appreciate the potentialities of using the transforming principle for the study of the chemistry of heredity determinants; since no other form or system of heredity determination offers such possibilities, most of the chemical work was indeed done on this substance. The transforming principle can be extracted from the cell, purified, chemically identified, and analyzed. It can be subjected in vitro to the action of physical and chemical agents stronger than those which can act on the living cell without killing it; after removal of excess reagent, the nature and the extent of changes induced in the DNA molecule can be estimated; and, finally, such changed transforming principle (DNA) can be re-

introduced into the cell to study the relationship between the change in structure and the change in function. In short, this mode of attack offers possibilities of applying to the problem of heredity the same rational approach which yielded elucidations in so many fields of biochemistry.

Although the above reasons for interest in the transforming principle appear to the author to be the most important ones, the biochemist's and biophysicist's interest may often come from the application of the biological activity of DNA to the study of DNA itself. In early chemical approaches the DNA was subjected to degradation, with complete disregard of the macromolecular nature of the substance. This error was compensated for in the last decade by extensive biophysical studies of the giant molecules of DNA. However, the biophysicist soon faced a dilemma as to whether his substance was indeed "native" or still degraded. The discovery that the DNA loses its transforming activity on the slightest degradation suggested a convenient yardstick of "intactness"; for, although no one can say whether his DNA preparation is in the same state as the DNA that exists in the cell, the "functionally intact" unit seems important enough to warrant study and constant enough to serve as a standard. In such a study subtle reactions of certain agents such as mutagenic, carcinogenic, or carcinostatic agents with the DNA can often be demonstrated, by studying the loss of transforming activity, long before these reactions can be discovered by any physical or chemical method.

In the following, the emphasis will be on the transforming principle itself and the transforming phenomena will be mentioned only in their role as detectors of activity. It must be remembered, however, that a transforming phenomenon may involve several steps, and at present it is not possible to decide which is or are responsible for inactivation.

The Chemical Nature of the Transforming Principle

In 1944 Avery, MacLeod, and McCarty found the purified transforming principle to have all the properties of a highly polymerized DNA. Their conclusion that the transforming principle *is* DNA was based on the following observations: (1) Elementary analysis of the transforming principle corresponded to that of DNA. (2) Chemical and physical tests revealed the presence of DNA as the only detectable substance. (3) Serological tests failed to detect the presence of any immunologically active substances (such as polysaccharides). (4) Of several enzymes tested only deoxyribonuclease was able to destroy the transforming activity. At the same time the physical study (vis-

cosity, ultracentrifugation) revealed that the substance was in highly polymerized form.

The conclusion that the transforming principle is DNA was criticized mainly on the following grounds: (1) The chemical methods were not sensitive enough to exclude the presence of an impurity (protein?) which might be responsible for the activity. (2) Even accepting the evidence that the destruction of DNA by DNAase destroys the activity, it could still be that DNA is not active alone but merely in combination with such a hypothetical protein so that the destruction of either moiety results in inactivation. (3) The non-destruction of activity by a few proteolytic enzymes, by itself, does not prove the non-protein nature of the transforming principle, since proteins are known which resist many proteolytic enzymes.

The methods of purification and analysis have undergone considerable improvement in recent years in partial answer to the first point of criticism. The transforming principle of *H. influenzae* has been purified to the point where it contains less than 0.4% of protein, immunologically active substance, or ribonucleic acid.[19, 21] No loss of biological activity occurred during the gradual removal of impurities. The transforming principle of pneumococcus has now been purified to where it contains less than 0.02% of protein.[22] The amount of DNA sufficient to transform one cell of *H. influenzae* is of the order of 10^{-8} μg. according to one estimate.[21] An impurity of 0.01% would correspond to about six molecules of molecular weight 10^5, or less than one molecule of molecular weight 10^6. Thus one approaches the situation where the probability of the transforming principle being protein in nature can be excluded on purely analytical grounds.

Other studies also offer further indications although not absolute proof that the transforming principle is DNA. Crystalline pancreatic DNAase in concentrations lower than 10^{-4} μg./ml. produces 10-fold decrease of activity within 20 minutes.[21] Upon heating, the temperature at which the transforming principle begins to lose its activity (81°, 1 hour) is the same as the temperature at which the viscosity of the bulk of the preparation begins to decrease.[21] Most known proteins cannot withstand these heating conditions. The pH values (on both acid and alkaline side) at which the activity begins to decrease are again the same as the pH values at which the viscosity begins to decrease. Thus, the active molecule of the transforming principle seems to behave like the average molecule of DNA.

In summary, the evidence favors the view that the transforming principle is DNA; no evidence to the contrary has ever been presented.

The Heterogeneity

A chemist who intends to analyze a purified preparation of the transforming principle will, of course, be concerned with the problem as to whether the preparation represents a single chemical species or a mixture of many. Both the biological and the chemical evidence indicates that the latter may be the case.

If one performs a transformation experiment using the DNA from a donor carrying several transformable characters ("markers"), each of the resulting transformed cells carries as a rule * only a single marker. Doubly transformed cells occur as rarely as predicted from the probability of two independent particles hitting the same cell. Thus, the DNA of each cell seems to consist of many *different* molecules, each of which determines a different hereditary character.

Chemical evidence has been furnished by the important discovery of DNA fractionation. It has been shown that DNA preparations from calf thymus or from *E. coli* can be separated into several fractions each differing in its proportions of individual purines and pyrimidines. The compositions of whole DNA preparations recorded in the literature are therefore not the compositions of individual molecules but the averages of compositions.

The differences in the proportions of purines and pyrimidines (or their nucleotides) may be not the only manifestation of non-identity of individual molecules; other differences may involve a difference in sequence of nucleotides, in length of the molecule which is asymmetrical,[7] or in some other unknown feature.

The Molecule of the Transforming Principle

If, as it seems, the molecules carrying different features are different, it becomes important to estimate how many molecules of one kind are necessary to transform one cell. Such an estimate has been made for *H. influenzae*. This species is more convenient than pneumococcus because the cultures of the latter contain DNAase, which tends to obscure quantitative study. The total amount of DNA necessary to transform one cell of *H. influenzae* was found to be 10^{-8} μg., which is five times more than the total amount of DNA per cell (2×10^{-9} μg.) in this species.[21] If each molecule of DNA is different, then the number of molecules of one kind necessary for transformation would be of the order of five. If this number could be further reduced, support would be gained for the hypothesis that practically all mole-

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* An interesting exception will be discussed on p. 332.

cules of one kind are active. If, in addition, every DNA molecule in the cell is assumed to be a potential transforming principle, then the physical and chemical behavior of the bulk of the DNA preparation are representative of the physical and chemical behavior of the active molecules. Obviously, more evidence is needed before such a view can be fully accepted, but even at the present status the "infectivity" of DNA particles is comparable to that of bacterial viruses.

If one assumes an arbitrary molecular weight of DNA, the above estimates can be expressed in terms of numbers of molecules. For a molecular weight of the order of 5×10^6, the number of DNA molecules of all kinds necessary to transform one cell would be of the order of 1000 and the total number of molecules of DNA in one cell of the order of 200. Even if one assumes that all the molecules of DNA in the cell are functional, it still appears that there are too few of them to serve as determinants of *all* the hereditary characters of the cell. One is tempted to speculate whether the DNA molecules do not determine all the characters or whether one molecule of DNA determines several characters. That the latter may be the case is suggested by the discovery of the multiple transformations in *H. influenzae* [23] and in pneumococcus.[24]

In *H. influenzae* a new strain *ab* has been obtained by exposing cells *b* to the transforming principle from cells *a* (TP_a). The new type produces two capsules (*a* and *b*) and yields a new transforming principle TP_{ab} capable of producing cells *ab* from any susceptible receptor cells. These results cannot be obtained by simply mixing TP_a with TP_b in vitro.

In pneumococcus, the exposure of sensitive cells to the transforming principle from cells bearing two genetic markers, mannitol utilization and streptomycin resistance, produces up to 15 times more cells bearing two markers than would be expected from randomly distributed *independent* transformations. Again, this result cannot be obtained by simply mixing the two transforming principles in vitro.

At present, no evidence exists for the possibility that the two features reside in separate molecules connected by some link such as protein, since extensive deproteinization and autolytic proteolysis did not abolish the linkage; in addition, no evidence for a double molecular weight of the doubly transforming particles has been obtained. Thus it appears more likely that one molecule of DNA can indeed determine more than one genetical marker. Just how one molecule of DNA can reproduce faultily in the presence of another one (in the same locus?) remains entirely in the domain of speculation.

The Resistance to Physical and Chemical Agents

The literature on the effect of physical and chemical agents on DNA is rather voluminous. In most cases the starting material for these studies was prepared by the methods which are now known to give denatured and therefore less resistant DNA. The following discussion will be limited to studies in which the starting material had full transforming activity.

1. *Heat and pH.* Quantitative study of the effect of these factors has been made for the transforming principle of *H. influenzae.*[21] Both the viscosity and the activity of the purified preparation remain unchanged after 1 hour of heating at temperatures up to 81° (in citrate buffer) or after incubation at 23° between pH 5 and 10. The remarkable stability to heat, which is higher than for most known proteins, is similar to the stability of human DNA and calf-thymus DNA when they are prepared by a similar method;[25] this stability is much higher than the values reported in the literature for DNA prepared by the previous, somewhat injurious methods. However, it should be pointed out that the inactivation at the low range of pH could be due to a removal of purines and/or hypothetical hydrogen bonds to these purines. A study[21] of the amount of such "depurination" of the transforming principle reveals that a 100-fold inactivation occurs with the removal of less than two purines per thousand; thus, practically every purine may be necessary for the activity.*

2. *Deoxyribonuclease.* Mammalian and bacterial DNAases in minute amounts destroy the activity of pneumococcal transforming principle.[3] A quantitative study on the transforming principle of *H. influenzae*[21] reveals that crystalline pancreatic DNAase in concentrations of less than 10^{-4} μg./ml. causes a 10-fold decrease of activity within 28 minutes and complete inactivation within 140 minutes. On the other hand, the drop of viscosity at the beginning of inactivation is insignificant. The reason for this discrepancy is still not clear, but the initial change might involve the breaking of a few phosphate bonds, sufficient to destroy the activity but insufficient to cause any decrease in the size of the molecule still held together by hydrogen bonds.

3. *Ionic strength.* A quantitative study of the effects of exposure to various ionic strengths on activity and viscosity has been made for the transforming principle of *H. influenzae.*[21] Previous exposure to lower or higher ionic strengths did not affect the viscosity (as measured

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* This may be true only on the assumption that the active molecule behaves like an average molecule of DNA.

in a standard buffer); on the other hand, the activities were irreversibly reduced by exposure to lower (but not to higher) ionic strength. This permanent damage could be due to the breakage of a few vital bonds, such as hydrogen bonds, during stretching caused by repulsion of anions in the DNA molecule in the absence of salts.

4. *Deamination.* The chemicals whose action on the transforming principle was studied [21] were chosen for two reasons: (1) because the nature and the extent of reaction with DNA could be determined; (2) because the agents themselves have important biological activity, either mutagenic, carcinogenic, or carcinostatic.

Nitrous acid belongs to the first group of reagents. Incubation of the transforming principle with 2 M $NaNO_2$ at pH 5.3 (very mild deaminating conditions) results in a very rapid inactivation. However, the viscosity remains constant indicating that the average DNA molecule is but slightly altered. The extent of deamination corresponding to a 1000-fold decrease of activity is found to be of the order of 0.1%. Thus it seems that practically all the primary amino groups are essential for activity.

5. *Mutagenic agents.* The agents in this very heterogeneous class are grouped together, although the mechanisms of their action may be entirely different; they all seem to prime a phenomenon of great importance and of unknown nature, namely, mutation. It has been suggested [26] that the processes leading to mutation and to death are essentially the same, with the exception that the latter is accompanied by more extensive molecular changes. If this is indeed so, then the *inactivation* of the transforming principle by these agents could be a demonstration of a "too strong mutation."

6. *Ultraviolet irradiation.* The ultraviolet irradiation of the transforming principle (transformation to type specificity) has been studied in *H. influenzae.*[27] The inactivating dose was found to be of the same order of magnitude as the one necessary to inactivate bacterial viruses. The action of ultraviolet irradiation on DNA is now believed to be due to free radicals $H\cdot$ and $OH\cdot$, and to peroxides formed during irradiation of organic molecules. McCarty [28] observed reversible inactivation of pneumococcal transforming principle by ascorbic acid and several other self-oxidizing agents.

7. *Ferrous ion.* Ferrous ion self-oxidizes even in the absence of H_2O_2. In concentrations as low as 10^{-5} M, Fe^{++} alone causes a 10-fold inactivation of the transforming principle of *H. influenzae;* [21] again, no change in viscosity is observed. The exact nature of the damage is still

unknown; an oxidative deamination of a few nitrogenous bases and/or breakage of a few vital labile bonds might be postulated.

8. *Mustards*. Herriott[29] studied inactivation of pneumococcal transforming principle by di-(2-chloroethyl)sulfide (mustard gas). The inactivation of the transforming principle (type specificity) of *H. influenzae* by various nitrogen mustards was studied by Zamenhof et al.[27] In both experiments the inactivating concentrations were as low as 10^{-5} M. For various nitrogen mustards the order of inactivating power for the transforming principle seems to be the same as the order of carcinostatic power of these compounds, thus suggesting a correlation between these phenomena.[27]

Induction of Changes in the Active DNA Molecule

The removal of less than 0.2% of the amino groups of the DNA molecule coincides with a total inactivation of the transforming principle. The treatment applied (deamination or depurination) is not known to act specifically on *few* amino groups or purines of special importance; thus it appears that practically *all* the amino groups are necessary for the activity of the transforming principle or, perhaps, DNA in general. This view gained support when Watson and Crick[30] proposed their model of the DNA molecule. In this model *all* the amino groups are indeed essential for maintaining the integrity of the molecule through the hydrogen bonds.

One might suspect that *any* change in the DNA molecule, whether it affects the pattern of electrical changes (in depurination and deamination) or not, results in a total inactivation or at least in a mutation. The study of the effect of various agents on the transforming principle in vitro thus far has not solved this problem because the extent of changes which can be induced in vitro before total inactivation occurs is rather small and because the nature of the changes is not always known.

It has been shown in special cases that a drastic change in the chemical composition of the DNA molecule can be induced in vivo. When the cells of *E. coli* were grown on a medium containing 5-bromouracil, the highly polymerized DNA from these cells had up to half of the thymine molecules replaced by 5-bromouracil.[31, 32, 33] 5-iodouracil can also replace thymine in the DNA, but to a smaller extent, undoubtedly because it is sterically less similar to the methyl group in thymine. Neither of these groups bears any charge; neither participates in the H-bond structure of the Watson and Crick model.[30] This substitution did not result in any demonstrable changes in the pheno-

type or the genotype of the cells; [32] for on a medium free of 5-bromo-uracil or 5-iodouracil, the substitution is reversed. Thus it appears that certain drastic changes in the DNA molecule may be without consequences, but, on the other hand, the maintenance of the original pattern of electrical changes or H-bonds may be essential for un-changed activity. As a working hypothesis one could even postulate that a mutation is a heritable change in such a pattern of electrical charges.[34]

DNA with bases deaminated or partially absent has never been found in nature. The change of pattern must therefore occur through some other mechanism such as change of sequence or proportion of nucleotides or change of length of the DNA molecule. At present any such process can be visualized only as a rare fault occurring during DNA reproduction. Such a process, then, might be the chemical basis of mutation.

Unstable DNA

The premise that mutation occurs only during DNA reproduction might appear inconsistent with the phenomenon of delayed mutations since in certain cases mutations have been found to occur long after the originally working mutagenic agent had been removed. It has been suggested [35] that in such cases, or perhaps in every case, the role of the mutagenic agent is to bring the gene into an unstable state from which it can either return to the previous stable state or change into a stable mutant gene. This secondary change might no longer require the presence of the mutagenic agent.

It is of interest to note that the process of unstabilization of heredity determinants can also be demonstrated on the transforming principle in vitro. When the transforming principle of *H. influenzae* is subjected in vitro to the sublethal action of heat, H^+ ion, deoxyribonuclease, ultraviolet irradiation, or nitrogen mustard, the surviving (active) molecules become very unstable to heat or even to storage in the cold, under conditions entirely harmless for intact molecules.[36] Study of the kinetics of inactivation of the transforming principle by heat indi-cated that at least two reactions are involved: the unstabilization and the actual inactivation.

When the transforming principle, made very unstable by heat treat-ment or by mustard treatment, was used for the transformation experi-ment, it was found to reproduce as completely stable. Thus, the injury to DNA (unstabilization) induced in vitro was not retained on repro-duction; the change was therefore not a "mutation in vitro." However,

such unstabilization processes may be actually as important as mutations because the injury may determine the part of the molecule where the fault in the reproduction (i.e., mutation) is most likely to occur.[34]

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