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ADVANCES IN

PROTEIN CHEMISTRY

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VOLUME II

Analytical Chemistry of the Proteins

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Contents

1. Introductory

An account of protein analysis is probably best introduced with a discussion of the purpose of the analysis. Work in recent years in the various fields of science grouped around biochemistry has demonstrated both the physicochemical homogeneity and specificity of the proteins and the multitude of their active, specific roles in living organisms. Attention has consequently been focussed on the problem of their detailed structures. In elucidating these, exact knowledge of the nature and number of the amino acid residues composing proteins plays a similar role to knowledge of the nature and number of the component atoms in structural studies

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of simpler molecules. The techniques of amino acid analysis are not yet, however, as reliable as those of elementary analysis. We attempt here to review their present condition, and to indicate probable directions of progress. Much improvement in recent years has undoubtedly been stimulated by the desire, variously, to prove or disprove the speculative hypothesis of protein structure of Bergmann and Niemann, insofar as it deals with overall amino acid composition. Present analytical methods are barely equal to this task, even for particular amino acids. There is good reason to hope, however, that in a few years the problems of analysis of protein hydrolyzates or amino acids will have been solved: the emphasis may well shift to the problem of the relation of the composition of the hydrolyzate to that of the protein from which it was derived (cf. paras. 3, 4).

The present review deals only with this problem of determining the nature and number of the component amino acid residues of proteins and related compounds. No attempt is made to deal with 'higher' aspects of protein structure. It should, however, be pointed out that many of the techniques for separating amino acids are also suitable for the much more difficult tasks of separating the peptides resulting from the partial hydrolysis of proteins; some of these techniques have in fact been developed with this as their primary aim. Studies of partial hydrolysis products are likely to be very fruitful for the detailed elucidation of protein structure (cf. 1).

That reliable methods of amino acid analysis should be available is important also for agricultural, clinical, and nutritional work. In these disciplines, accuracy may often profitably be sacrificed in favor of speed and simplicity of manipulation.

In connection with metabolic studies making use of isotopes it is desirable that methods should be available for isolating every amino acid in a high state of purity and from all kinds of biological material. Special methods are also required for checking the purity of 'pure' amino acids.

We attempt here to review advances that have been made in this branch of protein chemistry during the last 15 years. From the qualitative standpoint, Vickery and Schmidt (2), and from the quantitative, Mitchell and Hamilton (3), have given admirable accounts of the position at the beginning of this period.

We hope, by having made the bibliography as comprehensive as possible, to give this review a value separate from any it may have as an expression of our opinion on the most interesting and valuable directions of technical progress. On these topics we have written at length, while others have been dismissed with a briefness which in some cases does them less than justice.

Block and Bolling (3a) have very recently published a reference work bringing together many methods and results of amino acid analysis of

proteins, chiefly from the nutritional standpoint and grouped according to amino acids. Their work is in many ways complementary to the present review, in which we attempt a critical discussion, grouped according to techniques, of the means by which we may hope to arrive at absolute figures for the amino acid residues constituting individual proteins.

2. The Amino Acids Occurring in Nature

For some years there has been increased reason for believing that only few, if any, new amino acid constituents will be discovered in the better known proteins, or are at all widely distributed among living organisms, and we are confirmed in this opinion by the results of 'two-dimensional' partition chromatography (see para. 5.3.4) applied to hydrolyzates of a variety of protein materials. It seems certain, however, that numerous new amino acids will continue to be found that have a limited distribution — particularly in higher plants, fungi, and micro-organisms. In the present section of this review we discuss evidence bearing on this subject subsequent to Vickery and Schmidt's very full review (2) and Dunn's supplementary notes (4), cf. also (5). In the majority of cases, products isolated by procedures not themselves destructive to a postulated precursor may reasonably be regarded as structural components of the intact protein. Doubtful cases, and products derived from altered proteins, are discussed individually below.

Not considering amino acids of abnormal optical form, the only changes required to bring Vickery and Schmidt's 'accepted' list of protein constituents up to date are that threonine should be inserted and hydroxyglutamic acid removed.

Vickery and Schmidt do not mention Leuchs' later work establishing by synthesis that 'naturally occurring' *hydroxyproline* is one of the 2 stereoisomers of γ-hydroxy-*l*-proline (6). The configuration at the γ-C atom of 'natural' hydroxyproline is the only remaining structural problem concerning the 'accepted' protein constituents, and recent work (7, 8) suggests that the —OH and —COOH groups may lie *trans* in relation to the pyrrolidine ring.

The presence of a common '*l*-' configuration at the α-C atom has been demonstrated for the amino acids usually found in nature. The varied experimental approaches on which this conclusion is based fall outside the scope of this article (cf. 9).

Citrulline, $H_2N \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$. There seems no doubt that free *l*-citrulline occurs in nature. Citrulline was first described in water-melon press-juice by Wada (10). It is also probable (11, 12) that it is an important intermediary of animal metabolism. However, the citrulline isolated by Wada (13) from a tryptic casein digest may have

arisen by degradation of arginine residues in peptide linkage. There is reason (14) for disbelieving Wada's statement that proline is the main product on treating citrulline with hot mineral acid. The only other evidence that citrulline occurs in proteins seems to be Fearon's (15) color reaction, characteristic of substituted ureas and given by all proteins that have been tested (cf. 16). The possible occurrence of *carbamic acid*, $H_2N \cdot COOH$, as a protein constituent deserves serious consideration (17). Peptides of this amino acid would presumably give the Fearon reaction. Citrulline (δ-carbamyl-ornithine) is merely a special case of such a peptide. Model experiments (17, 14) suggest that on acid hydrolysis peptides of carbamic acid do not yield CO_2 or NH_3 stoichiometrically, so the CO_2 evolved in acid hydrolysis of proteins (18) does not set an upper limit to the carbamic acid residues possibly present. The whole problem of the possible occurrence of urea groupings in proteins deserves systematic study.

Wada (19) described '*prolysine*,' $HN \cdot CO \cdot CH \cdot (CH_2)_3 \cdot CH(NH_2) \cdot COOH$,

$$OC\text{----}NH$$

as occurring in casein and gelatin hydrolyzates. Nothing further has been published by other workers about this (cf. 20).

Ornithine, $H_2N \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$. This amino acid, like citrulline, is a known metabolite and is a constituent of ornithuric acid. δ-Monoacetylornithine has been isolated from plant material (21). The presence of ornithine in hydrolyzates of alkali-treated or otherwise altered proteins (cf. 22) can be attributed to the breakdown of arginine residues. The ornithine isolated from acid hydrolyzates of tyrocidine (14) and 'gramicidin S' (23, 109a) may also have originated by such a breakdown during autolysis of the parent bacteria. Failure to detect ornithine in protein hydrolyzates may often have been the consequence of inadequate analytical procedures; however, a recent thorough examination of the products of acid hydrolysis of egg albumin failed to reveal any (16).

Canavanine, $H_2N \cdot C(:NH) \cdot NH \cdot O \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$. Dunn (4) gives references to the isolation, proof of constitution and synthesis of this amino acid, which occurs free in soya-bean meal, etc. (cf. also 24–29).

Octopine, $H_2N \cdot C(:NH) \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(COOH) \cdot NH \cdot CH(CH_3) \cdot$ $COOH$. Irvin and Wilson (30) provide a bibliography of this compound which occurs free in octopus and scallop muscle. Syntheses (cf. also 31) have shown its structure. Karrer, *et al.* (32, 33), on the basis of enzyme and model experiments, have been unable to determine the optical configuration of the alanine moiety. The arginine moiety is *l*-arginine.

Hydroxylysine. Early reports of the isolation of a base of this character are mentioned by Dunn (4). Subsequently, Van Slyke and colleagues (34–36) have isolated from hydrolyzates of gelatin a base which may have

this constitution, although its carbon skeleton has not yet been identified. Its dissociation constants have been determined, and it has been shown, on treatment with periodate, to yield half its N as NH_3, together with one molecule of formaldehyde. Van Slyke and colleagues suggest that it is either $\alpha\delta$-diamino-ϵ-hydroxy-caproic acid or δ-hydroxylysine. They have determined it quantitatively in hydrolyzates of a number of proteins (37). The quantities present are small, even in the richest known source, gelatin. We have isolated material agreeing in properties (38) from the 'lysine' base-precipitation fraction of gelatin hydrolyzates by solvent extraction as its NN^1-diacetyl-O-benzoyl derivative.

If the compound is δ-hydroxylysine, it will be interesting to compare the configuration of the δ-C atom with that of the γ-C atom of hydroxyproline, which is perhaps formed *in vivo* from γ-hydroxyornithine, the next lower homologue of δ-hydroxylysine. In this connection, the possible occurrence (39) of a *hydroxyarginine* in clupein is of interest.

Dunn (4) gives references to the isolation, structural characterization and synthesis of *threonine*, $CH_3\cdot CH(OH)\cdot CH(NH_2)\cdot COOH$ (cf. also 40–42). Threonine has subsequently been isolated from myosin (43), an *Aspergillus* autolyzate (44) and from human blood-group A substance (45), and must accordingly be added to the list of 'accepted' protein constituents. Threonine has been recognized through its reaction with periodate (para. 5.6.1) as a very widely distributed protein constituent. Higher homologues of threonine were not detected in a number of proteins after a specific search (38).

The periodate reaction has at the same time made it probable that β-*hydroxyglutamic acid* is absent from casein (46, 47) and it is desirable that this amino acid, at least for the present, should be withdrawn from the 'accepted' list (cf. 5). Bailey, *et al.* (48) sum up the present situation, and suggest a possible explanation of some of the reports of its occurrence (cf. also 49).

Dakin (49a) mentions the isolation from a casein hydrolyzate of material that might be a *hydroxyleucine.*

Jacobs and Craig (50) obtained $\beta\beta$-dimethylpyruvic acid or pyruvic acid together with NH_3 by alkaline degradation of various ergot alkaloids, and suggested that these arose by deaminative degradation of unstable α-hydroxyvaline and α-hydroxy-alanine residues respectively. Since pyruvic acid can result from the alkaline degradation of serine (cf. para. 4) it is simpler to postulate β-*hydroxyvaline* and serine as the precursors of these keto acids (cf. 51). Earlier claims (2, 4, 52; cf. 53–55) to have isolated hydroxy-valine from proteins are inadequately supported, and require, like similar claims in respect of α-*aminobutyric acid* (2, 4, 55, 56; cf. 57), *norvaline* (α-aminovaleric acid) (2, 4, 55, 58) and *norleucine* (α-aminocaproic acid)

(2, 4, 55, 59–63) to be re-investigated by the newer, more specific methods described in para. 5. By the use of the two-dimensional qualitative partition-chromatographic technique referred to there, we have convinced ourselves that they are absent from some protein hydrolyzates so far studied, and a recent special study (63a) throws grave doubt on the claims for the natural occurrence of norleucine.

Wieland and Witkop (7) report the isolation of a *hydroxytryptophan* from phalloidin (a toxic crystalline constituent of the fungus *Amanita phalloides*). They bring forward evidence that its formula is:
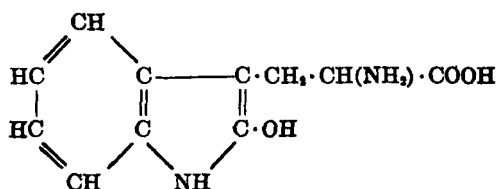
Earlier reports of hydroxytryptophan (cf. 2) are less convincing. The isolation of such a compound has special interest, since it has been postulated as an intermediate in the formation of *kynurenin* in nature (cf. 64, 65, 66). There is no evidence that the latter amino acid is a protein constituent. A synthesis of kynurenin has been reported (67, 68).

The fact that *thyroxine* can be recovered from hydrolyzates of proteins treated with iodine and alkali can be explained by the dismutation of iodinated tyrosine residues. Harington (69) has presented a stimulating review of this subject; in the same article recent data on the natural occurrence of *iodogorgoic acid* are discussed (cf. also 70).

There is now some evidence (71) for the absence of *3, 4-dihydroxyphenylalanine* from a number of proteins (cf. 2).

The view that *glutamine* and *asparagine* residues exist in proteins, inherently probable because of their natural occurrence in the free state, was partly based on the quantitative correspondence between the NH_3 and dicarboxylic amino acid content of hydrolyzates of some proteins. More direct evidence for it has now been obtained by the isolation of asparagine (72) and glutamine (73) from enzymic digests of edestin and gliadin respectively, and by the isolation of *l-αγ-diaminobutyric acid* from acid hydrolyzates of gliadin that had been subjected to Hofmann degradation (74).

β-Alanine, $H_2N \cdot CH_2 \cdot CH_2 \cdot COOH$, is found free in nature and in combination in carnosine, anserine, and pantothenic acid. It has been suggested that it arises by decarboxylation of aspartic acid (75; cf. 76). There is now direct evidence from microbiological tests that it is not a constituent of a number of proteins (77, 77a, cf. 77b).

Whether *cystine* residues exist in intact proteins as such, as *cysteine* or in other forms (*e.g.*, as thiazoline groupings, 78), is a complicated question which cannot be discussed here adequately. Readers are referred to Anson's article in the present volume and to Neurath and colleagues' recent review (79) for discussion of some aspects of the problem; the question of the mode of linkage of serine, threonine, etc. is subject to some of the same considerations. There seems no doubt that cystine residues exist preformed in some proteins (*e.g.*, keratins, insulin) and that in the protein they exhibit, often in enhanced degree, the tendency to dismutation, oxidation, reduction, etc. exhibited by cystine itself. Cysteine residues set free in keratin by reduction may be substituted by various reagents (cf. 80–84); particular interest attaches to the introduction into wool by such means (82, 83) of *djenkolic acid* (see below) and homologous residues.

Cysteic acid, $HOOC \cdot CH(NH_2) \cdot CH_2 \cdot SO_3H$, has been isolated from human hair oxidized with permanganate (96a) and normally occurs in the outer part of the sheep's fleece, where the wool is exposed to light and weather (313).

meso- and *dl-Lanthionine*, $HOOC \cdot CH(NH_2) \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot COOH$, have been isolated from hydrolyzates of wool and other proteins that had been treated with alkali, and have been identified with synthetic materials (85–91). Various views (92–94) have been put forward as to the mechanism by which lanthionine is formed. If the final stage is coupling of the thiol group of a cysteine residue with an α-aminoacrylic acid residue, there seems no reason why the latter should be derived from cystine rather than from serine. Nicolet and Shinn's (95) coupling of benzyl mercaptan with serine residues in alkali-treated silk fibroin (to give *S*-benzylcysteine residues) is of interest in this connection. Similarly, threonine residues might be expected to give rise to *β-methyllanthionine*. Küster and Irion (96) isolated a product from wool that had been treated with sodium sulfide, which they formulated as $HOOC \cdot CH(NH_2) \cdot CH_2 \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ but which from their evidence could equally be β-methyllanthionine. They had difficulty in repeating the preparation.

Selenium-containing material has been obtained from an extract of the vetch *Astragalus pectinatus* grown on seleniferous soil (97). The preparation was formulated as $HOOC \cdot CH(NH_2) \cdot CH_2 \cdot CH_2 \cdot S \cdot CH_2 CH(NH_2) \cdot COOH$ crystallizing with 2 molecules of its *Se* homolog; there was some evidence also for the separate crystallization of the two components. The natural occurrence of selenium in amino acid combination has special interest since selenium has been shown to be incorporated in the proteins of wheat grown on seleniferous soil.

It is desirable that more specific and more quantitative methods of isolation should be employed in future studies of these thio- and seleno-

ethers. The methods so far used depend entirely on the insolubility of the substances in aqueous media. It is also desirable that the structure of their carbon skeletons be rigorously established. Schöberl (97a) has made use of the insolubility of the Na salt of NN^1-dibenzoylcystine for separating it from the corresponding lanthionine derivative. He also employed cyanide reduction of free cystine for converting it to water-soluble compounds without affecting free lanthionine.

Partially racemic *l-djenkolic acid*, $HOOC \cdot CH(NH_2) \cdot CH_2 \cdot S \cdot CH_2 \cdot S \cdot CH_2 \cdot CH(NH_2) \cdot COOH$, was isolated by van Veen and Hyman (98) from the djenkol bean after low temperature alkali treatment, and from the urine of persons suffering from djenkol poisoning. The structure suggested by them was confirmed synthetically by du Vigneaud and Patterson (99).

Blumenthal and Clarke (100) have discussed evidence bearing on the possible occurrence of *thiolhistidine* in proteins. They examined the lability of the S of protein hydrolyzates to various reagents, and were unable to account for its behavior in terms of its existing solely in the form of cyst(e)-ine and methionine (cf. also 100a).

Amino Acids of Abnormal Steric Configuration. A number of amino acids stereoisomeric with those normally occurring have been isolated in recent years from hydrolyzates of bacterial and fungal materials. It seems unlikely that the abnormal configurations have arisen by Walden inversion during isolation, and interest attaches to the biological significance of these abnormalities.

l-Hydroxyproline 'B' — having abnormal configuration at the γ-C atom — has been isolated from phalloidin and identified (7) by comparison with a specimen of Leuchs' synthetic material. It is distinguished from the normal stereoisomer by the color reaction with isatin.

The following amino acids having abnormal configurations at the α-C atom have so far been reported:

d-Proline from ergot alkaloids (101, 102).

d-Glutamic acid from capsular material of *Bacillus anthracis* and related organisms (103–105).

d-Leucine (106–108) and probably *d-Valine* (109) from gramicidin.

d-Phenylalanine (14) from tyrocidine and 'gramicidin S' (109a).

3. RACEMIZATION

In such cases as those cited above, it is probable that residues of the *d*- form of the amino acids pre-exist in the original molecule. Where, however, an amino acid is isolated in a partially or completely racemic state, it seems preferable to regard this as having arisen from the optically active form by racemization during hydrolysis or isolation unless evidence

to the contrary (as 109) can be produced. The following discussion is mainly concerned with racemization of ultimate hydrolysis products of proteins by acids, since it is acid hydrolyzates which are usually employed in amino acid analysis, and it is from the products of acid hydrolysis that deductions are often made as to the optical state of amino acid residues in the intact molecule. Treatment by hot alkali causes extensive racemization of free amino acids, and amino acids in peptide linkage are in general still more readily racemized by alkali. We are not aware of any case of racemization by proteolytic enzymes acting in a medium that does not itself effect racemization. In view of the use of acetyl derivatives of amino acids and peptides for analytical purposes, reference should be made to investigations of racemization by acetylating agents (110–116).

A number of workers have disposed in detail of Kögl's claim that normal and malignant tissue proteins can be differentiated by the degree of racemization of amino acids in their acid hydrolyzates. A small degree of racemization seems to be usual in acid hydrolyzates of all proteins (117, 118–120, 121, 121a). This question has also been studied (122, 128a) by the 'isotope dilution' technique (cf. para. 5.2), which is likely to be of considerable value in studying the optical form of compounds obtained by hydrolysis of proteins. We have isolated (123) almost completely racemic phenylalanine from an acid hydrolyzate of wool by a procedure which did not (124) itself bring about significant racemization. There is evidence (125) that, as with alkali treatment, acid treatment racemizes amino acids more readily when they are in peptide linkage than when free. It may prove possible to avoid some racemization by the use of enzymes or by avoiding the application of high temperatures in the early stages of hydrolysis (cf. 109).

Detailed studies have been made of the racemization of free glutamic acid (126–128a) and cystine (129–132). Dunn (4) gives a bibliography of earlier work on the racemization of amino acids by hot mineral acid (cf. also 133).

It has been proposed (118) to determine whether D is substituted for H at the α-C atom of d-enantiomorphs obtained after hydrolysis in water containing excess deuterium. This might indicate whether racemization had or had not occurred in the course of hydrolysis. We are not aware of any experimental data obtained in this way.

The general implication from our present incomplete knowledge is that varying and unpredictable degrees of racemization are to be expected for different amino acids in the acid hydrolyzates of different proteins. Quantitative analytical procedures must be carried out with this in mind.

4. DESTRUCTION AND ALTERATION OF AMINO ACID RESIDUES UNDER CONDITIONS OF PROTEIN HYDROLYSIS

Most of the quantitative analytical procedures discussed below are applied to *hydrolyzates* of proteins. Alteration of amino acid residues during hydrolysis has been demonstrated in particular cases, and in general presents a greater obstacle than does racemization in relating analyses of hydrolyzates, however accurately performed, with the composition of the intact protein. It is usually necessary, when analyzing quantitatively for any protein constituent, to carry hydrolysis so far (i) that all of that constituent is set free and (ii) that any partial hydrolysis products that may interfere with the analysis are destroyed. These conditions are not always compatible with maximum conservation of the constituent in question.

Tryptophan undergoes some destruction whether acid or alkali is employed as hydrolytic agent. In the presence of carbohydrates its destruction by hot acid is usually complete (for bibliography cf. 134, 14). Tyrosine under these conditions is affected also (134) and cyst(e)ine is rendered more than otherwise labile by the presence of carbohydrate (135). Tyrosine may also undergo destruction during alkaline hydrolysis (136). There is a suggestion that in hot acid tryptophan may be less stable in the presence of some other amino acids than when by itself. Access of oxygen may also affect its stability, and certainly affects the quantity of humin produced. It would be reasonable to expect other amino acids also to be altered, if such interactions with tryptophan occur. On acid treatment, tryptophan perhaps gives rise to dicarboxylic amino acid (137). The 'artefact' bands noted on silica gel chromatograms (cf. para. 5.3.4) are of interest in connection with this question of amino acid destruction. Tristram (138) demonstrated destruction of arginine during acid hydrolysis in the presence of carbohydrate. Schein and Berg (121a) found very little destruction of basic amino acids during acid hydrolysis of casein. Destruction of phenylalanine during acid hydrolysis has also been noted (661a).

Serine is destroyed slowly by boiling acid (139–141). This destruction amounts to about 10 per cent under the conditions usually employed for hydrolyzing proteins (140, 142) but may be more for serine residues carrying a phosphoric ester group (139, 143). It has been shown that serine residues in silk fibroin (144) and casein (140) are more labile to alkali than is free serine. Threonine in the free state appears (145) to be more resistant to boiling mineral acid than serine, but some destruction occurs (146) and on autoclaving, this increases (148). Dismutation products of serine by alkali (149; cf. 150) and acid (139, 141) have been studied in some detail. For further data on destruction of hydroxyamino acids by acid, see (147).

Ammonia is liberated in these and other dismutations, and consequently, as is well known, the ammonia content of complete hydrolyzates of proteins always exceeds the 'amide-NH_3'. For determining amide-NH_3 kinetic studies and various milder acid hydrolysis procedures, not destructive of hydroxyamino acids etc. have been employed (18, 142, 151–155).

Warner and Cannan (156–158) have studied several aspects of the destruction of amino acids during alkaline hydrolysis.

Were destruction simply that of the free amino acids under the hydrolysis conditions, control experiments with made-up mixtures of amino acids would permit an approach to the original composition by successive approximation. Such experiments are indeed very useful in providing a measure of analytical errors introduced in the later stages of hydrolysis and in the analysis itself, and should always be employed in quantitative work. But the extent of destruction of residues in the early stages of hydrolysis, when they are still in peptide linkage, may be different and impossible to assess until much more is known of the structure of the protein. Nor are procedures of determination which avoid hydrolysis capable of surmounting this difficulty, since here the reactions of the group that is being determined may be affected by its unknown mode of linkage. This applies to all colorimetric, spectrometric and titrimetric analyses of incompletely hydrolyzed proteins.

Thus obstacles to analytical accuracy are encountered in colorimetric determinations of tryptophan (e.g., 159), while color reactions, titration values, and absorption spectra due to tyrosine residues in different proteins often diverge from those to be expected were all the phenolic groups of tyrosine free (160, 161, 162). Neurath, et al. (79) give other references to apparent linking of amino acid side-chains. However, we regard as too sweeping their conclusion "The side-chain polar groups of the pure native protein are, in general, strikingly and unexpectedly inert toward specific agents for these groups."

It is clear that some species of amino acid are subject to greater analytical errors from these sources than others, but one cannot be certain that any is immune, and there is therefore likely to be uncertainty as to the number of residues of any particular amino acid in a protein molecule when this number is at all large.

The difficulty can to some extent be overcome by analyzing protein hydrolyzates prepared in different ways, and by employing a number of analytical procedures for the same amino acid. A number of concordant results gives confidence in the significance of the figure arrived at. Brand and Kassell (162–165) by adopting this approach have achieved especially valuable conclusions respecting the minimum molecular size and numbers

of residues of particular amino acids in the molecules of certain well-characterized and homogeneous proteins.

The rest of this review is mainly devoted to discussion of quantitative amino acid analysis of protein *hydrolyzates*. In relating the results of such work to the constitution of the intact protein, the considerations brought forward in this and the previous section must never be overlooked.

5. QUANTITATIVE AMINO ACID ANALYSIS

5.1.   GENERAL

Mitchell and Hamilton in 1929 (166) reviewed very fully, as well as critically, the methods then available for the quantitative analysis of protein hydrolyzates. They summed up:

'By the use of all methods available, only half of the nineteen known amino acids can be determined with any degree of accuracy. Methods for the determinations of cystine, tyrosine, tryptophan, glutamic acid and proline have also been developed until the respective amounts of each of these amino acids in a protein may be determined with a fair degree of accuracy.'

During the last fifteen years intensive work has been directed towards the accurate analysis of protein hydrolyzates. Old methods have been critically studied and refined. New methods, based on entirely novel principles, have been introduced. Some few amino acids have been determined with accuracy, within say 2 per cent in a number of proteins, and with important theoretical results. These include arginine, lysine, tyrosine, cyst(e)ine and, recently, glutamic and aspartic acids. The time has nearly arrived, however, when it will be possible to determine with equal accuracy *all* of the amino acids in a protein hydrolyzate. The main types of method which will make this possible can already be outlined, and accordingly the present review is concerned more with analytical technique than with results, most of which remain to be secured. At present, for assessing our knowledge of the amino acid composition of any protein, a separate critical review of the literature is necessary. Published tables of the amino acid composition of proteins are of little value except as bibliographies. Already, however, a considerable number of proteins and protein-like substances have had well over 90 per cent of their N accounted for in the form of amino acids and ammonia. These include clupein (167), wool (168), gelatin (169–172), silk fibroin (173), β-lactoglobulin (173), insulin (173), fibrin (173a), and gramicidin (174). Extensive analytical data on several proteins of blood plasma (174a) are now available also.

There is unfortunately no agreed method of presenting the results of analysis of proteins. But one of the most widely used has been in terms

of grams of amino acid per 100 grams of protein. In view of the possible uncertainty of the meaning of 'dry' protein, and of the difficulty of handling it (cf. 175), and of the fact that many modern methods of measurement depend upon the Kjeldahl nitrogen, amino nitrogen or carboxyl group determinations, we believe that it would be a great advance if editors insisted on results being expressed in terms of grams of N (total N, α-amino-N, guanidino-N, indole-N, etc. for each amino acid) for 100 grams total N in the protein. Stoichiometric relations are thereby more evident, and a check on the completeness of the analysis is possible without making assumptions about the mode of linkage of the residues.

It is then simple, if desired, to express the residues of an amino acid (α-amino-N) as a fraction of the total amino acid residues of the protein (total α-amino-N). The latter may be determined by the ninhydrin-CO_2 procedure (correcting for aspartic acid), or by Chibnall's (176) difference method (i.e., subtracting determined non-α-amino-N from total N) or by other suitable procedure (corrected formol titration, etc.).

The determination of nitrogen is a problem of elementary analysis rather than protein analysis, and the use of a micro-Kjeldahl method is almost universal. Chibnall and colleagues (175) have shown, however, that of recent years the desire to hurry the estimation has led to low and variable results, a longer period of digestion being required than most recently modified procedures have claimed. It is obvious that the error of the N determination will appear in any analytical figures for amino ac ds. However, when properly carried out, the N determination is more accurate than any existing amino acid determination.

The success of micro-analysis in the elementary field is being followed by the introduction of micro-methods into protein analysis. It is probably true, except where fractional crystallization or fractional distillation is employed, that there is no reason why an analysis should require more than a few milligrams of amino acid for highly accurate results. In fact, most of the more revolutionary new methods have required only a few milligrams of protein, and in many cases the quantity used has been merely such as to fit it for the usual micro-Kjeldahl or Van Slyke apparatus, as good separations being readily obtained with far smaller quantities. Qualitative methods have been developed which require only a few micrograms of each amino acid, which should prove of great value in controlling more quantitative procedures since they take only negligible aliquots.

The present trend in amino acid analysis is away from separations involving solid phases, which attain equilibrium slowly, are to some extent unpredictable, and are manipulatively unsuited to repeated fractionation (e.g., fractional crystallization, which is a highly laborious procedure). Amino acids in solution can be made to move in a predictable way in an

electric field. Rapid equilibrium can be obtained in systems depending on ion exchange or adsorption from solution at a solid surface, or on partition between two liquid phases, permitting a chromatographic arrangement to be adopted. Thus the ionophoretic (para. 5.4) and chromatographic (para. 5.3) methods have inherent advantages, in that cumulative purification may be effected with relative ease of manipulation. In combination, they are likely to prove very powerful analytical weapons. They have the additional advantage of being 'comprehensive' analytical procedures, more likely to reveal unsuspected amino acids in a mixture than methods directed to the determination of particular amino acids.

At its present stage of development it is highly desirable, that analysis of protein hydrolyzates should in every case be controlled by repeating the complete analytical procedure with a mixture of amino acids simulating as closely as possible that present in the hydrolyzate. Successive approximation may then be used if necessary. The use of such a mixture is a considerable safeguard against the lamentable results that often arise from blindly applying to the hydrolyzate of one protein an analytical procedure that may have given satisfactory results with the hydrolyzate of a protein of different composition. The recent commercial availability of the amino acids makes this no longer a counsel of perfection. It has often been assumed that 100 per cent recovery from the hydrolyzate of an added sample of the amino acid being determined establishes the precision of the method. This is an extrapolation which is often unjustified.

Another general safeguard may be sought by choice of analytical methods. It is desirable that as many different amino acids as possible be simultaneously determined by *isolative* procedures in the *same portion* of hydrolyzate. (Of course this leads to great economies in the consumption of protein in analysis, which may be important.) By working in this way, any material getting into the wrong end-fraction is only contaminating *one* end-fraction. Were other portions of hydrolyzate analysed for other amino acids, the same impurity might appear several times. By applying further analytical procedures, which may often be semi-quantitative and non-isolative, to aliquots of end-fractions from a single portion of hydrolyzate, it is possible to obtain valuable secondary corrections, as well as to keep close control on the course of the analysis.

It is to be hoped that before long definite standards of accuracy, such as now prevail in elementary analysis, will have been accepted by protein chemists generally for the quantitative determination of amino acids in protein hydrolyzates.

In the following sections, the different analytical procedures are grouped according to their technical form rather than according to the amino acids for which they are employed. The least satisfactory aspects of quantita-

tive amino acid analysis seem at present to be (i) the determination of glycine and hydroxyproline; (ii) the differentiation of leucine and isoleucine. These problems can however be expected to be solved in the near future. We make no attempt to deal at length with minor modifications of the older established methods. Block and Bolling's recent compilation (3a; cf. 177) includes much of this extensive literature. Vickery (178) and Chibnall (176) have presented discussions of the present condition of amino acid analysis and of the deductions which may be made from its results.

5.2. 'ISOTOPE DILUTION'

By adding a substance containing an excess of a particular isotope to a mixture containing that substance with normal isotopic proportions, the quantity of the substance in the mixture can be inferred from the proportion of isotope in the substance after isolation and purification. This method of analysis has several advantages. The error in the determination is independent of the method of isolation of the pure substance or of the yield. Since the yield is unimportant, the difficulty of obtaining pure substances is decreased. Similarly, the proportion of the substance originally present in the mixture is unimportant, and without influence on the accuracy of the analysis. Finally, the errors of the analysis are all calculable. In this, the method has the advantage of almost all other methods at present employed in protein analysis.

Rittenberg and his colleagues have described the preparation, analysis, and stability of N^{15}-containing amino acids (179–181), and the analysis of amino acid mixtures (182) and the calculation of the errors. Analyses for glycine, glutamic and aspartic acids in cattle fibrin are also given. In another paper (183) the glutamic acid and its degree of racemisation in malignant tumours was investigated. Rather surprisingly (cf. para. 3) no significant amount of d-glutamic acid was found. The results of Wieland and Paul (128a), obtained by the same technique, show a more usual degree of racemization of the glutamic acid. The N^{15} method has also been employed by Shemin. See (174a).

Deuterium-rich amino acids have also been used (184), but seem less suitable (cf. 185–188). C^{13} does not yet seem to have been used.

The method in its present state, using N^{15} is capable of analyses with errors of 1 per cent, better probably than any single method of protein analysis in use at present. The apparatus required is a formidable deterrent to its use at present, however.

5.3. CHROMATOGRAPHIC AND RELATED METHODS

Though adsorption has long been used for biochemical separations, and many have noticed (189–200) that amino acids are adsorbed by various

substances, no attempt has been made until recent years to work out quantitative separations. With a view to chromatography elaborate studies of the adsorption on Al_2O_3 and active carbon have recently been made (201–202a). Adsorption on ion-exchange material has also received recent attention (203).

The application of chromatography to water-soluble substances has lagged curiously behind its use for fat-soluble substances. Thus in 1936 a few pages could deal with the chromatography of aqueous solutions (204). This is perhaps due, as was the small use at first made of charcoal, to the unfavorable adsorption characteristics, the adsorption isotherms being far from linear, and this rendering good separations more difficult to obtain (205, 206). Wieland (207) in 1943 reviewed the chromatographic separations of amino acids. He considered the separations under three headings: (i) exchange, (ii) adsorption, and (iii) partition. We cannot do better than to follow this division here.

We have found it convenient to define chromatography as 'the technical procedure of analysis by percolation of fluid through a body of comminuted or porous rigid material irrespective of the nature of the physicochemical processes that may lead to the separation of substances in the apparatus' (208). The chromatographic arrangement can be employed in connection with types of development (cf. para. 5.3.3.) other than the usual 'elution development.'

5.3.1. *Exchange*

Ion exchange materials, first consciously used as natural zeolites for water-softening, consist essentially of insoluble acids (or bases) which can form insoluble salts. Practically any soil or clay possesses these ion exchange properties, and fullers' earths, sometimes acid-activated, are familiar to the biochemist. Numerous synthetic ion exchange materials are now available, many of the recent examples being synthetic resins. Myers (208a) has presented a valuable review of the ion exchange properties of synthetic resins.

The ratio of the proportions of a given pair of ions, in the exchange material and in the solution, depends, no doubt, on as many complicated factors as does true adsorption but also, and overridingly, on the charge. Thus Whitehorn (209) found that a base weaker than $K_{bas} = 1 \times 10^{-7}$ was not adsorbed on permutit from a neutral solution. This dependence on charge and sign of charge explains the sharp separations that is possible to make between dicarboxylic, basic and neutral amino acids. (It should be remembered, however, that it is the charge that is important, not the number of potentially ionizable groups.)

Separation of Basic Amino Acids. F. Turba (210) has described in detail

the quantitative separation of arginine, lysine and histidine from each other and from all the remaining neutral or acid amino acids. He employs two adsorbents, 'Filtrol-Neutrol' and 'Floridin XXF' (both presumably of the fullers' earth type and obtained from H. Bensman, Bremen). A typical separation is shown below:

Mixture 25 mg. each of Histidine, Leucine, Arginine, and Lysine.

12 g. 'Floridin XXF extra' column, developed with 200 ml. water

FILTRATE	ADSORBED
(*histidine+leucine*)	
	Eluted with 100 ml. H_2SO_4-
Evaporated to 10–20 ml.	pyridine mixture. H_2SO_4 and
	pyridine removed. Evap. to
5 g. 'Filtrol-Neutrol' column	10 ml.
developed with 100 ml. water	(*Arginine+lysine*)

FILTRATE	ADSORBED	
(*leucine*)	eluted with 100 ml. mixture	
	of 5 vols. N H_2SO_4 and 1 vol.	
	pyridine. Former removed as	
	$BaSO_4$, latter by evaporation	
	(*histidine*)	
		25 g. 'Filtrol-Neutrol' col-
		umn made up with $M/6$
		KH_2PO_4, developed with 300
		ml. same solvent

FILTRATE	ADSORBED
KH_2PO_4 precipitated from	Eluted with 100 ml. H_2SO_4-
(*lysine*) with alcohol	pyridine mixture. H_2SO_4 and
	pyridine removed.
	(*arginine*)

The test for beginning or end of elution of monoamino acids or lysine is made with 5 drops of eluate, 0.5 ml. 1% ninhydrin solution and 0.5 ml. ($M/3$-pH 7) phosphate buffer.

The quantity of each amino acid is finally estimated by a Van Slyke amino-N determination. The recovery of monoamino acids reaches 100%, of histidine 98–100%, of lysine and arginine 98%. When a mixture of amino acids (alanine, valine, leucine, glutamic acid, serine, tyrosine, cystine and tryptophan) was used in place of leucine, similar recoveries were obtained.

Synthetic resins have been used also for the separation of basic amino acids by Block (211) and others (212). Wieland (213) has shown that a 'basic' Al_2O_3 column (*i.e.*, Al_2O_3 containing Na' ions) will retain arginine and lysine, while histidine and monoamino acids are eluted by a neutral solution. Wieland (212c) has also used the synthetic resin 'Wofatit C'. In its free acid form it will retain lysine, arginine and histidine. As its

potassium salt it will retain only lysine and arginine. A resin column can hold 20 times as much as a 'basic' Al_2O_3 column of the same weight.

Schramm and Primosigh (212d) have used silica gel and found it more convenient than 'Filtrol-Neutrol' for adsorbing the basic amino acids. Elution is readily achieved with HCl. They have found it possible (212e) to make the amino acid bands visible by virtue of their competitive displacement of colored substances from the adsorbent (methyl red, bromthymol blue, Co^{++}).

Archibald (212a) has employed acidic resins for the separation of citrulline from other plasma constituents. Strain (212b) mentions the use of titanium dioxide for adsorbing amino acids.

Separation of Dicarboxylic Amino Acids. Wieland (213–216a) introduced Al_2O_3 (Brockmann) pre-treated with acid for the adsorption of the dicarboxylic acids. The Al_2O_3 acquires a positive charge and holds a number of the ions of whatever acid has been used to activate it. In neutral or acid solution these can be exchanged for other ions. In alkaline solution the charge on the Al_2O_3 is reversed, and thus the acid ions are eluted.

Not only aspartic and glutamic acids are adsorbed by acid-activated Al_2O_3 but also cystine (214). Wieland suggests that this is the result of its insolubility at pH 4–5. But perhaps the rather acid isoelectric point of cystine (pI $= 5.02$) also plays a part. Cystine is elutable by H_2S saturated water, which does not affect glutamic or aspartic acids. Wieland elutes the latter with saturated $Ba(OH)_2$. Most of the humin is left at the top of the columns, which is removed before elution, and apparently contains no glutamic or aspartic acids. The $Ba(OH)_2$ eluate is dealt with by conventional methods. The total dicarboxylic acids for a number of proteins are given (215), in substantial agreement with other authors' figures.

Turba and Richter (217) employ an analogous procedure. They were unable to obtain fully quantitative results using Al_2O_3 activated by N HCl as used by Wieland. By the use of N acetate buffer (pH 3.3) to activate the alumina they were able to get fully quantitative separations of glutamic and aspartic acids from the other acids, the monocarboxylic acids being eluted with water (except cystine, eluted with $N/10$ acetate buffer (pH 3.3)) and the dicarboxylic acids with $N/20$ NaOH. Glutamic and aspartic acids were also separated, using larger quantities of Al_2O_3, the glutamic acid being preferentially eluted with N acetate buffer (pH 3.3). (5 g. Al_2O_3 required for 10–15 mg. dicarboxylic acid for separation from monocarboxylic acid: 30 g. Al_2O_3 required for 5 mg. aspartic acid for separation from 5 mg. glutamic acid.) Wieland and Wirth (216) report a similar, but perhaps more convenient, separation.

Appropriate synthetic resins have also been used for separations of dicarboxylic acids (212).

Cannan (218) has used the polyamine-formaldehyde resin 'Amberlite IR4' (Resinous Products and Chemical Company, Philadelphia) to separate glutamic and aspartic acids from protein hydrolyzates. Rather surprisingly, he does not use it chromatographically, but instead stirs the acid (HCl) hydrolyzate with enough resin (in free base form) to bring the pH to 7. After filtration more acid and resin are added, a total of three treatments being given, leaving only a negligible amount of the dicarboxylic acids unadsorbed. The resin is washed with water, and the dicarboxylic acids eluted with a moderate excess of HCl. The presence of bases in the hydrolyzate increases the quantity of resin and number of treatments required, and in one method put forward, the greater part of the bases are removed previously by precipitation with phosphotungstic acid. The glutamic and aspartic acids are separated by conventional means, the residue in the mother liquor being estimated by the method of Kibrick.

Kibrick (219) used Cannan's method of separating the acids and then estimated glutamic and aspartic acid from electrometric titration without and with formaldehyde (giving the quantity of dicarboxylic acids); the ninhydrin-CO_2 determination (220; para. 5.6.1) gives the proportion of aspartic to glutamic acid. Both Cannan's and Kibrick's figures on various proteins are in reasonable agreement with Chibnall and colleagues' 'classical' figures (221), and the total dicarboxylic acid figures are in excellent agreement.

It seems probable that a chromatographic procedure using not the 'free base' resin, but some salt, say the acetate, would be more convenient and require less resin and smaller volumes of solution, at least where bases had not first been removed from the hydrolyzates.

Separation of Monoamino-monocarboxylic Acids. Wieland (215) showed that acid-activated Al_2O_3 would adsorb the sodium salts of all amino acids from 80–90 per cent alcoholic solution, the amino acids carrying a net negative charge under these conditions, whereas glucose is not held under these conditions. He attributed this adsorption to the more acid character of the amino acids in alcohol. In fact, however, the amino acids are less acidic in alcohol than in water. Presumably the high concentration of alcohol interferes with the reversal of charge on the alumina that the high pH would be expected to bring about. The acids are elutable by water.

Schramm and Primosigh (222) have obtained adsorption on acid-activated Al_2O_3 by weakening the basic group by combination with formaldehyde, so that the amino acids acquire a net charge at neutral pH. In 10 per cent formaldehyde solution the basic groups of glycine and serine are much more affected than those of the other monocarboxylic acids (a difference of about 1 unit of pK_2). A mixture of glycine, serine, alanine, proline, valine, leucine and isoleucine was run on a 10 g. Al_2O_3 column and

developed with 50 ml. of a 10 per cent formaldehyde solution (pH 7–8). The glycine and serine alone were retained in the column and eluted with alkali. The micro-Kjeldahl method showed 100 per cent recovery of (glycine+serine) and 101 per cent recovery of the other amino acids. About 5 mg. (glycine+serine) and 10 mg. other acids were used.

Turba, Richter, and Kuchar (223) have made use of acetic acid-activated Al_2O_3 to separate cystine from alanine, valine, leucine and proline. 50 per cent alcohol-water is used for development, the cystine remaining in the column and being eluted later with $N/20$ NaOH. 10 g. Al_2O_3 is sufficient for 5 mg. cystine; 200 ml. are required for development, 25 ml. for elution.

They have also been able to separate proline, alanine and glycine from valine and leucine on 'Filtrol-Neutrol' using 50 per cent alcohol at pH 5.5 as developing medium. This separation is due, they suggest, to the more basic character of the former amino acids. A 30 g. column of 'Filtrol-Neutrol' handles 5 mg. of each amino acid. The adsorbed acids (proline, glycine, and alanine) are eluted with water.

Proline and alanine they separate on acetic acid-treated Al_2O_3 using 50 per cent alcohol. (50 g. Al_2O_3 for 5 mg. of each amino acid. 140 ml. 50 per cent alcohol is used for development. Alanine is eluted with 100 ml. water.) In all these separations yields of 90–100 per cent are obtained.

5.3.2. *True Adsorption*

Wachtel and Cassidy (224, 225) have used active charcoal for the separation of glycine, leucine, phenylalanine and tyrosine, with excellent yields, except for the phenylalanine and tyrosine.

Tiselius studied the adsorption of amino acids on active carbon, but as his methods are so original, we will discuss them below separately (para. 5.3.3). He showed that different acids are adsorbed to widely different extents, and probably stimulated thereby some of the work described here.

Schramm and Primosigh (222) in the paper quoted above also studied the separation of various monoamino-monocarboxylic acids. In order to obtain quantitative recovery of the amino acids put on, they found it necessary first to poison the carbon with KCN (as suggested by Tiselius) and then to modify its adsorption properties by treatment with 5 per cent acetic acid; the acids were not elutable with 5 per cent acetic acid unless this pre-treatment was performed. (This irreversibility is difficult to understand and it is greatly to be hoped that some theory will replace the present empirical approach to the problem of altering the adsorption properties.) With carbon so prepared the aromatic amino acids phenylalanine, tyrosine, tryptophan were retained and all others eluted with 5 per cent acetic acid. (2 g. of carbon handles 15 mg. aromatic amino acid; 50 ml. are used for
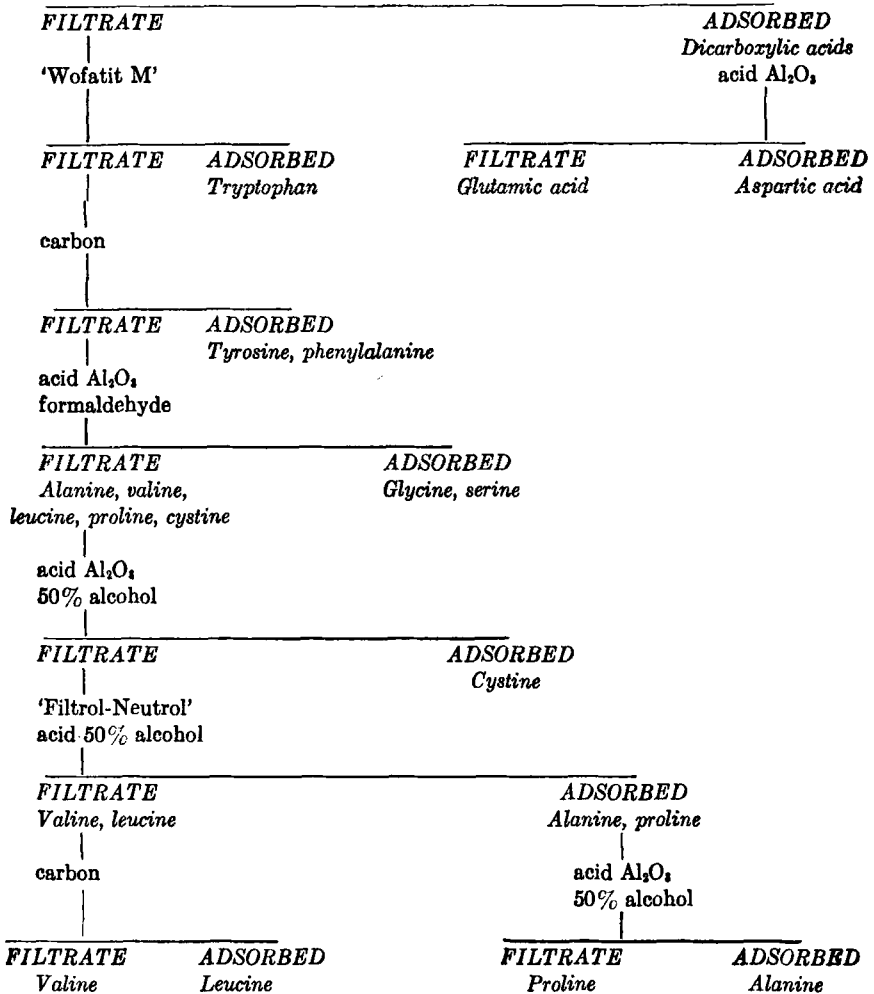
development; 100 ml. 5 per cent phenol in 20 per cent acetic acid eluted the aromatic acids.)

Turba, Richter and Kuchar (223) in the paper described above employ active carbon for the separation of tyrosine and phenylalanine from the aliphatic amino acids (0.5 g. animal charcoal, treated with KCN, employed for 5 mg. of each amino acid). The columns were developed with 100 ml. of water, and the aromatic acids eluted with a pyridine-acetic acid mixture. Valine and leucine were also separated on a 4 g. carbon column, using 5 mg. of each amino acid (valine eluted with 300 ml. $M/15$ phosphate buffer — pH 5.6). Valine and methionine were similarly separated on a 6 g. column (methionine eluted with 400 ml. water, valine with pyridine-acetic acid-water, 10:0.5:100). The recoveries on these last pair of separations ranged from 93–98 per cent, lower values than obtained with other separations, perhaps because of the absence of pre-treatment of the carbon with acetic acid. (This might well, however, have interfered with the separations as they carried them out.) See also (223a).

They have found that the synthetic resin 'Wofatit M' is a specific adsorbent for tryptophan from other monoamino-monocarboxylic acids. (8 g. of 'Wofatit M,' pre-treated with $N/5$ acetic acid is required for 5 mg. tryptophan; developed with 200 ml. water; the tryptophan is removed with aqueous pyridine or alcohol, and is contaminated with some nitrogenous material from the resin. This may be removed by a 'Filtrol-Neutrol' column.)

Turba and his collaborators suggest the following scheme, based on the various chromatographic separations so far outlined, for the analysis of amino acid mixtures:

Mixture of Amino Acids

'Floridin XXF'

FILTRATE			ADSORBED
			Arginine, lysine
			'Filtrol-Neutrol'
'Filtrol-Neutrol'			

FILTRATE	ADSORBED	FILTRATE	ADSORBED
	Histidine	*Lysine*	*Arginine*

acid Al_2O_3

```
FILTRATE                                                        ADSORBED
   |                                                         Dicarboxylic acids
'Wofatit M'                                                     acid Al₂O₃
   |                                                               |
   |                                                               |
FILTRATE      ADSORBED          FILTRATE              ADSORBED
   |          Tryptophan        Glutamic acid         Aspartic acid
   |
carbon
   |
   |
FILTRATE      ADSORBED
   |          Tyrosine, phenylalanine
acid Al₂O₃
formaldehyde
   |
FILTRATE                        ADSORBED
Alanine, valine,                Glycine, serine
leucine, proline, cystine
   |
acid Al₂O₃
50% alcohol
   |
FILTRATE                        ADSORBED
   |                            Cystine
'Filtrol-Neutrol'
acid·50% alcohol
   |
FILTRATE                                        ADSORBED
Valine, leucine                                 Alanine, proline
   |                                                |
carbon                                          acid Al₂O₃
   |                                            50% alcohol
   |                                                |
FILTRATE      ADSORBED          FILTRATE              ADSORBED
Valine        Leucine          Proline               Alanine
```

Schramm and Primosigh (212d) suggest separation into five groups:

(1) Tyrosine, tryptophan and phenylalanine by adsorption on charcoal (pretreated with acetic acid);

(2) Arginine, lysine and histidine by adsorption on silica gel;

(3) Aspartic and glutamic acids by adsorption on acid-activated Al_2O_3;

(4) Serine, threonine, cystine and glycine by adsorption on Al_2O_3 in presence of formaldehyde;

(5) Alanine, valine, leucine, isoleucine, proline and hydroxyproline are unadsorbed.

They find it necessary to have H_2S present throughout to prevent precipitation and oxidation of cystine.

Karrer, Keller, and Szönyi (226) have used a radically different approach for the separation of the aliphatic amino acids. The methyl esters of the N-p-phenylazobenzoyl derivatives are developed on a basic zinc carbonate column with 5:95 benzene-petrol ether mixture. Coloured bands of the glycine, alanine, leucine and valine derivatives, with the glycine at the top, are formed. No explanation of the anomalous position of the valine has been found. The method at present is not quantitative.

5.3.3. The Work of Tiselius

Tiselius has introduced two important new methods of chromatographic analysis — 'front analysis' and 'displacement development.' He has used principally active carbon as his adsorbent.

Front Analysis. In this method (227–232) there is no development as normally employed. Instead, the solution to be investigated is supplied continuously to the column and the effluent is examined by one of the 'Schlieren' methods that have been devised for the ultracentrifugal or electrophoresis techniques (233, 234). The curves obtained by these
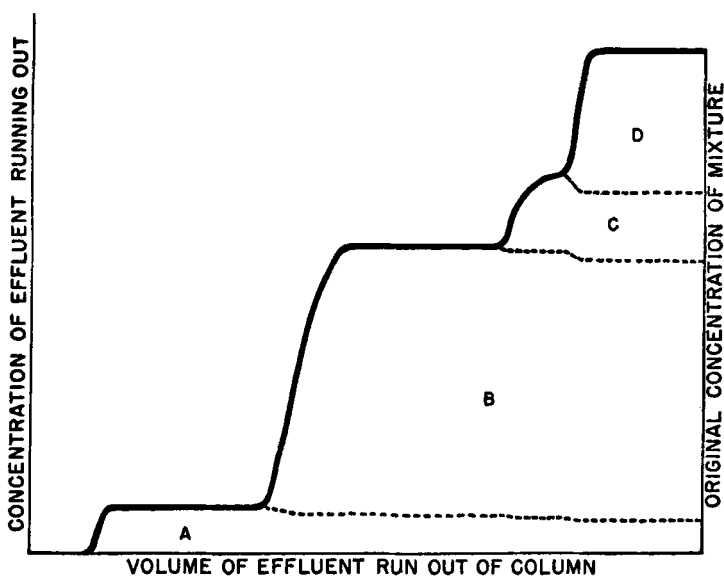
FIG. 1.   Front analysis of mixture A+B+C+D

methods, so familiar from electrophoretic protein investigations, are of course those of *gradients* of refractive index (gradients of concentration), not of refractive index (concentration). Figs. 1, 2, 3 are of refractive
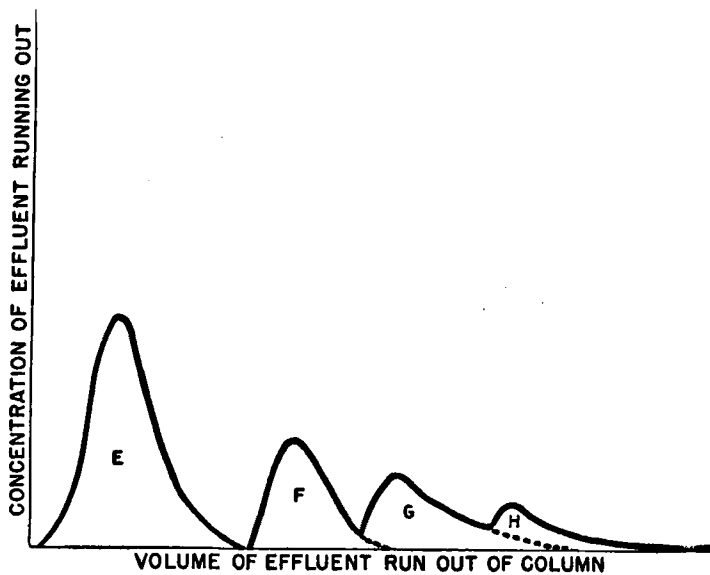
FIG. 2.   Normal or elution development of mixture E+F+G+H
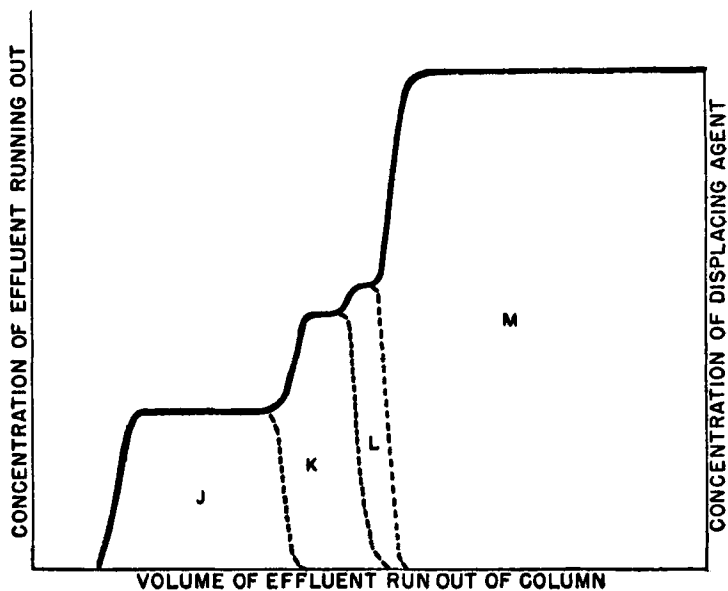
FIG. 3.   Displacement development of mixture J+K+L with displacing agent M

index (concentration) as obtained from the interferometric method mentioned below.

In this way a series of steps in the concentration of the effluent may be observed. The rate of travel of the front of a given solute down the column is a function of the amount adsorbed by unit quantity of adsorbent. Tiselius expressed the adsorption of the various solutes as 'specific retardation volumes,' these being the volumes of liquid that pass through a column containing 1 g. of adsorbent before the particular solute appears in the effluent. If the adsorption isotherm be linear, these specific retardation volumes will be the same for all concentrations of solute in the original solution. If the adsorption isotherm be not linear (if, for instance, as is usually the case, the amount adsorbed is relatively greater at low concentrations), the specific retardation volume changes with concentration, becoming in the case supposed less at high concentrations (cf. 235–237).

In the method of front analysis this increase of retardation volume with decrease of concentration plays a most important part; since when the concentration is low, the rate of travel is slower; the front is thus maintained relatively sharp, diffusion being automatically counteracted. This is in sharp contrast to the usual chromatogram: the less linear the adsorption isotherm, the better the analysis. If, for simplicity, we assume that the various solutes are without influence on the adsorption of one another, then the concentration of each solute in the effluent at any time (assuming the fronts to be absolutely sharp) is either zero or the value in the original solution. In practice the concentration of a given solute will be either zero or higher than the concentration of that solute in the original solution, as each front will tend to elute some portion of the solutes already adsorbed. Fig. 1 diagrammatically shows how the effluent from the column changes with time, the original solution containing solutes A, B, C, D .

In order to be able to detect lower concentrations than those adapted for the 'Schlieren' methods, and to experiment with elution (which, by reason of the decreasing density of the effluent, would cause instability in the cuvette) Tiselius and Claesson (238) have used an interferometric method for refractive index determination. In this, the determination was made in a cell of about 0.1 ml. capacity, through which the effluent flowed on leaving the column. This method should be of value in almost all chromatographic work (in which the solvent is not frequently varied) dealing with colourless substances. Its only disadvantage is the need for good temperature control and the relatively complicated apparatus required; cf. also 238a, 238b.

Displacement Development. Later Tiselius (239–242) showed that by developing a chromatogram with a solution of a suitable strongly adsorbed substance, a steady state was set up in the chromatogram, the band of the

least adsorbed substance being immediately followed and eluted by the band of the next least adsorbed substance, and so on. The concentration of each band automatically adjusts itself until the rate of travel of each front is the same. Thus in practice each band shows a higher concentration than the preceding one. The distance between successive fronts is a measure of the quantity of that material present, for a given concentration of eluting substance. This phenomenon Tiselius calls 'displacement development.' It is of course a familiar experience with chromatograms, it often being observed in the first chromatographing of a mixture that a particular substance runs fast as a narrow band, and that this band, when again run on a fresh chromatogram runs much more slowly and is more diffuse; displacement development being predominant during the first run and normal development during the second (Figs. 2 and 3). Tiselius is the first to make systematic use of this phenomenon.

As in the case of front analysis a non-linear adsorption isotherm is desirable in using displacement analysis, a sharp front depending on this. The sharpness of the front will unfortunately depend not only on the rate of movement of the band and the size of the adsorbent particles and the spaces between them, but also on the difference between the adsorption coefficients of the substances on opposite sides of the front in question. Thus, when very large numbers of substances are to be separated, large volumes will have to be employed in order to obtain a clear gap between the different fronts, most of which cannot be sharp, since the adsorption coefficients of many of the substances will be very similar.

So far, few analyses have been reported using these methods (242) — just separations of a comparatively few synthetic mixtures. Amino acids, peptides and carbohydrates have been reported as being separated by displacement development, and phenol and ephedrine have been used as displacing agents. The methods appear to offer the future possibility of extremely rapid and accurate estimation of many if not all of the amino acids in protein hydrolyzates.

5.3.4. *Partition*

After preliminary studies (243–247) on the separation of acetylamino acids, making use of differences in their partition coefficients between chloroform and water, Martin and Synge (248) introduced the partition chromatogram. In this, in place of the normal solid adsorbent, an inert powder is used which can serve as a mechanical support for a liquid phase. The column is run with a second liquid phase which is immiscible with the first. In such a chromatogram, partition between the two liquid phases replaces adsorption on an adsorbent as the physical property on which the separations depend.

For the separation of acetylamino acids precipitated silica, which can take up about half its weight of water while still remaining apparently dry, was found suitable as the supporting powder. Water containing methyl orange was used as the stationary phase and chloroform as the moving phase. The acetylamino acids are sufficiently strong acids to change the color of methyl orange while quite dilute, so that the passage of a band of acetylamino acid down the column can be observed as a red band travelling against a yellow background. The bands as they run from the column are collected and titrated.

Gordon, Martin, and Synge (249–251) have developed the method to a nearly routine protein analysis procedure, and have found it possible to determine, in the hydrolyzate from 25 mg. of protein, phenylalanine, (leucine, isoleucine), valine, alanine, proline, tyrosine, methionine, and tryptophan. Four solvent mixtures — chloroform containing 1 per cent and 17 per cent of n-butanol and cyclohexane containing 5 per cent and 30 per cent of n-propanol — are employed in development.

The scheme of separation is as shown on page 28.

The slower-moving acetylamino acids cannot be dealt with if all are present. But if only one or two be present they can sometimes be estimated (250, 252, 253).

When 17 per cent butanol-CHCl₃ or 30 per cent propanol-cyclohexane is used, methyl orange is unsatisfactory as indicator, as it is too soluble in the moving phase, is leached from the column, and contaminates the product. Some anthocyanins have been found satisfactory as indicators (249–251) and Liddell and Rydon (254) have synthesized a new indicator for this purpose which is probably the most satisfactory available.

The preparation of the silica seems to be a rather chancy process and is not yet fully understood (251). The difficulty lies in the fact that adsorptions on to the silica can completely alter the color and range of the indicator, and a gel suitable for use with one indicator is not necessarily suitable with another. Two other conditions must be fulfilled — free base must be completely washed from the silica and the silica must not adsorb the acetylamino acids. All the gels so far prepared strongly adsorb the acids from pure chloroform or cyclohexane, and the addition of butanol, etc. is required to reduce the adsorption to negligible proportions. The adsorption is troublesome because it is of the Freundlich type and spreads the bands. Being greater for the faster-moving acids it still further reduces their separation. With higher concentrations of alcohols, the acetylamino acids are rendered more extractable by the organic solvent phase.

The accuracy of the determination is about ±5 per cent except when the amount of a particular amino acid is very small. The errors seem to be involved rather in preparing the acetylamino acid mixture than in

Acetylamino Acids from 25 mg. of Protein

3 g. silica gel

Developed with | ←—1% butanol-CHCl₃—→   ←——17% butanol-CHCl₃—→ |
Band No. | (1)   (2)   (3)   (4)   (5) |

2 g. silica
propanol-cyclo-
hexane

2 g. silica
propanol-
cyclohexane

*Remaining
acids left
in column*

A   A   *Tyrosine*   A

Phenylalanine

2 g. silica
propanol-
cyclohexane

2 g. silica
propanol-
cyclohexane

A   *Tryptophan*   A   *Alanine*

Leucine-isoleucine

3 g. silica
propanol-
cyclohexane

Valine   *Methionine*   A   A   *Proline*

The substances represented as 'A' are artefact acids produced during hydrolysis and acetylation. A mixture of amino acids in proportions similar to those of the protein, when treated in the same way cannot be distinguished in its behavior on the columns from the protein itself.

analyzing it. The errors on the determination of mixtures of pure acetyl-amino acids are very small, and duplicate columns on aliquots from the same acetylation are much closer than when entirely separate determinations are made. Tristram (255; cf. 251) has found that mineral acid present during the final evaporation of the acetylamino acids before putting them on the columns causes esterification and low recoveries, and he has modified the procedure to avoid this. Errors from these sources may to some extent be offset by determining correction-factors with known mixtures of amino acids.

The method has been used for the analysis of wool (248, 249), gelatin

(249, 253), gramicidin (252, 256), tyrocidine (250), myosin and fibrin (173a), and in studying hydrolysis products of methylated proteins (257, 258). Quite recently it has been used for analyzing 'gramicidin S' (109a).

Dakin's widely used method of butanol extraction for the separation of monoamino-monocarboxylic acids from the rest has been shown not to be very specific (259, 260). The determination of the partition coefficients for a number of amino acids by England and Cohn (261) suggested, however, that with a suitable chromatographic technique many of the amino acids should be readily separable (cf. 262). Leland and Foster (263) by butanol extraction have estimated thyroxine and diiodotyrosine in thyroid glands. The difference in partition coefficient is so great that a simple extraction procedure followed by iodine determination gives nearly quantitative results. Consden, Gordon and Martin (264) have now applied partition chromatography to separating amino acids without previous acetylation. In this method, cellulose is used as the support for the stationary phase, and a wide variety of moving phases has been tried. The cellulose is used in the form of filter-paper; a strip of filter-paper bearing some amino acids near the top is allowed to hang from a trough containing the solvent, and the whole is enclosed in a chamber so that the atmosphere is maintained saturated with water and solvent vapor. The solvent syphons out of the trough by capillarity and flows slowly down the strip; the individual amino acids move down at rates determined by the ratio of water to solvent and by the partition-coefficient. When water-saturated n-butanol is used as solvent, the partition coefficients calculated from the rate of movement of the amino acids agree with those directly determined by England and Cohn (261) indicating that the cellulose is playing a passive role as mechanical support only.

The order of the amino acids down the strip is very different with different solvents. By making use of this fact and of the mechanical properties of paper a 2-dimensional separation of the amino acids is possible. A drop of a solution of amino acids is placed near the corner of a sheet of filter-paper (18″×22″). Hanging by one edge from a long trough containing collidine, the chromatogram is allowed to develop for 48–72 hrs. The amino acids are now spread along a line close and parallel to one edge of the paper. The sheet is dried, and this edge is then put into the trough, and a second run is made using water-saturated phenol, in an atmosphere containing NH_3. After development the sheet is again dried. The acids are then spread in a characteristic pattern across the paper, and their position may be revealed by spraying the paper with ninhydrin solution, drying, and heating. Fig. 4 shows a photograph of a chromatogram made in this way.

A pair of solvents that will separate all the amino acids into separate
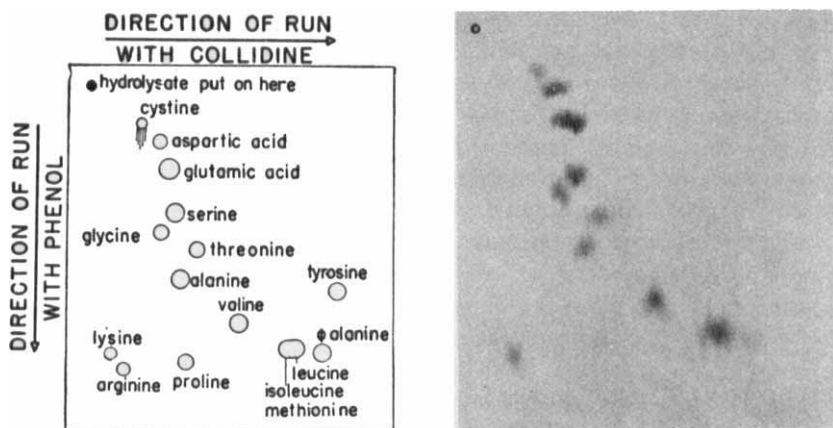
FIG. 4. Two dimensional chromatogram of a wool hydrolyzate
First run with collidine. Second run with phenol. Quantity of protein used: 300 μg.
The proline spot is a strong yellow color, but does not show well in the photograph.

spots has not yet been found, but by a suitable choice of solvents any desired amino acid may be so obtained. Collidine, phenol, n-butanol and benzyl alcohol are among the most useful solvents.

The quantity of material required is remarkably small. 0.2–0.4 mg. of protein is required for each 2-dimensional sheet and less for a 1-dimensional experiment. 1 μg. of amino acid gives a clearly visible spot with ninhydrin. The method has not yet been made quantitative, but is already most useful qualitatively. Cf. (63a.)

Synge (256) has used partition chromatography on raw potato starch with n-butanol as a preparative method, using ordinary chromatogram tubes. Here again the starch has been shown to act merely as a mechanical support for the water phase. Hence information as to the behavior of materials gained upon paper should be available for prediction of their behavior upon starch, and vice versa.

Wieland and Fremerey (264a) have employed partition chromatography with silica gel in phenol-chloroform-water and similar systems for separating the Cu 'salts' of monoamino acids. They describe nearly quantitative separations of valine and alanine and other amino acid pairs. The Cu in the solution issuing from the column is titrated iodimetrically. Here, presumably, adsorption of the amino acids (which in our experience prevents effective partition chromatography of free amino acids on silica gel) is prevented owing to their amino groups being involved in complex formation with Cu. The Cu derivatives have the additional valuable property of being colored.

5.3.5. *Comparison of the Chromatographic Methods*

So far the chromatographic methods have not been sufficiently widely used to permit of any final assessment of their value, although the beginning is most promising. Of the adsorption chromatographic methods, Wieland's acid-alumina method for the dicarboxylic acids is the only one to have been used on proteins; his recoveries of glutamic and aspartic acids seem low compared with Chibnall's values for similar substances. It is very doubtful if Turba's scheme will give the accuracy on protein hydrolyzates that he obtains with simple mixtures, since so many operations are involved. However, further experience will no doubt reduce the labor and permit of a true estimate of the accuracy of the analysis. Karrer's method is also in its infancy, but appears at present less promising than the other methods. Partition chromatography of acetylamino acids is relatively simple and unlaborious, and can be used with small quantities of material. It is not highly accurate, but investigation of the sources of error might well render it so. Partition chromatography on paper and starch have not yet been brought to a quantitative stage but there is no reason to believe this to be impossible. The paper method is already a valuable tool for identification of small quantities of substances, checking purities, and investigating for unknown or doubtful amino acids. The methods introduced by Tiselius have not yet been extensively applied, but should be capable of yielding accurate results. Their chief disadvantage would appear to be the rather elaborate apparatus required.

However, the most important field in protein chemistry for the chromatographic methods is almost certain to be the separation of peptides, and for this it is already clear that successive use of the different methods will give especially valuable separations. The following studies have already been reported: partial hydrolysis products of clupein (on bleaching earths) (265, 266; cf. 267); of gelatin (as acetyl derivatives on silica gel) (253); of wool on paper chromatograms (268); of gramicidin on starch chromatograms (256). With these extremely complicated mixtures it is one of the virtues of the chromatographic methods that they permit some idea to be formed of the problem to be tackled. Thus Consden, *et al.* (268) have evidence of more than 40 substances (other than amino acids) present in considerable quantity in a partial hydrolyzate of wool (3 days in 10 N HCl at 37° C.).

5.4. IONOPHORETIC METHODS

The terms electrolysis, electrophoresis, electrodialysis, electroosmosis, fractional electric transport and ionophoresis have all been used more or less interchangeably.

We propose to use *ionophoresis* to describe processes concerned with the

movement in an electric-field of relatively small ions, *electrophoresis* for movement of large molecules and particles and *electrodialysis* for the removal of smaller ions from large molecules and particles. Ionophoresis is the subject of what follows; we are concerned with electrophoresis and electrodialysis only because of the similarity of the apparatus sometimes employed. A. Löddesol (269) has reviewed the use of electrodialysis for soil analysis: Other reviews (270, 271) cover electrophoresis and electrodialysis. Zhukov (271a) has reviewed the electrokinetic phenomena associated with electrodialysis. The analytical protein methods originated by Tiselius are of course outside the scope of the present article.

In 1909, Ikeda and Suzuki (272) obtained a patent for the preparation of glutamic acid by ionophoresis. They used a 3-compartment cell, glutamic acid being placed in the anode compartment and a protein hydrolysis mixture in the middle and cathode compartments. A potential of 4–6 v. was applied between a corrodible anode (Zn, Fe or Al) and an iron cathode. The membranes were of canvas impregnated with gelatin which was subsequently rendered insoluble with formaldehyde. The hydrolysis of the protein was performed with H_2SO_4 which was removed as $CaSO_4$ and $BaSO_4$ before ionophoresis. This method of using a soluble anode avoids most of the troubles such as change of pH, liberation of chlorine and destruction at the anode that render the ordinary 3-compartment cell method only of value for the basic fraction. No one seems to have followed up this lead.

Foster and Schmidt (273, 274) used a 3-compartment cell, in its essentials like Ikeda's save that the electrodes were carbon, for the preparation of the basic amino acids. Barium hydroxide was added at intervals to maintain the middle compartment neutral, and CO_2 was bubbled through the cathode compartment to prevent excessive alkalinity. After the first run (the end being represented by disappearance of arginine from the middle compartment) the cathode material was treated again, as it still contained much monoamino acid. If the pH of the centre compartment was maintained at 5.5, all the basic amino acids would go to the cathode; if at 7.5, only the lysine and arginine would migrate. After the second run, the bases were found to be substantially free from monoamino acids.

Cox, King, and Berg (275) used essentially the same method, but had parchment paper instead of formolized gelatin, and determined the end point by the rise in resistance of the cell (cf. also 275a).

Albanese (276) has applied a similar method to the estimation of the basic amino acids, using Ruppel's membrane arrangement (see below). Two runs were made, the catholyte from the first run being returned to the centre compartment after neutralization, and no attempt being made to control the pH of the center compartment during the run except indirectly by emptying the anode compartment at intervals. The cathode

material from the second run is finally analyzed into arginine, lysine and histidine by conventional methods. High accuracy in the analysis of a test mixture of amino acids was obtained, and analyses for bases of a number of proteins are given.

Theorell and Åkeson (276a) have developed this technique as a micro method, which can be used for analysis of the hydrolyzate from 20–30 mg. of protein. Their apparatus is 70 mm. long and 65 mm. in outer diameter. The choice of material for the membrane was found to be very important; genuine parchment, after soaking in water and removing the barium sulfate-treated subcutaneous layer, was found to be greatly superior to any other material tested. They report determinations of histidine, arginine, lysine, dicarboxylic acids and amide N in purified crystalline preparations of horse-radish peroxidase and horse-liver catalase, with control determinations on a series of artificial amino acid mixtures of similar composition.

The 3-compartment cell has also been used by Gawrilov and colleagues (277–281) for the separation of amino acids from diketopiperazines. Both middle and cathode compartments are maintained acid. Under these conditions deamination of amino acids occurs at the cathode unless the current density there is kept very low. Only the amino acids go to the cathode compartment.

Gordon, Martin, and Synge (282, 283) have used the 3-compartment cell to study the basic and neutral fractions of partial hydrolyzates of proteins. A number of other workers have used the same arrangement (284–289a).

Ruppel (290, 291) and colleagues, using chromed gelatin as anode membrane and parchment paper as cathode membrane, used a 3-compartment cell to electrodialyze electrolytes from serum. Pauli (292, 293), also preparing salt-free proteins, conducted the dialysis slowly to avoid large pH changes (cf. 294).

These investigators believed that the various membranes possessed a different permeability to anions and cations (270, 294, 295). This differential permeability may conveniently be attributed to two mechanisms:

(a) *Difference of transference numbers* for cations and anions in the pores of the membrane from corresponding values in the free solution. This will be manifested whenever a large fraction of the pore-contents is occupied by the diffuse part of the electrical double layer at the pore wall. Thus in a membrane having a positive ζ-potential the transference number for anions will be increased. This effect will in practice be greatest with fine-pored membranes and in low concentrations of electrolyte (271a, 295a).

(b) *Increased velocity of the ions* migrating in the same direction as the electroendosmotic stream will increase their transference number. The electroendosmotic stream will be decreased by raising the electrolyte

concentration, but will be little affected by the pore diameter. A stream
due to a pressure difference across the membrane will, of course, have a
similar effect, and changes in permeability to ions due to mechanism
(b) may presumably be controlled by applying an adequate pressure.
For membranes of reasonable thickness the transfer of uncharged mole-
cules across the membrane will be due almost entirely to the electro-
endosmotic stream and therefore amenable to control in this way.

Most investigators have employed a cathode membrane having a nega-
tive ζ-potential and an anode membrane having a positive ζ-potential. This
arrangement maintains the pH of the middle compartment more or less
neutral and promotes the elimination of ions from the middle compart-
ment. However, the reverse arrangement should be superior for the
separation of basic, neutral and acidic amino acids, etc. Since the electro-
endosmotic stream will be directed towards the middle compartment
through each membrane, losses of uncharged molecules from the middle
compartment will be greatly decreased. With this arrangement, external
pH control must be adopted and the current efficiency will of course be
lower. This is unimportant in analytical work.

Watson (296) using coarse membranes realized that the effect of the
different membranes was only due to the electroendosmotic pumping of
more or less of the material from one compartment to another. He then
chose his membranes, of rigid siliceous or alundum type, on the grounds
of their mechanical properties and provided a siphon to permit flow of
liquid from catholyte etc. to maintain the pH of the centre compartment
at the required value.

There does not appear to be any clear description of the qualitatively
essentially simple processes taking place in and between the membranes.
Let us consider a single membrane. Three processes are occurring simul-
taneously — diffusion, ionophoresis and endosmosis. Provided enough
other ions are present for diffusion potentials to be small, the rate of trans-
fer by diffusion of a given ionic species (or uncharged molecule) depends
directly on the mobility, the effective cross-section of the membrane and
the difference in concentration on the two sides of the membrane, and
inversely on the thickness of the membrane. The rate of transfer by
ionophoresis depends directly upon the effective cross-section, concentra-
tion, mobility and potential gradient. The rate of electroendosmosis (in
say ml./sec.) depends upon the effective cross-section, the potential of the
membrane relative to the solution and, inversely, on the ionic strength of
the solution and the potential gradient. The application of a suitable
pressure will eliminate any endosmotic flow. Water is also carried by the
ions, and in strong solution the transport of water from this cause is likely
to be greater than that due to endosmosis. If flow of solution occurs, and

the membrane be not very thin, then diffusion may play a negligible role, and the rate of travel of a given ion will be determined by the factors discussed above. Since the flow through the membrane can always be adjusted by the application of an external pressure, it should be possible to control diffusion from say middle to cathode compartment merely by increasing the pressure in the latter. If the solution in the cathode compartment was kept dilute, this should not unduly lengthen the run.

The change of pH that the middle compartment experiences is the result of the addition and loss of H· and OH′ to and from the other compartments, and the creation and destruction of H· and OH′ from weak acids and bases and their ions as the result of the movement of their ions into and out of the compartment. The final pH of the middle compartment after the current has been run for a long time depends upon diffusion and electro-endosmosis of electrolytes from the other compartments.

Loading a given compartment with electrolyte reduces the transport of any given ion (other than those added, of course) because it reduces the potential gradient in that compartment and the neighbouring membranes. Similarly, denudation of a particular compartment of ions increases the potential gradient across it, usually decreasing that across other compartments, since the total potential difference across the cell is limited by practical considerations. Thus usually a run is terminated when a given compartment has been denuded of ions, since the potential gradient across the others is then too low to permit of further useful separation.

The potential that can usefully be applied to a given apparatus is limited by the problem of dissipating the heat produced. This cooling is usually done by cold tubes, fingers, etc. In all the designs of apparatus we have so far seen a very excessive distance between the membranes is allowed, resulting in needless heating of the solutions. It should be possible to design an apparatus in which the current is limited only by the heating within the diaphragm, the diaphragms being as close to each other as mechanical considerations permit, and cooled by the forced circulation through external coolers of the liquids between them. Under these circumstances, the maximum current density should then be used, which would be proportional to the permissible temperature rise in the centre of the diaphragm, the thermal conductivity and effective cross-section of the diaphragm, and to the conductivity of the electrolyte, and inversely to the square of the thickness of the diaphragm. The effective cross-section should clearly be as low as possible, since the occluded part is available to conduct heat away. The thickness should be as small as possible, since contamination by diffusion is proportional inversely to the thickness but the permissible current is proportional inversely to the *square* of the thickness. Finally, and very important, the pressures of all compartments must

be so adjusted that no, or controlled, electroendosmosis occurs. If, however, a pressure is employed to force liquid against the direction of the relevant migrating ions, thickness of diaphragm is not a critical matter.

The pore-size of the membrane must be uniform, or differences in endosmotic pressures will be set up, with resulting mixing notwithstanding a back-pressure; the absolute pore size is however not critical, provided it be below that necessary to give a negligible flow with the differences of pressure present within a single compartment, and large enough to permit the passage of the ions in question.

An ionophoresis apparatus designed on these lines should be capable of giving almost complete separations in one operation, and would moreover be flexible as to the quantity of material handled. Since the distance the ions have to be transported is small, a high potential gradient can be obtained with a small total potential.

Multi-compartment cells have been used by several workers (297–300). Williams and Waterman (301) desired to maintain a multi-compartment apparatus with a gradient of pH along its length, and hoped that any ampholyte would be found at the conclusion of a run in the compartment with pH nearest to the isoelectric point of the ampholyte. Ions and ampholytes were indeed found more or less in the expected place, but by no means sharply confined to the appropriate compartment. Since, however, the maintenance of the range of pH's was the result of diffusion and convection of acid back from the anode compartment and alkali back from the cathode compartment, the main drop of potential occurred across the compartment most denuded of ions, with the result that the gradient was small across other compartments. Hence the lack of sharp divisions is not surprising.

With their dilute solutions electroendosmosis was of course severe, and in later papers Williams and his collaborators (302–305) used membraneless apparatus, of beakers connected by siphons or a tube divided by perforated glass plates. Very high potentials, sometimes 15,000 v., were applied.

The essential trouble with this method, as employed at present, is that if steps are taken to reduce diffusion and convection, the resistance of the centre compartments becomes altogether excessive. A small regular supply of cations at the anode and anions at the cathode reduces the potential required considerably (cf. 300).

This type of arrangement was also used by du Vigneaud and colleagues (306–310) for separations of pituitary hormones; later, apparatus with sintered glass membranes was used, with mercury bubblers to maintain the pressure necessary to prevent electroendosmosis. Spies, *et al.* (311) have

used a modification of the Williams apparatus for a carbohydrate-protein complex. It has also been used for amino acids (312).

Consden, Gordon and Martin (313) have approached the problem in a different way. They have performed the ionophoresis in a thin slab of dilute silica gel. The gel contains a buffer solution, and the protein hydrolyzate is inlaid, in fresh silica gel, in a narrow strip at right angles to the length of the slab. A carbon anode and metal cathode at the ends of the slab are in a current of acid and alkaline buffer respectively, of about the same strength as that in the gel. In this way the pH of the slab remains uniform and unchanging. 1–4 volt/cm is applied for 24–48 hrs. A 'print' may be taken from the slab when required by laying a strip of dry filter-paper on it. Ninhydrin shows the location of the amino acids. Buffer in the gel is necessary since where the pH is such that a given substance is partially ionized, ionophoresis will result in pH changes, since ions are moved and then de-ionize and neutral molecules are left behind, some of which then ionize. Since the buffer behaves similarly if suitably chosen, no pH changes need result in the presence of buffer. Various precautions are required to minimize the effects of electroendosmotic flow and to prevent local flooding or drying of the gel throughout the length of the slab.

The method has been applied to the partial hydrolysis product of wool, and the fact that five fractions with little overlapping have been obtained suggests that this is an advance over previous methods of ionophoresis. It is, however, only adapted for small quantities of material, and the diaphragm type of cell is likely to continue to be the most satisfactory for larger preparations. On the other hand it is capable of a type of separation practically impossible with the diaphragm type: where the dissociation ranges of two ampholytes of weak acids or bases (e.g., glycine and serine, pK_b 9.78 and 9.15) are appreciably different, but nevertheless overlap, at a suitable pH a larger proportion of one than the other carries a net charge (e.g., at pH 8.5 several times as much serine as glycine carries a net negative charge). In the slab of gel its rate of movement is correspondingly faster, and it may be completely separated as a result. It should be noticed that this separation does not make use of a difference of iso-electric point. It may perhaps be remarked here that the small differences in the iso-electric points of the various monoamino-monocarboxylic acids are not likely to be made the basis of a separation, since the proportion carrying a net charge is so small near the iso-electric point. If the ranges of the amino- and carboxylic groups were approximated by alcohol, dioxane or formaldehyde, similar differences in iso-electric point should make separations possible.

Similar separations can be effected in many cases by acid and base

exchange adsorbents and by ionophoresis — perhaps in many cases more conveniently with the adsorbents. Whereas, however, ionophoresis is almost solely concerned with the net charge on the molecule (and very slightly with the molecular volume, which will alter mobility), many structural features may also play a part in the adsorption. Thus the two methods may well be of value when used together. For large scale separations ionophoresis is obviously the more convenient if a suitable diaphragm apparatus can be used.

5.5. PRECIPITATION METHODS: THE WORK OF BERGMANN AND COLLEAGUES

For isolating particular amino acids or groups of amino acids not many new precipitants have been introduced during the last fifteen years by workers other than the Bergmann group, whose work is separately discussed below. Nitranilic acid and cuprous oxide have now definitely established themselves beside the traditional reagents for quantitative work. The main advance, however, in this field has been the recognition, as the result of critical studies, that: (i) the presence of other amino acids increases the solubility of amino acid-precipitant complexes in an often unpredictable way (but cf. discussion of Bergmann's work below); (ii) precipitates may carry down, by formation of mixed crystals, adsorption, or otherwise, amino acids other than those which it is desired to precipitate; (iii) mixed crystallization may render the precipitation of some amino acids in mixtures more complete than would be expected from experiments with the pure amino acid ('co-precipitation'). When working by precipitation methods it is therefore especially important to control analysis by the use of mixtures of amino acids of known composition. The order in which the different operations are carried out is often vital to the success of the analysis, and methods of working may have to be modified in accordance with variations in the amino acid composition of the protein being analyzed. Little attention has so far been paid to the effect of partial racemization (cf. para. 3) of amino acids on analytical results obtained by these methods, except in the case of glutamic acid (314).

It is by precipitation methods that some of the most accurate and generally accepted figures for the amino acid content of proteins have been obtained. Some of these figures are known for certain to be accurate within 2 per cent. Results of this kind are mainly confined to the basic and dicarboxylic amino acids. To achieve them requires great skill and experience from the analyst, as well as an excessive number of man-hours.

Employing precipitation methods, Chibnall and colleagues (315) have evolved a system where 'the cardinal point in the procedure is that no reagent may be introduced into the main hydrolyzate unless it can be quantitatively removed at a later stage without appreciable loss of nitro-

gen, for in an extended scheme such as we have adopted the working losses are cumulative, and can soon amount to several per cent of the total protein nitrogen. It follows that certain reagents either cannot be used at all, or only at a stage where there is reason to believe they comply with this provision.'

To adhere rigidly to the approach recommended above is a counsel of perfection, which would preclude at present the use of certain methods of proved value. Nevertheless, it is an aim which should continually be borne in mind, particularly when planning the order in which analytical procedures are to be used.

Not being ourselves experienced in these methods, we have not attempted to criticise the massive data in the literature on the analysis of particular proteins.

Precipitation of Free Amino Acids, etc.

Direct precipitation of amino acids from the neutralized hydrolyzate (with or without the addition of further salt) has not proved generally useful as a quantitative analytical procedure. Part of the *tyrosine* was determined in silk fibroin by Bergmann and Niemann (316), but the purity of their product has been criticised (317; cf. 318, 315). Fox (319) found commercial (precipitated) *leucine* to be difficult to free from methionine by direct recrystallization, and recommended recrystallization as the formyl derivative. Baptist and Robson (320) have employed salting out for the preparation of *mixed monoamino acids* (cf. 321, 322).

Glutamic acid is usually isolated in the last stage by precipitation of its hydrochloride in the presence of excess HCl. Reference has already been made to studies of the relative solubilities of the different optical forms.

Phosphotungstic Acid

This precipitant in strong acid solution has for long been widely used for the basic amino acids *arginine, histidine,* and *lysine.* Ammonia, if present, is also precipitated. It is not, however, liberated when the phosphotungstates are decomposed with amyl alcohol-ether at acid pH (323). Decomposition with solvents appears preferable to the use of baryta, since it can be accomplished with very little loss of nitrogen (*e.g.,* 315).

Recently Van Slyke and colleagues (324, 325) have made detailed studies of the conditions for optimum precipitation of single and mixed basic amino acids by phosphotungstic acid, and of the composition of the precipitates. The occurrence of mixed crystallization was demonstrated. This leads to better precipitation of *small* amounts of *histidine* or *hydroxylysine* in the presence of the other basic amino acids than would be calculated from the solubilities of their pure phosphotungstates. Once-recrystallized phosphotungstic acid precipitates from hydrolyzates are practically free

from monoamino acids. However, *cystine* is partially precipitated, and should therefore be removed at an earlier stage (see below). The actual residual solubilities of the basic amino acids after phosphotungstic acid precipitation in an analysis of β-lactoglobulin have been studied (315).

Ornithine seems to be difficult to precipitate completely by phosphotungstic acid (326, 327). It would be interesting to know what its behavior is in the presence of other basic amino acids.

Proline phosphotungstate is somewhat insoluble, and has been used (328) for purifying proline.

Flavianic Acid

This acid has been employed for precipitating *arginine* with considerable success. It was introduced by Kossel and Gross (329; cf. 330), who also studied the properties of the flavianates of a number of other bases. It has found widespread use as an almost quantitative precipitant of arginine from complete protein hydrolyzates as its monoflavianate, and in the later stages of determining arginine by other methods. The monoflavianate can be recrystallized free from impurities with very slight losses. Vickery (331) has rendered the procedure more quantitative by conducting an initial precipitation of *arginine diflavianate* from the hydrolyzate and recrystallizing as the monoflavianate. He compares the arginine content of a number of proteins determined in this way and by other methods (cf. 332). Chibnall, *et al.* (315) have studied this procedure and conclude that significant quantities of histidine and lysine may be carried down, and require repeated recrystallization for their elimination (cf. 333). The use of flavianic acid thus appears to be more valuable for the direct determination and isolation of arginine from protein hydrolyzates than for its quantitative removal with a view to subsequent detailed analysis for other amino acids (333). Cf. (333a).

Diflavianates have been employed for the characterization and for the final stages of the isolation of *histidine* (334–336) and $\alpha\gamma$-diaminobutyric acid (337). Dakin (49a) has described glycine flavianate.

Albanese (338) has removed flavianic acid quantitatively by ionophoresis from the mother-liquors from precipitation of arginine monoflavianate. Other workers have usually employed precipitation as its Ba salt or have extracted it with *n*-butanol from acid solution.

Other Organic Acids

A number of other acids have been employed as precipitants for amino acids; they are usually applied for quantitative analysis after the amino acid under investigation has been selectively concentrated from the hydrolyzate by other methods. The slightly soluble salts and complexes have additional

value as reference compounds for the characterization of amino acids. When used preparatively, it is desirable that the acid should be easily removable after decomposition of the salt. Below is given an incomplete but illustrative bibliography of the main precipitants of this class:

PICRIC ACID	Lysine	e.g., 335, 323
	Ornithine	327
	Proline	339–341
	Hydroxyproline	339, 342
	Hydroxylysine	343, 344
(Decomposition of picrates 345)		
PICROLONIC ACID	Histidine	346
	Phenylalanine	347, 320
(Use of bromopicrolonic acid 348)		
NITRANILIC ACID	Glycine	349 (cf. 350)
	Histidine	350, 351, 351a (cf. 352, 315)
REINECKE ACID	Proline	339, 353, 354
	Hydroxyproline	339, 352, 353, 355, 354, 355a, 355b
	Histidine	346
RUFIANIC ACID		356
3,4-DICHLOROBENZENE- SULFONIC ACID	Histidine	357, 358, 358a, 358b
'STYPHNIC ACID'	Lysine	359

('Purpurinsulfonic acid' 360; 'Sozoiodolic acid' 361)

Note by Editor. An important distinction should be pointed out between two classes of acid precipitants discussed here. The very strong acids, of which the aromatic sulfonic acids are typical, may be considered completely dissociated at all pH values. Thus the stoichiometric concentration of the anion (S^-) of such a sulfonic acid may be considered equal to the concentration of the added sulfonic acid or of its sodium salt. If the pH of the system is sufficiently acid, so that all of the amino acid being precipitated is in the form of the cation (A^+), then the solubility product of the slightly soluble salt (A^+S^-) should be well defined in a given medium, and should be nearly independent of moderate variations in pH, provided that the concentrations of other components in the system are held practically constant. This greatly simplifies the definition of conditions and the making of reproducible measurements in such systems. These principles are of great importance in the success of Bergmann's solubility product method of amino acid determination (page 45) in which certain aromatic sulfonic acids have proved particularly valuable reagents.

On the other hand, the situation is considerably more complex when somewhat weaker acids, such as picric acid, are employed as precipitants. Here the acid cannot be regarded as existing completely in the form of the anion, except at hydrogen ion concentrations so small that the amino acid is not completely in the form of the cation. The precise definition of the state of such a system, therefore, is considerably more complex than in the other case, and equilibria between the solid phase and the solution are more likely to show marked sensitivity to pH variations.

Calcium and Barium Compounds

Minor modifications of the method of Foreman (362) — precipitation with alcohol of the calcium salts — have been until quite recently the only methods available for approximately quantitative isolation of *glutamic and aspartic acids*. Bailey, *et al.* (314) (cf. 315, 333) have critically reviewed the developments and modifications which this technique has undergone, and on the basis of their long experience, and of numerous control experiments, have been able to define precautions which must be taken in employing the method for accurate quantitative work. They conclude: 'These findings show without any equivocation that the lime-ethanol method of Foreman, as applied by us and by all other workers in the past, must have given results that were far from quantitative and hence of no value whatsoever from the point of view of the Bergmann-Niemann hypothesis.'

Cupric Compounds

Copper 'salts' of amino acids have been employed for isolation and characterization since the earliest days of protein chemistry. The highly insoluble copper salt is today in general use for the last stages of isolating *aspartic acid* (314).

The different solubilities of the various pure aminoacid copper salts in water and alcohols led to the proposal of solvent extraction of dried copper salts as a method of separating the amino acids in protein hydrolyzates (340). This method of separation is theoretically unsound, owing to mixed complex formation and to the mixed crystallization and mutual solubility effects already mentioned. In addition it has the practical disadvantages associated with all procedures involving quantitative extraction of solids by liquids. Subsequent work (320, 341, 363–366) has demonstrated its limitations. Bailey, *et al.* (314) mention a striking experiment on the effect of Cu serine on the solubility of Cu aspartate (cf. 367, 368). Copper salts have been satisfactorily employed in the preparative isolation of *proline* (341, 353), and *hydroxyproline* (342) and for the elimination of monoamino acids from solution with a view to subsequent work with residual small quantities of basic and dicarboxylic amino acids (315). An isolation of *serine* by a copper salt procedure has recently been reported (368a).

Cuprous Compounds

Cuprous oxide was early (369; cf. 370) employed for improving the appearance of protein hydrolyzates. Its use in analysis is comparatively recent, and may be said to date from Hopkins' (371) use of it for the isolation of reduced glutathione as its slightly soluble cuprous mercaptide. Pirie (372) first prepared pure *cysteine* cuprous mercaptide and showed

that unlike the mercaptides of several related compounds it was insoluble in the presence of excess Cu_2O. Vickery and White (373) employed Cu_2O in excess for quantitatively precipitating the cysteine resulting from the acid hydrolysis of proteins in the presence of tin. They determined total S in the washed mercaptide precipitate, and in this way obtained *cystine* figures in substantial agreement with those obtained by other workers. Rossouw and Wilken-Jorden (374) precipitated cysteine as its cuprous mercaptide with Cu_2Cl_2 from 'dirty' hydrolyzates and decomposed the mercaptide before determining the cysteine colorimetrically. They were the first to notice that, as with glutathione (374a), the cuprous compound by itself is capable of reducing cystine to cysteine (cf. 375). Graff, *et al.* (376) employed Zn for the reduction, precipitated with Cu_2O, and estimated cyst(e)ine by N and S determinations (which were equivalent) on the mercaptide precipitate. Zittle and O'Dell (377) used no extraneous reducing agent. Their total S and 'Sullivan' figures checked with a hydrolyzate of horse serum albumin, but not with one of spermatozoa. Beach and Teague (378) have described an indirect method for determining *methionine*. They determined (a) S precipitated by Cu_2O after Zn reduction of an HI hydrolyzate (*i.e.*, cysteine S); (b) S precipitated by Cu_2O after Zn reduction of an HI hydrolyzate subsequently treated with alkali. The alkali opens the ring of the homocysteine thiolactone, which is then precipitated as homocysteine cuprous mercaptide. The S precipitated thus represents (cysteine S + methionine S). The difference of (b) and (a) is supposed to represent methionine S.

Cuprous oxide precipitation has recently proved very useful as a preliminary treatment for protein hydrolyzates, to free them from cyst(e)ine, and incidentally, humin. Cyst(e)ine and its dismutation products interfere seriously with subsequent analysis for basic, dicarboxylic and other amino acids. Two groups of workers, Lucas and Beveridge (379, 333) and Bailey, *et al.* (314; cf. 315) have independently from somewhat different starting-points arrived at similar methods of working. They use excess Cu_2O as reducing and precipitating agent simultaneously in hydrolyzates largely freed from HCl by previous evaporation. There seems no doubt that cyst(e)ine is quantitatively precipitated by this procedure. The main disadvantage seems to be that a considerable quantity of other amino acids are carried down in the precipitate. Lucas and Beveridge specifically mention glycine, arginine and methionine as contaminants. Bailey, *et al.* deny that arginine is precipitated but mention methionine, and possibly leucine, phenylalanine and other amino acids.

The fact that the procedure may precipitate other amino acids indicates that quantitative analyses of hydrolyzates treated in this way should be interpreted with circumspection. Nevertheless it has already proved a

valuable analytical technique particularly in connection with the determination of glutamic and aspartic acids. What implications the finding of contaminants in the precipitate has for the quantitative cystine and methionine methods mentioned above is uncertain, since cupric copper is present under the Lucas-Bailey conditions and may be responsible for the contamination.

Silver, Mercury, and Cadmium Compounds

Precipitation with silver compounds was employed in the early studies which led to the discovery of the basic amino acids (380). This use of silver still forms the essential part of a system of base analysis (*e.g.*, in chronological order 381–383, 335, 336, 384–394, 352, 395, 315, 333) which has been worked out in great detail and with many modifications. In this connection the studies of Vickery and his colleagues have been outstanding, and have led to very accurate figures for *arginine, histidine* and *lysine* in a number of proteins.

Histidine and arginine are separated from lysine etc. by silver precipitation at alkaline pH (but cf. 395) and histidine is separated from arginine at neutral reaction. The histidine precipitation has been carried out both after and before the alkaline precipitation. The precipitations have been carried out directly on protein hydrolyzates, on protein hydrolyzates partially or completely freed from cyst(e)ine, on basic fractions prepared by phosphotungstic acid precipitation, etc. Arginine has usually been determined gravimetrically as its monoflavianate after removal of Ag.

The histidine fraction may require removal of contaminating cyst(e)ine by $CuCO_3$ or $Cu(OH)_2$ (384). A mercury precipitation is then usually, but not always, carried out, and histidine is then determined gravimetrically as diflavianate, nitranilate or dichlorobenzenesulfonate (see above) or colorimetrically (cf. para. 5.6.4).

Lysine is usually isolated from the silver precipitation mother liquors by successive precipitation as phosphotungstate and picrate.

The methods in their latest forms have been submitted to critical review by Tristram (394) and Chibnall, Rees and Williams (315).

Supplementary references to the mercury precipitation of *histidine* are 338, 396, 381, 397, 398. Mercury precipitation is also used for *methionine* as a preparative procedure (399; cf. 399a) and for *tryptophan* both preparatively and in connection with colorimetric work (cf. para. 5.6.4).

Precipitation of *proline* (and its separation from hydroxyproline) as its cadmium chloride complex was introduced by Kapfhammer and Eck (339) and has subsequently been employed in various isolations of proline (*e.g.*, 340, 341, 353, 400, 363).

The Work of Bergmann and Colleagues

By the use of variously substituted aromatic sulfonic acids, etc. Bergmann and his colleagues (355, 401–407) have introduced a new and powerful method of amino acid analysis. It depends essentially upon the experimental fact that while the solubility of an amino acid, and the solubility product of the amino acid and sulfonic acid, are sensitive to the presence of amino acids or other substances in the solution, the ratio of the solubility products for two, given, different proportions of amino acid and sulfonic acid, is little affected by other amino acids.

By determining the solubility of the amino acid salt of the sulfonic acid, first in a protein hydrolyzate and then in a protein hydrolyzate containing nearly sufficient of the sulfonic acid to precipitate the amino acid salt, the quantity of the amino acid present in the hydrolysate may be calculated, provided that the ratio of the solubility products in each determination be known. A preliminary analysis is required so that the concentration of the amino acid may be adjusted near to that for which the ratio of the solubility product has been determined.

Bergmann and his colleagues argue that since the sulfonic acid salt of a pure amino acid is dissolved, the method must be specific; 'it is obvious that if *l*-leucine is the only amino acid in the solid phase, then it is the only amino acid taking an active part in the equilibrium between solid phase and solution, and the analysis is fundamentally specific for *l*-leucine' (407). This seems to gloss over the question of the formation of a mixed crystal layer, a few molecules thick, containing various amino acids on the surface of the added salt which, in addition to rendering the attainment of equilibrium slow would introduce errors in the solubility determination. These would be influenced by the state of subdivision of the added salt, and there is a possibility of a change of crystal habit, owing to the presence of adsorbed substances involving solution and deposition of an appreciable fraction of the crystals. These criticisms may perhaps be shown experimentally to be without weight, but *l*-leucine should be estimated in the presence of *d*-leucine, isoleucine, valine, and methionine, — all substances more likely to form mixed crystals with *l*-leucine than those included in the test mixtures so far published.

There is a further difficulty introduced by racemization, which normally accompanies hydrolysis. The solubility method of Bergmann deals with one optical isomer, or a racemic mixture, but two analyses are required for a partially racemized mixture. Where the *dl*-amino acid salts are racemic compounds, less soluble than the active salts, analysis of partially racemized hydrolyzates must be difficult or impossible.

Much further work, principally in the investigation of new sulfonic acid reagents, is required before the full range of this method for amino acid

analysis is realized. Already, however, a number of amino acid figures for various proteins have been reported (408, 406), and the new reagents have permitted the isolation of *l*-serine (405), glycyl-*l*-alanine and *l*-alanylglycine (318) from silk fibroin.

Amino acid precipitants introduced earlier by Bergmann, *et al.* (408–410, 355) seem now to have been superseded (cf. 318).

5.6. NON-ISOLATIVE PROCEDURES

Most of the methods of analysis so far discussed lead to the isolation of amino acids either in the free state, or as derivatives from which the amino acid can be regenerated and then tested by any desired method. The non-isolative procedures have the inherent disadvantage that this cannot be done. It is therefore more than ever desirable that control experiments on appropriate mixtures should be employed in connection with these methods. Procedures based upon non-stoichiometric or incomplete reactions require a specially cautious approach, since slight changes in the environment may have large effects. To avoid offence to others, we quote an example from our own work (411) where use of a correction factor not determined with the proper mixture led to an illusorily high figure for the serine content of silk fibroin, the hydrolyzate contributing the glycine and other amino acids which Van Slyke, *et al.* (412) showed to be necessary for stoichiometric recovery of NH_3 (cf. 413, 414).

Nevertheless, many non-isolative procedures are highly specific and accurate, and included among them are valuable methods for determining the aggregate of particular classes of amino acids in mixtures. For some of these methods protein chemists owe a debt of gratitude to Van Slyke and his school which can best, perhaps, be repaid by emulating their critical and well controlled approach to every analytical problem. The various methods employing ninhydrin and periodic acid are destined to play a big part in protein chemistry.

5.6.1. *Methods Involving Chemical Degradation*

Use of Nitrous Acid. Van Slyke's pioneer studies early demonstrated the value of nitrous acid for quantitative determination of amino groups in amino acids and peptides by measurement of the nitrogen gas evolved. Determinations based on the other reaction products (hydroxy acids, unsaturated acids, chloro acids, etc.) have proved less satisfactory. Some are mentioned below.

In 1929 Van Slyke (415) described a manometric modification of his originally volumetric amino-N determination; this is now almost universally employed by protein chemists, and has numerous advantages over the earlier method. Kendrick and Hanke (416; cf. 417–419) have pre-

vented the reaction from giving high values for glycine, cystine and some of their peptides by adding KI to the reaction mixture, and have suggested various minor modifications. Tryptophan amino-N cannot, however, be determined under these conditions. Schmidt (420) has studied the reaction of HNO_2 with amino acids giving anomalous values, by allowing the reaction to proceed at 45°. Kendrick and Hanke (418) give data on the evolution of CO_2 during the reaction.

The behavior of ammonia (420a), ω-amino acids (421, 422), arginine and related compounds (419, 423, 424), uramino acids, etc. (425), lysine and intact proteins (424, 426) has also been studied. Carter and Dickman (427) report positive reaction from a number of non-nitrogenous compounds. Such interference has been too little recognized in the literature, and many workers are unaware of the deleterious effects that such a common substance as alcohol exerts on the amino-N determination. The effect of light on the reaction of tyrosine in the determination has recently been discussed (428, 428a).

The use of Van Slyke's original method for determining *proline* and *hydroxyproline* by difference between total N and amino-N in hydrolyzate fractions free from basic amino acids has in recent years had limited application (*e.g.*, 429) and is likely to give place to direct determination of these amino acids. Olcott (430) has proposed to estimate *glutamic acid* by the decrease in amino-N due to pyrrolidone-carboxylic acid formation on autoclaving a protein hydrolyzate, and gives a bibliography of other attempts to determine glutamic acid by pyrrolidone-carboxylic acid formation. None of these can be regarded as successful.

An ultra-micro modification of the amino-N determination has been proposed (431). Richardson (432) has discussed the relative value of the Van Slyke nitrous acid procedure and various titrations (cf. para. 5.6.3) in work with crude plant extracts.

Rapoport (433) proposed to determine (*glycine+serine*) by oxidation of their nitrous acid deamination products to oxalic acid and (434) serine as glyceric acid by colorimetry after deamination. Kendall and Friedemann (435) determined *alanine* as lactic acid (by $KMnO_4$ oxidation to acetaldehyde) after deamination. Fürth, *et al.* (436) described some necessary precautions in applying the procedure to protein hydrolyzates. High values (437, 438, cf. 439) resulted in recent applications of the method (cf. also 440). Fromageot and Heitz (441a) employed a specific color reaction for determining the acetaldehyde liberated, and prevented serine and aspartic acid from contributing acetaldehyde by the addition of mercuric acetate. Desnouelle (441b) describes some modifications and applications of their procedure. Braunshtein and Bychkov (441c) have shown that this alanine

procedure is not specific, threonine being simultaneously determined (cf. also 441d, 441e, 444).

Fromageot and Heitz (441) proposed to determine *leucine* and *valine* by oxidation of their deamination products to acetone with chromic acid. The two amino acids gave different yields under different conditions of oxidation, from which, it was claimed, the quantities of each amino acid could be calculated. The method has been criticized (442) and modified by Block and colleagues (443, 444) to determine *isoleucine* as well (cf. 445).

It is unlikely that any of these procedures will find general application, since more specific methods, in most cases isolative, are now available for these amino acids. For several of them, microbiological methods of considerable accuracy are now available also (see the review by Snell in this volume).

Use of Ninhydrin, Chloramine T, etc.   The reagents discussed here bring about oxidative degradation of the type:

$$O + R \cdot CH \Big\langle {}^{COOH}_{NH_2} \quad \rightarrow \quad R \cdot CHO + CO_2 + NH_3$$
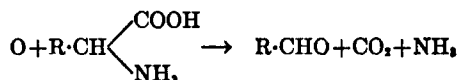
Van Slyke and colleagues (446) give an admirable review and bibliography of the various attempts previously made to apply such reagents quantitatively to the determination of amino acids by estimation of the various reaction products. In the same paper they describe in detail the most generally useful procedure to which they arrived on the basis of their own experiments, namely determination of the CO_2 liberated on heating amino acids under specified conditions with ninhydrin. The particular value of this determination is that free a-amino acids (and proline and hydroxyproline) yield CO_2 whereas ordinary peptides do not. Free amino acids can thus be determined with some confidence in partial hydrolyzates of proteins and of peptides and in biological fluids, etc. Hitherto no satisfactory method had been available for this purpose. An additional use of the Van Slyke ninhydrin-CO_2 method is for the determination by difference of *aspartic acid* in mixture with glutamic acid and other amino acids, since it alone of the usual amino acids yields 2 molecules of CO_2. Examples of the use of the ninhydrin-CO_2 method for the study of the evolution of free amino acids in proteolysis are (446–450), for aspartic acid estimation (446, 437, 451–453; cf. 454) and for *glutamine* (455, 455a).

Van Slyke, MacFadyen and Hamilton (456) describe a titrimetric modification of the ninhydrin-CO_2 determination. Schott, Rockland and Dunn (456a) report high values for certain amino acids in the manometric procedure, which they attribute to the presence of volatile aldehydes. They advocate the use of hydrazine for eliminating this source of error, and dis-

cuss some other aspects of the determination. Techniques for determination of amino acids in plasma (457), blood filtrates (458; cf. 459) and urine (460; cf. 461) have also been reported.

MacFadyen (462) describes the precautions necessary for quantitative determination of the NH_3 evolved in the ninhydrin-amino acid reaction, and some applications of this to the determination of particular amino acids by difference methods.

Only Virtanen and colleagues seem to have attempted to determine the aldehydes evolved in the reaction (463–466). The possibilities of further exploiting this approach, for determining the liberation of particular amino acids during proteolysis by estimating particular aldehydes seems worthy of investigation (cf. 411). Braunshtein and Bychkov (441c) have employed isatin as oxidizing agent, and determine the acetaldehyde liberated (from alanine and aspartic acid) by the nearly specific color reaction with p-hydroxydiphenyl. Quite recently MacFadyen (466a) has stated that *glycine* may be determined specifically through the formaldehyde evolved in reaction with ninhydrin.

As a quantitative colorimetric reagent, ninhydrin has found only limited application (446, 463, 467, 468, 469).

Cohen (470) has developed a specific determination of glutamic acid, employing chloramine T followed by HCl to convert it to succinic acid which he determines by an enzymic method (cf. also 471). A less specific conversion of glutamic acid to succinic acid for analytical purposes (successive use of $NaNO_2$ and $KMnO_4$) has been described by Arhimo and Laine (471a).

Use of Periodic Acid, etc. Periodate, introduced by Malaprade as a specific oxidizing agent for α-glycols, has found widespread use in structural studies of carbohydrates. Nicolet and Shinn (472) discovered that, among the amino acids, periodate attacks in the same way those possessing the structure $R \cdot CHOH \cdot CHNH_2 \cdot R'$, yielding NH_3 and aldehydes thus:

$$IO_4' + R \cdot CH(OH) \cdot CH(NH_2) \cdot R' \longrightarrow IO_3' + R \cdot CHO + R' \cdot CHO + NH_3$$

Of the usual amino acids *serine, threonine,* and *hydroxylysine* may be determined by the volatile aldehyde and ammonia liberated in these reactions. Some other amino acids reduce periodate, but neither NH_3 nor volatile aldehydes are liberated (472, 412). The aldehydo-acids which are supposedly formed at the same time have not so far been much studied (cf. 437).

Threonine gives acetaldehyde on treatment with periodate. This may readily be separated from the formaldehyde resulting from serine, hydroxylysine, etc. by vaporization in a stream of gas at low temperature. This forms the basis of Shinn and Nicolet's (473; cf. 411) method for determin-

ing threonine. Winnick (474) has described a modification employing micro-diffusion.

The formaldehyde (from serine and/or hydroxylysine) remaining in the reaction mixture is determined by Nicolet and Shinn (475) by precipitation as its dimedone derivative. Boyd and Logan (476) distil out the formaldehyde and determine it by a specific colorimetric procedure. We (411) encountered difficulties in quantitative precipitation of formaldehyde-dimedone from protein hydrolyzates, and Mr. M. W. Rees (private communication) informs us that this has been his experience also. He distils out the formaldehyde and determines it by bisulfite titration, having previously removed acetaldehyde according to Shinn and Nicolet (473). He has encountered low yields of formaldehyde when tryptophan is present in the reaction mixture. Neuberger (476a) mentions the possibility of similar interference by histidine.

Van Slyke and colleagues (412) have studied the use for quantitative analysis of the NH_3 evolved from the reaction. Their procedure was worked out specifically for use with hydroxylysine but is equally applicable to serine, threonine, β-hydroxyglutamic acid, etc. For quantitative recovery of NH_3 somewhat different conditions of reaction are needed from those in the aldehyde determinations. A 'Conway' modification has been described (477, 478).

The NH_3 determined in this way is usually equivalent to the formaldehyde+acetaldehyde determined by the methods referred to above. This affords a valuable check that the aldehydes do not originate from carbohydrate, etc. Carbohydrate is so altered by the usual conditions of protein hydrolysis that it does not yield significant quantities of aldehyde (476, 479). Interference by glucosamine is a more serious problem (412, 476).

Conversely, the fact that the ammonia resulting did not exceed the volatile aldehydes formed enabled Nicolet and Shinn (479) to exclude the presence of significant amounts of β-hydroxyglutamic acid in a casein hydrolyzate (cf. para. 2).

Among results so far obtained by the use of periodate are estimations of hydroxyamino acids in:

Egg·albumin (411, 476); Serum proteins (480); Muscle proteins (411); Hemoglobins (476); Casein (473, 474, 475, 476, 411, 479); Collagen, gelatin (473, 475, 476, 481); Elastin (480); Lactalbumin (475, 479); Lactoglobulin (474, 479, 480); Wool, hair (411, 474, 481); Silk proteins (411, 477, 482, 483); Insulin (484); Salmine (476); Various purified enzymes (480); Various plant proteins (411, 474, 485, 481); Myosin and fibrin 173a); Various animal organs (485a); Blood plasma proteins (174a). See also (3a).

Van Slyke, *et al.* (412) have determined hydroxylysine in a number of proteins.

It is questionable if the figures obtained by these methods can be employed for exact stoichiometric calculations. The destruction of hydroxy-amino acids during acid hydrolysis of proteins has already been discussed in para. 4. Results of periodate determinations on partial acid hydrolyzates (481) showed higher values in many cases than on corresponding complete hydrolyzates.

Block and Bolling (486; cf. 445) have employed lead tetracetate in acetic acid for determining threonine. Periodic acid seems, however, to be a more convenient and specific reagent, and the Block and Bolling method has not therefore been widely used (cf. 487). Block and Bolling (444) give the threonine content of a number of materials, obtained presumably by this method.

Tsuverkalov (487a) has determined guanidino groups (as in *arginine*) by the evolution of nitrogen on treatment with hypobromite. Block and Bolling (444) following Arhimo (445a) describe (without exprimental results) the determination of *aspartic acid* by a bromine oxidation procedure; cf. (3a).

5.6.2. *Methods Involving Degradation by Agents of Biological Origin*

Methods of this type have one especially marked characteristic in contrast with most other techniques of amino acid analysis — that is their high specificity in dealing with optical stereoisomers. This is a disadvantage for estimating the aggregate of any particular amino acid in a hydrolyzate, in view of its unknown degree of racemization (cf. para. 3). (Hunter and Dauphinee (488) have suggested that it is on account of such racemization that maximum yields of arginine by the arginase method are obtained before acid hydrolysis of the protein is complete.) On the other hand, optical specificity makes this type of reagent especially useful for the experimental detection of small amounts of one stereoisomer in the presence of large quantities of another. We can expect that, when more such reagents have been introduced, and have had their specificity more accurately defined, they will prove of great use in the microchemical exploration of phenomena connected with racemization and stereoisomerism.

Methods depending on the growth of micro-organisms are reviewed elsewhere in the present volume, and we will not discuss them further here, except to stress the necessity for caution where the possibilities of interference between one amino acid and another or its own optical enantiomorph are so great. (Thus Hegsted and Wardwell, 489, mention a case where *d*-leucine gives 9 per cent of the response given by *l*-leucine, but the response to a mixture of the enantiomorphs is not additive; cf. also

490, 490a). We would emphasize Ryan and Brand's (490) sound recommendation that new microbiological assay techniques should, before they are applied generally, be tested with proteins for which there exist reliable figures obtained by other procedures. We would also ourselves recommend, as for other branches of amino acid analysis, the more general use of complicated known mixtures of amino acids for control purposes.

Not much use has so far been made of suspensions, etc. of living microorganisms for effecting specific degradations stoichiometrically. Such work as that of Woods and Trim (491) on the degradation of amino acids by *Clostrid um welchii* suggests that such methods may have value for rapid and semi-quantitative routine work, where an equivalent enzyme preparation is not available (cf. 492).

Several of the 'biological reagents' discussed here have proved of great value in preparative amino acid chemistry, and especially for the resolution of racemic mixtures. We make no attempt to review this application.

Arginine by Arginase. Hunter and Dauphinee (488) who gave a bibliography of earlier work in this direction showed in great detail and with carefully controlled experiments, that degradation of *l*-arginine by liver arginase, with determination of the resulting urea by a urease method, can serve as a specific method for determining arginine in protein hydrolyzates as accurate as any of the other available methods. Subsequent modifications of their procedure have been proposed as follows:

gasometric urea determination (493)

gravimetric determination of urea with xanthydrol (494, 495)

use in connection with silver precipitation (496)

volumetric determination of urea with xanthydrol (497)

desirability of removing canavanine from urease preparations employed (497a).

Vickery (498) has compared results by the arginase method with those by other methods for a number of proteins.

Liver arginase has a high degree of specificity for *l*-arginine (499, 500). It attacks argininic acid and a number of related compounds slowly (500) and also canavanine (501–504). It apparently attacks arginine residues in peptide linkage which have a free —COOH group, and has consequently been employed in structural studies of protein partial hydrolysis products, which fall outside the scope of this review. A recent study has been made (504a) of the inhibition of arginase by amino acids.

Lysine by Decarboxylase. Gale and Epps (505, 505a) prepared and studied a bacterial decarboxylase specific for *l*-lysine and hydroxylysine. Neuberger and Sanger (506) have extended somewhat the data on the specificity of this material, and have described a few experiments on its application (by manometric determination of the CO_2 evolved) to the

quantitative determination of lysine. A more detailed study by Zittle and Eldred (506a) of the analytical application of the enzyme has recently appeared.

It appears probable (cf. 507, 507a, 507b) that decarboxylase preparations applicable to the determination of further individual amino acids may become available.

d-Amino Acids by d-Amino Acid Oxidase. The mammalian *d*-amino acid oxidase of Krebs (508), which attacks most of the *d*- stereoisomers of the naturally occurring amino acids, has been applied to the detection of *d*-amino acids in acid hydrolyzates of proteins and of normal and malignant tissues (509; cf. 510), to the determination of *d*-amino acids in hydrolyzates of gramicidin and tyrocidine (511), and to the determination of the activity of *d*-peptidases (512).

The specificity of the enzyme has been studied by a number of workers (513–521, 506).

Recently a *d*-amino acid oxidase from a *Neurospora* attacking a more limited range of amino acids has been described (522).

Various *l*-amino acid oxidase systems have been described, but we are not aware that they have been employed for analytical purposes (523–526; 526 b, c, d; cf. 506, 514, 516). The preparation of a 'glycine oxidase' has also been described (526a).

5.6.3. *Titration Procedures*

Much work has been done on the titration under various conditions of amino acids and peptides. Many methods give accurate results upon pure amino acids, but no method appears to give satisfactory and interpretable answers for complicated biological fluids. Except for routine titrations of mixtures of known type there appears to be a considerable advantage in using electrometric means of determining the end-point, rather than indicators.

An excellent review was given by Calvery in 1938 (527). Van Slyke and Kirk (528) and Richardson (529) give useful comparisons of methods.

By following the titration electrometrically and applying corrections determined in a blank run, titrations are possible in aqueous solutions. It is more general, however, to reduce these corrections by performing the titration in a non-aqueous medium, or altering the dissociation range of a particular group, or both.

In solvents of relatively low dielectric constant the dissociation of carboxylic acids and phenols is reduced, since more energy is required to separate the —COO′ and H· than in water (530–533). The dissociation of the amino group is not much altered.

In titration with alkali in strong alcohol using indicators (534, 529, 530)

the sharp end-point is due to a shift in the indicating range of the indicator to over pH 12, and the small amount of alkali required to reach this pH. Differentiation between amino acids and peptides (534) is unsatisfactory. Balson, Earwicker and Lawson (536) were able, using electrometric methods, to estimate leucylglyclglycine in the presence of amino acids, but complicated mixtures could scarcely be handled so easily. Micro-titrations in alcohol have been described (535, 537).

Acetone titrations with HCl (538, 528, 529, 539) have also been used. In this case the shift of the pK of the carboxyl group enables an end point to be reached at comparatively high pH. Rask and Eckles (539a) have studied the solution and glass electrode potentials of amino acids in 95 per cent acetone on addition of butylsulfonic acid.

Perhaps the main difficulty in the use of non-aqueous solvents is in the low solubility of many of the substances to be titrated. It has been claimed (540) that this trouble is largely overcome by using dioxane as solvent. The use of ethylene glycol has been studied by Loiseleur (540a).

Formol Titration. The formol titration as a means of estimating amino or imino groups in amino acids and peptides depends upon a large shift in the "apparent dissociation constant" of the basic group on combination with formaldehyde. This reaction has been extensively studied by Harris (533, 541, 542), Levy (543–546) and others (547–550). For greatest accuracy, the use of a glass electrode is recommended. Dunn and colleagues (551, 552) have shown that precisions of ±0.1 per cent are attainable with pure amino acids. All solutions should be as strong as possible; a pH of 6 is used before addition of formaldehyde, and the end point is about pH 9. The concentration of formaldehyde at the end of the titration is 6–9 per cent (544, 545). Micro-titration methods in which the glass electrode is the titration vessel have been developed (553, 553a). The reactions of formaldehyde with amino acids and proteins are further discussed elsewhere in this volume (p. 277).

Titrations in Acetic Acid. Conant and his colleagues (554–559) studied the titration of bases in glacial acetic acid and developed suitable electrodes for use in this medium. L. J. Harris (560) titrated amino acids and peptides in glacial acetic acid with perchloric acid, using brilliant cresyl blue as indicator. Nadeau and Branchen (561) used indicators (crystal violet) and a chloranil electrode. The titrations show an extremely high reproducibility (see also 562). Toennies and Callan (563) in a thorough investigation determined the effect of water on the blank value and sharpness of end point. They used crystal violet as indicator. They also show that formic acid is of value in the dissolving of the more insoluble acids. They conclude that though highly reproducible, the method is less suited

to purity determinations than the formol titration of Dunn and Loshakoff (551).

Later, Kolb and Toennies (564) followed the course of acetylation by acetic anhydride of amino acids dissolved in glacial acetic acid by titration with perchloric acid. Acetylation was complete in a few hours and was inhibited by the presence of strong acid. Sakami and Toennies (565) showed that the acetylation of hydroxy groups occurred in the presence of strong acid, and that in the presence of excess perchloric acid hydroxy-amino acids could be quantitatively converted to acetoxy compounds. The consumption of acetic anhydride was measured by the addition of an excess of anthranilic acid. The unacetylated anthranilic acid was then titrated with perchloric acid. Toennies and Kolb (566) developed this into a titration method for hydroxy groups. This method gives fairly accurate results and also estimates any arginine and tryptophan as acetyl derivatives of their non-basic nitrogen atoms. Water of crystallization and cystine or cysteine interfere, the latter by reduction of the perchloric acid.

Gas Titrations. Titrations can be carried out in the entire absence of solvent. Bancroft and Barnett (567) studied the uptake of NH_3 and HCl by a wide variety of dry substances including amino acids and proteins. Czarnetzky and Schmidt (568, 569) studied the uptake of NH_3, HCl, CO_2, and H_2S by amino acids and proteins. The end point of 'chemical' combination and beginning of 'adsorption' is taken to be the point at which the pressure rises gradually, rather than remaining constant or rising sharply to a new constant value on addition of successive small quantities of gas. With most of the pure substances a constant pressure region was found, as was to be expected from the phase rule. It is of interest that tryptophan and various peptides take up 2 molecules of HCl under a few mm. pressure. Bancroft (567, pp. 2433 *et seq.*) finding no straight-line portion of the curve for uptake of HCl by zein comes to the remarkable conclusion that zein contains no peptide bonds!

We feel that the significance of a continuous (non-stepwise) change of pressure with uptake of gas has been missed by these workers. A sharp step is only to be expected where the solid phase changes sharply to another solid phase on combination with the gas. The dissociation pressure for a mixed crystal of, say, leucine and norleucine would be expected to be dependent on the amount of NH_3 combined if combination with each isomer proceeded independently. If the crystals of the ammonium salt grew at the expense of the crystal of amino acids, then a constant pressure intermediate between that given by pure leucine and pure norleucine would be expected. The curves shown by Czarnetzky and Schmidt (568) have sharp steps in them, and thus appear to show that leucine and norleucine do not form mixed crystals, which seems remarkable. (Since the optically

active natural products were compared in these experiments with mixed *racemic* leucine isomers of synthetic origin, more experimental work would be required before rigorous conclusions could be drawn.) The same argument of course applies to the interpretation of the gas titration of gelatin hydrolyzate by Belden (570), in which the gradual rise of pressure should not be regarded as evidence of 'adsorption.' Since there can be no question of recrystallizing protein during the titration, and the various nitrogen atoms will have different dissociation pressures for HCl, the curves cannot in these cases either be regarded as evidence of 'adsorption.'

Experimentally, this method is slow and inconvenient, but may be valuable for proteins, being equivalent to extending the titration range to extremely low pH values.

Copper Titration. Kober and Sugiura (571; cf. 572-576) determined the amount of copper dissolved from $Cu(OH)_2$ by amino acids and peptides in a solution of pH 8.8. They showed that amino acids dissolved half a mole of Cu and peptides one mole of Cu per mole. By fractional precipitation of $Cu(OH)_2$ with $Ba(OH)_2$ they decided the proportion of amino acids to peptide in a mixture. Pope and Stevens (577) substituted copper phosphate for $Cu(OH)_2$ and measured only total Cu dissolved. Albanese and Irby (578) have applied the latter method to urine. Both users of the copper phosphate method allude to it as a determination of amino-N, omitting reference to the fact that a mole of peptide dissolves twice as much Cu as one of amino acid (cf. 579). The method gives highly reproducible results, the Cu being estimated iodimetrically, and entails very little work. It seems practically specific for the amino and imino acids and peptides. Histidine alone of the amino acids gives more than ½ a mole of Cu.

5.6.4. *Colorimetric and Spectrometric Methods*

The special advantages of the procedures here described are mainly (i) that they require less time and manipulation than most other analytical methods, this rendering them useful for routine clinical work, foodstuff analysis, etc.; (ii) that they usually consume only minute quantities of material. They can therefore be employed in the analysis of material available only in small quantity. The combined advantages of speed and ultra-micro scale make them useful for controlling the various steps of an isolative operation by analysis of small aliquots of each fraction.

On the other hand it is difficult to convince oneself of the accuracy and specificity of colorimetric methods. Moreover, the extremely close control of conditions which is normally recommended as essential, and the generally polemical character of the literature suggest that considerable experience is necessary before even repeatable results can be obtained.

General Color Reactions for Amino Acids, etc. Reference has already been made (para. 5.6.1) to the use of ninhydrin colorimetrically. It has given rather disappointing results. β-Naphthoquinonesulfonic acid was introduced in 1922 by Folin (580), and has since found wide application in clinical chemistry (581–582b). A recent study of the method (582, 582a) shows that the procedure is capable of giving semi-quantitative determination of amino and imino-N with mixed amino acids, peptides and aliphatic and aromatic amines. It has definite potentialities as an ultra-micro method in protein analysis for quantitatively determining compounds isolated by various analytical procedures, and for controlling these procedures. (Cf. 583.)

Ley and Arends (584) proposed to employ Cu salts of amino acids for this purpose. Alloxan (585) gives widely varying color intensities with the various amino acids. Color reactions with *p*-nitrobenzoyl chloride (586; cf. 587) and with 'bindone' (588) have been reported.

Arginine. Sakaguchi's color reaction for arginine (589) is specific for mono-substituted guanidines (cf. 590, 591) and has proved itself useful in protein chemistry since Weber (591) succeeded in rendering it quantitative. Numerous modifications of the conditions have been proposed (*e.g.*, 592–601a). Results with protein hydrolyzates in good agreement with those obtained by other methods have been described by Thomas, *et al.* (597) and by Brand and Kassell (598). Intact proteins give the reaction, but less strongly than would be calculated from their arginine content (cf. para. 4).

Glycocyamine has also been determined by this reaction. Methods have accordingly been worked out for separating glycocyamine from arginine (599, 602–605.).

Arginine has been estimated colorimetrically by Lang (606) employing acetylbenzoyl (cf. 596).

Histidine. By Coupling with Diazotized Sulfanilic Acid. Histidine and tyrosine give a color under these conditions. Histidine must therefore be freed from tyrosine by one or other of the methods mentioned above before carrying out the determination. The reaction, originally due to Pauly, was first applied quantitatively by Koessler and Hanke (607, 608). Several modifications have subsequently been proposed [(351a, 609, 610, 614, 600, 611, and 612 (carnosine)].

By Color Reaction with Bromine. Knoop's color reaction with bromine for histidine (cf. especially 612–615 for data on its specificity) was rendered quantitative by Kapeller-Adler (616; cf. 617) for the determination of histidine in urine (cf. 613, 615, 618, 619, 620) and in proteins. The method has been employed by various workers with protein hydrolyzates, the histidine being first concentrated by one or other precipitation procedure

(*e.g.*, 601, 621–623). Neither this method, nor titrimetric determinations of histidine (and of tyrosine and tryptophan) by their absorption of bromine (624–628) have found general favor among protein chemists.

Glycine. Zimmermann's (629) more or less specific color reaction of *o*-phthaldialdehyde with glycine (cf. 630) was employed quantitatively by Klein and Linser (631), whose procedure was modified by Patton (632; cf. 632a). The method has not found favor generally, and has failed in at least one case (633; cf. 634) to reveal the presence of glycine in a hydrolyzate.

Hydroxyproline and Proline. Lang (635) described a method which purported to determine proline and hydroxyproline. Waldschmidt-Leitz and Akabori (636) showed that Lang's proline control was contaminated with hydroxyproline, and that in fact only hydroxyproline is determined. Pyrrole, formed from hydroxyproline by hypochlorite treatment, was steam-distilled and determined colorimetrically. They gave hydroxyproline figures for a few proteins. The method has not been at all widely accepted. Morse (637) described a color reaction for hydroxyproline. A method employing peroxide oxidation has also been described (638; cf. 601). Guest (639) has proposed a similar method for proline in the absence of hydroxyproline. (Cf. also 639a.)

The Aromatic Amino Acids. Direct Ultra-Violet Absorption Spectrometry. Holiday (640) has reviewed earlier literature on the subject, and has proposed a quantitative micro-method for the determination of tyrosine and tryptophan in proteins. Since the spectrometric determination is done on the undecomposed protein, the method is subject to the limitations discussed in para. 4. However, Crammer and Neuberger (641) found the 'abnormalities' of the egg albumin spectrum to disappear in alkaline solution, and Holiday recommends $N/10$ NaOH as solvent in his method. Results obtained in this way have in fact agreed fairly well with analyses of protein hydrolyzates (cf. also 642, 601).

Phenylalanine gives a characteristic many-banded spectrum. It is difficult, however, to employ this for analysis in the presence of tyrosine and tryptophan, whose absorption bands predominate in this region of the spectrum (643, 644).

The Folin Methods for Tyrosine and Tryptophan. The methods for determining tyrosine and tryptophan in alkaline protein hydrolyzates developed by Folin and his colleagues (645, 646) depend on precipitation of tryptophan as its mercuric complex, and determination of the tryptophan with the Folin-Denis phenol reagent; the tyrosine in the supernatant is determined either with the same reagent, or by the quantitative Millon reaction. Results have been obtained by these methods on a very wide range of proteins, and when adequately controlled the methods have proved reliable

in the hands of a number of protein chemists. Criticisms have however been made (647, 648) of the specificity for this purpose of the Folin-Denis reagent. Lugg (649, 650), particularly, has critically studied the conditions for successful application of the Folin procedures, and most subsequent workers have acted on his recommendations. Lugg determined the tryptophan as well as the tyrosine by the quantitative Millon reaction. Tyrosine may also be determined by this reaction directly in acid hydrolyzates, since tryptophan is usually completely absent from these. Modified conditions for the quantitative Millon reaction have been described (651–652a), as well as photometric adaptations of the Folin-Lugg procedure (653, 654; cf. also 655).

Tsuverkalov (656) described conditions for applying the Millon reaction to determining the tyrosine content of unhydrolyzed proteins (but cf. para. 4). Calvery and colleagues (657) studied the tyrosine and tryptophan colors evolved at different stages in peptic and alkaline hydrolysis of egg albumin.

Other Methods. Tyrosine. A method based on reaction with nitrosonaphthol has been described (658, 658a).

Phenylalanine. The color produced by the reaction of hydroxylamine with nitrated phenylalanine affords the basis of a quantitative determination. Tyrosine, which interferes, is destroyed by preliminary oxidation with $KMnO_4$. The method, due to Kapeller-Adler (659), has recently been further studied by Block and colleagues (660, 661, 661a), and employed by a number of protein chemists (601, 662–666b, 351a).

Hess and Sullivan (666c) report a new colorimetric procedure based on the reduction of nitrophenylalanine to the corresponding amino compound.

Iodogorgoic Acid and Thyroxine. Brand and Kassell (654) describe a complicated colorimetric 'difference' procedure for determining these amino acids and tyrosine separately, which in their hands gave results comparable with the more direct butanol extraction method of Leland and Foster (667; cf. para. 5.3.4).

Dihydroxyphenylalanine. Arnow (651) described a nitrate-molybdate colorimetric method fairly specific for *o*-diphenols. This has enabled him and his colleagues to demonstrate the absence of dihydroxyphenylalanine from various protein hydrolyzates (cf. para. 2).

Tryptophan. The numerous modifications of the various 'aldehyde' procedures that have been proposed for determining tryptophan demonstrate how few workers are satisfied with them. The following aldehydes, *inter alia*, have been employed at comparatively recent dates:

p-dimethylaminobenzaldehyde	668–675 ι
formaldehyde	672, 672a
vanillin	676
glyoxylic acid	677, 678, 675, 679

Shaw and MacFarlane's critical work (678, 675) suggests that many of the inconsistencies may be due to the use of incompletely hydrolyzed proteins (cf. para. 4). Tryptophan residues in different combinations may give different colors (cf. also 680). The glyoxylic acid-copper method is perhaps preferable to the others since it gives concordant results on intact proteins and their alkaline hydrolyzates. See also (680a).

Other color reactions of tryptophan have recently been proposed for its determination (681, 682; cf. 658). It would be premature to attempt to assess the value of these. (Cf. 681a, 682a.)

5.7. METHODS FOR SULFUR-CONTAINING AMINO ACIDS

Cystine and Cysteine

Methods of Hydrolysis. It has been found that the amount of cystine recovered from an insulin hydrolyzate depends greatly upon the acid used for the hydrolysis. When a mixture of formic and hydrochloric acids is used, half as much again cystine may be found as when hydrochloric acid alone is used (683, 684; cf. 685, 686). Baernstein (687) has advocated the use of HI, but this is regarded as inferior to the formic acid mixture by du Vigneaud, *et al.* (683). Most other proteins are certainly not as sensitive to the hydrolysis conditions as is insulin. The use of tin (688) has been condemned by Lugg (689) as leading to low recoveries of cysteine.

It would seem that by careful attention to the details of procedure that several methods may be made to give highly consistent and agreeing results, in the hands of any given workers. However, the differences between the values obtained by various workers are frequently greater than would be expected on the basis of the suggested errors.

Sullivan Reaction. The literature on the Sullivan reaction (690) for cysteine has attained formidable proportions, and a number of modifications have been proposed (691–704); considerable accuracy is claimed for most of them by their authors, though regarded as inadequate by others. The reaction, which is highly specific for cysteine [though colors are given with some peptides of cysteine (705)], gives a red color with 1,2-naphthoquinone-4-sulfonate in an alkaline reducing medium.

Cystine is estimated by previous reduction to cysteine (by cyanide reducing one half (690) or by some other reducing agent reducing all (692, 706)). The Sullivan reaction has been reported as unsuitable for estimating cystine or cysteine in the presence of each other (691, 701). However, this is contested (708, 707). In a late modification (706) Sullivan, Hess and Howard report the former non-equivalence of cysteine and cystine when used as the standard as due to the presence of insufficient reagent, and that with adequate amounts of reagent, cystine and cysteine can be used interchangeably, with a factor of 1 if cyanide is used as the reducing agent,

or with a factor of 2 if sodium amalgam is used. By the use of both methods, both cystine and cysteine can be estimated in the same mixture. Substances inhibiting the reaction are present in urine (690, 696, 697, 702, 701) and studies of interfering substances have been made (699, 704, 709, 710, 711, 711a). Previous precipitation as cuprous mercaptide is recommended by (712, 702, 703; cf. para. 5.5). The method has been applied to chemically altered proteins (713, 714). See also (712a).

Reduction of Phosphotungstic Acid. The blue color obtained by the reduction of phosphotungstic acid (Folin's uric acid reagent) by cysteine or by cystine in the presence of sulfite has been used as a method of estimation by many workers. Lugg (715) greatly improved the previous methods (716–721). Shinohara (722–725) and others (726, 727) made further studies. The rate of development of color is different for different peptides of cystine, and a maximum in color has been found during hydrolysis (728). Kassell and Brand (729) suggest that this is due to more rapid reaction of the peptides and show that the same amount of color is finally developed with all. The method in its later forms (*e.g.*, 729) gives highly reproducible results, and is convenient for the estimation of cystine and cysteine in the presence of each other.

Iodimetric Determination. Okuda (730) oxidized cystine to cysteic acid by bromate in acid solution. Other amino acids interfere with this as a method of estimation. Later (731–733) he titrated the charcoal-decolorized hydrolyzate with KIO_3 in acid solution containing KI, oxidizing cysteine to cystine. By reduction of the cystine by Zn dust (cystine+cysteine) could be measured. Teruuchi and Okabe (734) modified the method slightly (cf. also 734a). Baernstein (735), to avoid losses in charcoal, added excess iodine and used a gasometric method to measure the residual iodine. This however led to high values for cysteine (cf. 736). Lucas and King (737) made a thorough study of iodine uptake by various thiol groups (cf. 738). The iodimetric method has been used for urine (739) and glutathione (740) and widely by Sullivan and Hess. Baernstein (741) recommends the Okuda method on HI hydrolyzates though of course only (cystine+cysteine) can be estimated under these conditions, but humin is absent, and there is no necessity to use charcoal or other decolorizing agent. Very reproducible results are obtained by the Okuda (733) and the Baernstein (741) methods. Titration of cysteine in non-aqueous solvent has been tried (742) and stoichiometric results are claimed (743) in 70 per cent or stronger acetic acid.

Polarographic Determination. Brdička (744, 745) showed that various thiols and disulfides, including cystine and cysteine, could be estimated by the polarograph if divalent Co was used as a catalyst. He examined a number of protein hydrolyzates. Sladek and Lipschutz (746; cf. 747)

showed however that phenylalanine, histidine, arginine and tryptophan all cause interference. Smith and Rodden (748) found that methionine and S-benzylcysteine do not interfere and djenkolic acid does so only slightly. Stern, Beach and Macy (749) estimate the inhibiting effect of the various acids present by adding cystine to the solution and recording the increase of current. 15 mg./ml. can be measured with an accuracy of ± 5 per cent. Stern (750) also showed that homocystine could be similarly measured, though not distinguished from cystine.

Vassel's Method. Vassel (751), following Fujita and Numata (751a), has used the blue color obtained by the reaction of cysteine with p-amino-dimethylaniline in the presence of Fe and Zn as the basis of a colorimetric method. An error of ± 3 per cent is claimed. Mecham (752) has modified the method to increase the accuracy, particularly in the presence of detergents.

Methionine

Baernstein (753, 754) has shown that on heating with HI methionine is demethylated, and may be estimated as volatile iodide. In a later paper (755) he has shown that methionine, which has been converted to the thiolactone of homocysteine (cf. 756) by boiling with hydrogen iodide may be estimated by opening the ring in concentrated ammonia solution and allowing the homocysteine so produced to reduce tetrathionate. After acidification the thiosulfate formed is titrated iodimetrically. Baernstein obtains practically quantitative results with this method, lower when applied to proteins than those obtained with the volatile iodide method (cf. 743). Other workers (684, 683, 757, 757a) have not found complete recoveries, and the hydrolysis with HI has been criticized on the grounds that it causes greater evolution of volatile iodide and H_2S from insulin (which contains no methionine) than does HCl hydrolysis (683). The volatile iodide was not methyl iodide in this case. Beach and Teague (758) have estimated the homocysteine produced by HI hydrolysis as already described (para. 5.5).

Lavine (759) has found that methionine may be estimated by combination with 2 atoms of iodine at pH 7 in molar KI solution. Excess iodine is added, and back-titrated. A blank is done on a hydrolyzate in which the methionine has all been oxidized to sulfoxide by iodate in N HCl. Modifications are needed in the presence of tryptophan, or homocystine. Kolb and Toennies (760) present a method for use in simple mixtures in which methionine is oxidized to sulfoxide in N perchloric acid by H_2O_2. (Cf. 760a.)

Two color reactions for methionine (761, 762) involving the use of cupric Cu in strong acid solution have been described.

McCarthy and Sullivan (763, 764) have introduced a method of estimation in which the hydrolyzate is allowed to react with sodium nitroprusside in strongly alkaline solution. After acidification, the color is matched against a similarly treated methionine standard. It has been applied to a number of proteins (763, 765). Interference by cystine has been noted (765a).

Lanthionine

Sullivan and Hess (766) report that on boiling with HI 1 mole of lanthionine is converted quantitatively to 1 mole of cysteine. Lindley and Phillips (767) are unable to confirm the production of any cysteine under these conditions. Schöberl (767a) observed only 5 per cent of this theoretical yield.

Distribution of Sulfur in Proteins

Elementary analysis for S provides a valuable check on the total of S-containing amino acids. The distribution of S between these has been studied by various workers (e.g., 687, 702, 743, 765). Bailey (768) gives a valuable review and experimental critique of the various methods from this angle. Total recoveries of S are seldom more than 95 per cent, and often less than 90 per cent. Lugg, assuming cyst(e)ine and methionine to be the only S-containing amino acids present (cf. 100), estimates (cystine+cysteine) as SO_4'' produced by oxidation with fuming HNO_3, and methionine as S not so oxidizable. He compares results obtained by various methods on leaf proteins (769, 770) and tobacco mosaic virus (771).

REFERENCES

References which contain especially useful bibliographies, are denoted with an asterisk.

Paras. 1–4

1. Synge, R. L. M. (1943). *Chem. Revs.* **32**, 135.
2. *Vickery, H. B., and Schmidt, C. L. A. (1931). *Chem. Revs.* **9**, 169.
3. *Mitchell, H. H., and Hamilton, T. S. (1929). The Biochemistry of the Amino Acids. Chemical Catalog Co., New York.
3a. *Block, R. J., and Bolling, D. (1945). The Amino Acid Composition of Proteins and Foods; Analytical Methods and Results. Thomas, Springfield, Ill.
4. *Dunn, M. S. (1938). Chemistry of the Amino Acids and Proteins (ed. C. L. A. Schmidt), p. 21. Thomas, Springfield, Ill
5 *Vickery, H. B. (1941). *Ann. N. Y. Acad. Sci.* **41**, 87.
6. *Leuchs, H., and Bormann, K. (1919). *Ber.* **52**, 2086.
7. Wieland, H., and Witkop, B. (1940). *Ann.* **543**, 171.
8. Kaneko, T. (1940). *J. Chem. Soc. Japan* **61**, 207 (through *Chem. Abs.*).
9. *Dunn, M. S. (1941). *Ann. Rev. Biochem.* **10**, 91.
10. Wada, M. (1930). *Biochem. Z.* **224**, 420.
11. Krebs, H. A. (1942). *Biochem. J.* **36**, 758.

12. Gornall, A. G., and Hunter, A. (1943). *J. Biol. Chem.* **147**, 593.
13. Wada, M. (1933). *Biochem. Z.* **257**, 1.
14. *Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37**, 313.
15. Fearon, W. R. (1939). *Biochem. J.* **33**, 902.
16. Chibnall, A. C., Rees, M. W., and Williams, E. F. (1943). *Biochem. J.* **37**, 372.
17. Corwin, A. H., and Damerel, C. I. (1943). *J. Am. Chem. Soc.* **65**, 1974.
18. *Dunn, M. S. (1925). *J. Am. Chem. Soc.* **47**, 2564.
19. Wada, M. (1933). *Biochem. Z.* **262**, 57.
20. Hoppe-Seyler, F. A. (1933). *Z. physiol. Chem.* **214**, 267.
21. Manske, R. H. F. (1937). *Can. J. Research* **15B**, 84.
22. Kossel, A., and Weiss, F. (1913). *Z. physiol. Chem.* **84**, 1.
23. Belozersky, A. N., and Passhina, T. S. (1944). *Lancet.* **247**, 716.
24. Kitagawa, M., and Tsukamoto, J. (1937). *J. Biochem.* (Japan), **26**, 373.
25. Borek, E., and Clarke, H. T. (1938). *J. Biol. Chem.* **125**, 479.
26. Damodaran, M., and Narayanan, K. G. A. (1939). *Biochem. J.* **33**, 1740.
27. Ackermann, D., and Appel, W. (1939). *Z. physiol. Chem.* **262**, 103.
28. Cadden, J. F. (1940). *Proc. Soc. Exptl. Biol. Med.* **45**, 224.
29. Müller, E. (1941). *Z. physiol. Chem.* **268**, 245 (through *Chem. Abs.*).
30. *Irvin, J. L., and Wilson, D. W. (1939). *J. Biol. Chem.* **127**, 555.
31. Knoop, F., and Martius, C. (1939). *Z. physiol. Chem.* **258**, 238.
32. Karrer, P., Koenig, H., and Legler, R. (1941). *Helv. Chim. Acta* **24**, 127, 861.
33. Karrer, P., and Appenzeller, R. (1942). *Helv. Chim. Acta* **25**, 595.
34. Van Slyke, D. D., Hiller, A., Dillon, R. T., and MacFadyen, D. (1938). *Proc. Soc. Exptl. Biol. Med.* **38**, 548.
35. Van Slyke, D. D., Hiller, A., MacFadyen, D., Hastings, A. B., and Klemperer, F. W. (1940). *J. Biol. Chem.* **133**, 287.
36. Klemperer, F. W., Hastings, A. B., and Van Slyke, D. D. (1942). *J. Biol. Chem.* **143**, 433.
37. Van Slyke, D. D., Hiller, A., and MacFadyen, D. A. (1941). *J. Biol. Chem.* **141**, 681.
38. Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35**, 294.
39. Felix, K, and Mager, A. (1937). *Z. physiol. Chem.* **249**, 111.
40. Carter, H. E., Handler, P., and Melville, D. B. (1939). *J. Biol. Chem.* **129**, 359.
41. Maeda, S., Higasi, T., and Matuoka, H. (1938). *J. Agr. Chem. Soc. Japan* **14**, 689. (through *Chem. Abs.*).
42. Carter, H. E., and West, H. D. (1940). *Org. Syntheses* **20**, 81.
43. Sharp, J. G. (1939). *Biochem. J.* **33**, 679.
44. Woolley, D. W., and Peterson, W. H. (1937). *J. Biol. Chem.* **121**, 507.
45. Freudenberg, K., Walch, H., and Molter, H. (1942). *Naturwissenschaften* **30**, 87.
46. Dakin, H. D. (1941). *J. Biol. Chem.* **140**, 847 (footnote).
47. *Nicolet, B. H., and Shinn, L. A. (1941). *J. Biol. Chem.* **142**, 139.
48. *Bailey, K., Chibnall, A. C., Rees, M. W., and Williams, E. F. (1943). *Biochem. J.* **37**, 360.
49. Beveridge, J. M. R., and Lucas, C. C. (1944). *Biochem. J.* **38**, 88.
49a. Dakin, H. D. (1944). *J. Biol. Chem.* **154**, 549.
50. Jacobs, W. A., and Craig, L. C. (1938). *J. Biol. Chem.* **122**, 419.
51. Botvinnik, M. M., Prokof'ev, M. A., and Zelinskii, N. D. (1941). *Compt. rend. acad. sci. U.R.S.S.* **30**, 120.
52. Brazier, M. A. B. (1930). *Biochem. J.* **24**, 1188.
53. Abderhalden, E., and Heyns, K. (1934). *Ber.* **67**, 530.

54. Prokof'ev, M. A., and Botvinnik, M. M. (1939). *Compt. rend. acad. sci. U.R.S.S.* **25,** 488.

55. Abderhalden, E., and Bahn, A. (1937). *Z. physiol. Chem.* **245,** 246.

56. Oikawa, S. (1925). *Japan J. Med. Sci.* **1** (II), 61 (through *Chem. Abs.*).

57. Tobie, W. C. (1943). *Nature* **152,** 249.

58. Abderhalden, E., and Heyns K. (1931). *Z. physiol. Chem.* **202, 37.**

59. Nuccorini, R. (1930). *Boll. ist. super. agrar. Pisa* **6,** 227.

60. Nuccorini, R. (1931). *Boll. ist. super. agrar. Pisa* **7,** 147.

61. Nuccorini, R. (1934) *Ann. chim. applicata* **24,** 25.

62. Yaginuma, T., Arai, G., and Hayakawa, K. (1932) *Proc. Imp. Acad.* (Tokyo) **8,** 91.

63. Abderhalden, E., and Heyns, K. (1933). *Z. physiol. Chem.* **214,** 262.

63a. Consden, R., Gordon, A. H., Martin, A. J. P., Rosenheim, O., and Synge, R. L. M. (1945). *Biochem. J.* (in press).

64. *Kotake, Y. (1935). *Erg. Physiol.* **37,** 245.

65. Butenandt, A., Weidel, W., and Becker, E. (1940). *Naturwissenschaften* **28, 447** (through *Chem. Abs.*).

66. Becker, E. (1941). *Naturwissenshaften* **29,** 237 (through *Chem. Abs.*)

67. Butenandt, A., Weidel, W. and v. Derjugin, W. (1942). *Naturwissenschaften* **30, 51** (through *Chem. Abs.*).

68. Butenandt, A., Weidel, W., Weichert, R., and v. Derjugin, W. (1943). *Z. physiol. Chem.* **279,** 27 (through *Chem. Abs.*).

69. *Harington, C. R. (1944). *J. Chem. Soc.,* 193.

70. Bardkoll, A. E., and Ross, W. F. (1944). *J. Am. Chem. Soc.* **66,** 898.

71. Sborov, A. M., Peters, L., and Arnow, L. E. (1942). *Proc. Soc. Exptl. Biol. Med.* **49, 698.**

72. *Damodaran, M. (1932). *Biochem. J.* **26,** 235.

73. Damodaran, M., Jaaback, G., and Chibnall, A.C. (1932). *Biochem. J.* **26,** 1704.

74. Synge, R. L. M. (1939). *Biochem. J.* **33, 671.**

75. Virtanen, I., and Laine, T. (1937). *Enzymologia* **3,** 266.

76. Schenk, J. R. (1943). *J. Biol. Chem.* **149,** 111.

77. Pollack, M. A. (1943). *J. Am. Chem. Soc.* **65,** 484.

77a. Schenk, J. R. (1943). *Proc. Soc. Exptl. Biol. Med.* **54,** 6.

77b. Abromova, N. M. (1944). *Biokhimiya* **9,** 59.

78. Linderstrom-Lang, K., and Jacobsen, C. F. (1941). *J. Biol. Chem.* **137, 443.**

79. *Neurath, H., Greenstein, J. P., Putnam, F. W., and Erickson, J. O. (1944). *Chem. Revs.* **34,** 157.

80. Pillemer, L., Ecker, E. E., and Mariensen, E. W. (1939). *J. Exptl. Med.* **70, 387.**

81. Blackburn, S., Consden, R., and Phillips, H. (1944). *Biochem. J.* **38,** 25.

82. Patterson, W. I., Geiger, W. B., Mizell, L. R., and Harris, M. (1941). *J. Research Natl. Bureau Standards (Wash.)* **27,** 89.

83. Harris, M., Mizell, L. R., and Fourt, L. (1942). *Ind. Eng. Chem.* **34, 833.**

84. Goddard, D. R., and Michaelis, L. (1935). *J. Biol. Chem.* **112,** 361.

85. Horn, M. J , Jones, D. B., and Ringel, S. J., (1941). *J. Biol. Chem.* **138, 141.**

86. du Vigneaud, V., and Brown, G. B. (1941). *J. Biol. Chem.* **138,** 151.

87. Horn, M. J., and Jones, D. B. (1941). *J. Biol. Chem.* **139,** 473.

88. Brown, G. B., and du Vigneaud, V. (1941). *J. Biol. Chem.* **140, 767.**

89. du Vigneaud, V., Brown, G. B., and Bonsnes, R. W. (1941). *J. Biol. Chem.* **141, 707.**

90. Horn, M. J. Jones, D. B., and Ringel, S. J. (1942). *J Biol. Chem.* **144,** 87, 93.

91. Kuhn, R. and Quadbeck, G. (1943). *Ber.* **76,** 527 (through *Chem. Abs.*).

92. Mizell, L. R., and Harris, M. (1943). *J. Research Natl. Bureau Standards* **30, 47.**

93. Schöberl, A. (1942). *Biochem. Z.* **313**, 214 (through *Chem. Abs.*).
94. Stoves, J. L. (1942). *Trans. Faraday Soc.* **38**, 254.
95. Nicolet. B. H., and Shinn, L. A. (1941). *J. Biol. Chem.* **140**, 685.
96. Küster, W., and Irion, W. (1929). *Z. physiol. Chem.* **184**, 225.
96a. Lissizin, T. (1928). *Z. physiol. Chem.* **173**, 309.
97. Horn, M. J., and Jones, D. B. (1941). *J. Biol. Chem.* **139**, 649.
97a. Schöberl, A. (1943). *Ber.* **76**, 964, 970.
98. vanVeen, A. G., and Hyman, A. J. (1935). *Rec. trav. chim.* **54**, 493.
99. *du Vigneaud, V., and Patterson, W. I. (1936). *J. Biol. Chem.* **114**, 533.
100. *Blumenthal, D., and Clarke, H. T. (1935). *J. Biol. Chem.* **110**, 343.
100a.*Thomas, M. D., and Hendricks, R. H. (1944). *J. Biol. Chem.* **153**, 313.
101. Jacobs, W. A., and Craig, L. C. (1935). *J. Biol. Chem.* **110**, 521.
102 Smith, S., and Timmis, G. M. (1937). *J. Chem. Soc.* p. 396.
103. Iwanowicz, G., and Bruckner, V. (1937). *Z. Immunitätsforsch,* **90**, 304.
104. Bruckner, V., and Iwanowicz, G. (1937). *Z. physiol. Chem.* **247**, 281.
105. Bovarnick, M. (1942). *J. Biol. Chem.* **145**, 415.
106. Hotchkiss, R. D. (1941). *J. Biol. Chem.* **141**, 171.
107. Christensen, H. N., Edwards, R. R., and Piersma, H. D. (1941). *J. Biol. Chem.* **141**, 187.
108. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37**, 86.
109. Synge, R. L. M. (1944). *Biochem. J.* **38**, 285.
109a. Synge, R. L. M. (1945). To be published.
110. du Vigneaud, V., and Sealock, R. R. (1932). *J. Biol. Chem.* **96**, 511.
111. du Vigneaud V., and Meyer, C. E. (1932). *J. Biol. Chem.* **98**, 295.
112. du Vigneaud, V., and Meyer, C. E. (1932). *J. Biol. Chem.* **99**, 143.
113. Jackson, R. W., and Cahill, W. M. (1938). *J. Biol. Chem.* **126**, 37.
114. Neuberger, A. (1938). *Biochem. J.* **32**, 1452.
115. Synge, R. L. M. (1939). *Biochem. J.* **33**, 1918.
116. Cahill, W. M., and Burton, I. F (1940). *J. Biol. Chem.* **132**, 161.
117. *Chibnall, A. C., Rees, M. W., Williams, E. F., and Boyland, E. (1940). *Biochem. J.* **34**, 285.
118. Lipmann, F., Behrens, O. K., Kabat, E. A., and Burk, D. (1940). *Science* **91**, 21.
119. Konikova, A. S. (1940). *Biokhimiya* **5**, 316.
120. Wieland, T. (1942). *Ber.* **75**, 1001.
121. *Bailey, K., Chibnall, A. C., Rees, M. W., and Williams, E. F. (1943). *Biochem. J.* **37**, 360.
121a. Schein, A. H., and Berg, C. P. (1943). *Federation Proceedings* **2**, 69.
122. *Graff, S., Rittenberg, D., and Foster, G. L. (1940) *J. Biol. Chem.* **133**, 745.
123. *Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35**, 91.
124. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37**, 92.
125. Levene, P. A., and Steiger, R. E. (1930). *J. Biol. Chem.* **86**, 703.
126. Arnow, L. E., and Opsahl, J. C. (1940). *J. Biol. Chem.* **133**, 765.
127. Arnow, L. E., and Opsahl, J. C. (1940). *J. Biol. Chem.* **134**, 649.
128. Johnson, J. M. (1940). *J. Biol. Chem.* **134**, 459.
128a. Wieland, T., and Paul, W. (1944). *Ber.* **77**, 34.
129. Andrews, J. C. (1932). *J. Biol. Chem.* **97**, 657.
130. Andrews, J. C. (1933). *J. Biol. Chem.* **102**, 263.
131. Loring, H. S., and du Vigneaud, V. (1933). *J. Biol. Chem.* **102**, 287.
132. Loring, H. S., and du Vigneaud, V. (1934). *J. Biol. Chem.* **107**, 267.
133. *Borchers, R., and Berg, C. P. (1942). *J. Biol. Chem.* **142**, 693.

134. *Lugg, J. W. H. (1938). *Biochem. J.* **32**, 775.
135. Lugg, J. W. H. (1933). *Biochem. J.* **27**, 1022.
136. Brand, E., and Kassell, B. (1939). *J. Biol. Chem.* **131**, 489.
137. Wieland, T. (1942). *Naturwissenschaften* **30**, 374.
138. Tristram, G. R. (1939). *Biochem. J.* **33**, 1271.
139. Damodaran, M., and Ramachandran, B. V. (1941). *Biochem. J.* **35**, 122.
140. Boyd, M. J., and Logan, M. A. (1942). *J. Biol. Chem.* **146**, 279.
141. Abderhalden, E., and Broich, F. (1933). *Biochem. Z.* **262**, 321.
142. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35**, 1369.
143. Posternak, S., and Posternak, T. (1928). *Compt. rend.* **187**, 313.
144. Nicolet, B. H., Shinn, L. A., and Saidel, L. J. (1942). *J. Biol. Chem.* **142**, 609.
145. Winnick, T. (1942). *J. Biol. Chem.* **142**, 461.
146. Private communication from Mr. M. W. Rees.
147. Botvinnik, M. M., and Morozova, E. A. (1943).. *Compt. rend. acad. sci. U.R.S.S.* **41**, 70.
148. Borchers, R., Totter, J. R., and Berg, C. P. (1942). *J. Biol. Chem.* **142**, 697.
149. Daft, F. S., and Coghill, R. D. (1931). *J. Biol. Chem.* **90**, 341.
150. Plimmer, R. H. A., and Lowndes, J. (1938). *Compt. rend. trav. Lab. Carlsberg* (Sér. chim.) **22**, 434.
151. Vickery, H. B. (1922). *J. Biol. Chem.* **53**, 495.
152. Shore, A., Wilson, H., and Stueck, G. (1935). *J. Biol. Chem.* **112**, 407.
153. Bailey, K. (1937). *Biochem. J.* **31**, 1406.
154. Steinhardt, J. (1941). *J. Biol. Chem.* **141**, 995.
155. Chibnall, A. C. (1942). *Proc. Roy. Soc.* (London) **131B**, 136.
156. Warner, R. C. (1942). *J. Biol. Chem.* **142**, 705.
157. Warner, R. C., and Cannan, R. K. (1942). *J. Biol. Chem.* **142**, 725.
158. Warner, R. C. (1942). *J. Biol. Chem.* **142**, 741.
159. Shaw, J. L. D., and MacFarlane, W. D. (1940). *J. Biol. Chem.* **132**, 387.
160. *Philpot, J. St. L., and Small, P. A. (1938). *Biochem. J.* **32**, 542.
161. *Crammer, J. L., and Neuberger, A. (1943). *Biochem. J.* **37**, 302.
162. *Brand, E., and Kassell, B. (1942). *J. Biol. Chem.* **145**, 365.
163. Kassell, B., and Brand, E. (1938). *J. Biol. Chem.* **125**, 435.
164. Brand, E., and Kassell, B. (1939). *J. Biol. Chem.* **131**, 489.
165. Brand, E., and Kassell, B. (1941). *J. Gen. Physiol.* **25**, 167.

Para. 5.1 and 5.2

166. *Mitchell, H. H., and Hamilton, T. S. (1929). The Biochemistry of the Amino Acids. Chemical Catalog Co., New York.
167. Felix, K., and Mager, A. (1937). *Z. physiol. Chem.* **249**, 111.
168. *Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35**, 91.
169. Dakin, H. D. (1920). *J. Biol. Chem.* **44**, 499.
170. Bergmann, M. (1935). *J. Biol. Chem.* **110**, 471.
171. Bergmann, M., and Stein, W. H. (1939). *J. Biol. Chem.* **128**, 217.
172. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37**, 92.
173. Private communication from Prof. A. C. Chibnall.
173a. Bailey, K. (1944). *Advances in Protein Chemistry* **1**, 289.
174. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37**, 86.
174a. Brand, E., Kassell, B., and Saidel, L. J. (1944). *J. Clin. Investigation* **23**, 437.
175. Chibnall, A. C., Rees, M. W., and Williams, E. F. (1943). *Biochem. J.* **37**, 354.
176. *Chibnall, A. C. (1942). *Proc. Roy. Soc.* (London) **131B**, 136.

177. *Block, R. J. (1938). The Determination of the Amino Acids. Burgess, Minneapolis. (1940) (With D. Bolling). Revised Edition.
178. *Vickery, H. B. (1941). *Ann. N. Y. Acad. Sci.* **41**, 87.
179. Schoenheimer, R., and Ratner, S. (1939). *J. Biol. Chem.* **127**, 301.
180. Keston, A. S., Rittenberg, D., and Schoenheimer, R. (1939). *J. Biol. Chem.* **127**, 315.
181. Rittenberg, D., Keston, A. S., Rosebury, F., and Schoenheimer, R. (1939). *J. Biol. Chem.* **127**, 291.
182. Rittenberg, D., and Foster, G. L. (1940). *J. Biol. Chem.* **133**, 737.
183. Graff, S., Rittenberg, D., and Foster, G. L. (1940). *J. Biol. Chem.* **133**, 745.
184. Ussing, H. H. (1939). *Nature* **144**, 977.
185. Stekol, J. A., and Hamill, W. H. (1937). *J. Biol. Chem.* **120**, 531.
186. Foster, G. L., Rittenberg, D., Keston, A. S., and Schoenheimer, R. (1938). *J. Biol. Chem.* **124**, 159.
187. Rittenberg, D., Keston, A. S., Schoenheimer, R., and Foster, G. L. (1938). *J. Biol. Chem.* **125**, 1.
188. Münzberg, F. K., and Haurowitz, F. (1938). *Z. physiol. Chem.* **256**, 271.

Paras. 5.3, 5.3.1 and 5.3.2

189. Sadikov, V. S., and Lindkvist-Ru sakova, E. V. (1934). *Compt. rend. acad. sci. U.R.S.S.* **1**, 575 (through *Chem. Abs.*).
190. Abderhalden, E., and Fodor, A. (1919). *Fermentfors.* **2**, 74, 151, 211.
191. Ito, T. (1930; 1932; 1936). *Bull. Agr. Chem. Soc. Japan* **6**, 13; **8**, 59; **12**, 27 (through *Chem. Abs.*).
192. Nefelein, E. (1923). *Biochem. Z.* **142**, 493.
193. Mashino, M., and Shikazono, N. (1936). *J. Soc. Chem. Ind. Japan (suppl. bind.)* **39**, 54B; 88B; 136B.
194. Fuchs, H. (1937). *Z. physiol. Chem.* **246**, 278.
195. Wunderly, K. (1934). *Helv. Chim. Acta*, **17**, 523.
196. Bartell, F. E., and Miller, E. J. (1923). *J. Am. Chem. Soc.* **45**, 1106.
197. Phelps, H. J., and Peters, R. A. (1929). *Proc. Roy. Soc. (London)* **124A**, 554.
198. Cristol, P., and Foucade, J. (1939). *Compt. rend. soc. biol.* **131**, 414 (through *Chem. Abs.*).
199. Folin, O., and Bell, R. D. (1917). *J. Biol. Chem.* **29**, 329.
200. Ackermann, D., and Fuchs, H. (1936). *Z. physiol. Chem.* **240**, 198.
201. Lottermoser, A., and Edelmann, K. (1938). *Kolloid Z.* **83**, 262.
202. Cheldelin, V. H., and Williams, R. J. (1942). *J. Am. Chem. Soc.* **64**, 1513.
202a. Schaaf, E., and Reinhard, O. (1943). *Ber.* **76**, 1171.
203. Englis, D. T., and Fiess, H. A. (1944). *Ind. Eng. Chem.* **36**, 604.
204. *Koschara, W. (1936). *Z. physiol. Chem.* **239**, 89.
205. DeVault, D. (1943). *J. Am. Chem. Soc.* **65**, 532.
206. Weiss, J. (1943). *J. Chem. Soc.*, 297.
207. *Wieland, T. (1943). *Die Chemie* **56**, 213.
208. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1944). *Biochem. J.* **38**, 65.
208a. *Myers, R. J. (1942). *Advances in Colloid Science* **1**, 317.
209. Whitehorn, J. C. (1923). *J. Biol. Chem.* **56**, 751.
210. Turba, F. (1941). *Ber.* **74**, 1829.
211. Block, R. J. (1942). *Proc. Soc. Exptl. Biol. Med.* **51**, 252.
212. Freudenberg, K., Walch, H., and Molter, H. (1942). *Naturwissenschaften* **30**, 87.
212a. Archibald, R. M. (1944). *J. Biol. Chem.* **156**, 121.

212b. Strain, H. H. (1942). Chromatographic Adsorption Analysis, p. 88. Interscience, New York.

212c. Wieland, T. (1944). *Ber.* **77**, 539.

212d. Schramm, G., and Primosigh, J. (1944). *Ber.* **77**, 417.

212e. Schramm, G.. and Primosigh, J. (1944). *Ber.* **77**, 426.

213. Wieland, T. (1942). *Z. physiol. Chem.* **273**, 24.

214. Wieland, T. (1942). *Ber.* **75**, 1001.

215. Wieland, T. (1942). *Naturwissenschaften* **30**, 374.

216. Wieland, T., and Wirth, L. (1943). *Ber.* **76**, 8˙3.

216a. Wieland, T., and Paul, W. (1944). *Ber.* **77**, 34.

217. Turba, F.. and Richter, M. (1942). *Ber.* **75**, 340.

218. Cannan, R. K. (1944). *J. Biol. Chem.* **152**, 401.

219. Kibrick, A. C. (1944). *J. Biol. Chem.* **152**, 411.

220. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P. (1941). *J. Biol. Chem.* **141**, 627.

221. Chibnall, A. C., Rees M. W., and Williams, E. F. (1943). *Biochem. J.* **37**, 372.

222. Schramm, G., and Primosigh, J. (1943). *Ber.* **76**, 373.

223. Turba, F., Richter, M., and Kuchar, F (1943). *Naturwissenschaften* **31**, 508.

223a. Koschara, W. (1944). *Z. physiol. Chem.* **280**, 55.

224. Wachtel, J. L., and Cassidy, H. G. (1942). *Science* **95**, 233.

225. Wachtel, J. L., and Cassidy, H. G. (1943). *J. Am. Chem. Soc.* **65**, 665.

226. Karrer, P., Keller, R., and Szonyi, G. (1943). *Helv. Chim. Acta*, **26**, 38.

Para. 5.3.3

227. Tiselius, A. (1940). *Arkiv Kemi, Mineral., Geol.* **14 B**, No. 22.

228. —— (1941). *Arkiv Kemi, Mineral , Geol.* **14 B**, No 32.

229. —— (1941). *Arkiv Kemi, Mineral., Geol.* **15 B**, No. 6.

230. —— (1941). *Science* **94**, 145.

231. —— (1941). *Advances in Colloid Science* **1**, 81.

232. —— (1942). Kemisk Analys. genom Adsorption, *Tek. Samfund Handl.* pp. 85–104. Göteborg (not seen).

233. Svensson, H. (1939). *Kolloid-Z.* **87**, 181.

234. Philpot, J. St. L. (1938). *Nature* **141**, 283.

235. Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35**, 1358.

236. Weiss, J. (1943). *J. Chem. Soc.* 297.

237. DeVault, D. (1943). *J. Am. Chem. Soc.* **65**, 532.

238. Tiselius, A., and Claesson, S. (1942). *Arkiv Kemi, Mineral., Geol.* **15 B**, No. 18.

238a. Claesson, S. (1944). The Svedberg — 1884. $\frac{30}{8}$ 1944 — p, 82. Uppsala.

238b. Dutton. H. J. (1944). *J. Phys. Chem.* **48**, 179.

239. Tiselius. A. (1943). *Kolloid-Z.* **105**, 101.

240. —— (1943). *Kolloid-Z.* **105**, 177.

241. —— (1943). *Arkiv Kemi, Mineral., Geol.* **16A**, No. 18.

242. —— (1944). The Svedberg — 1884 $\frac{30}{8}$ 1944 — p. 370. Uppsala.

Paras. 5.3.4 and 5.3.5

243. Synge, R. L. M. (1939). *Biochem. J.* **33**, 1913.

244. —— (1939). *Biochem. J.* **33**, 1918.

245. —— (1939). *Biochem. J.* **33**, 1924.

246. —— (1939). *Biochem. J.* **33**, 1931.

247. Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35,** 91.
248. —— (1941). *Biochem. J.* **35,** 1358.
249. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37,** 79.
250. —— (1943). *Biochem. J.* **37,** 313.
251. —— (1944). *Biochem. J.* **38,** 65.
252. —— (1943). *Biochem. J.* **37,** 86.
253. —— (1943). *Biochem. J.* **37,** 92.
254. Liddell, H. F., and Rydon, H. N. (1944). *Biochem. J.* **38,** 68.
255. Private communication from Dr. G. R. Tristram.
256. Synge, R. L. M. (1944). *Biochem. J.* **38,** 235.
257. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37,** 538.
258. Blackburn, S., Consden, R., and Phillips, H. (1944). *Biochem. J.* **38,** 25.
259. Calvery, H. O. (1932). *J. Biol. Chem.* **94,** 613.
260. Sharp, J. G. (1939). *Biochem. J.* **33,** 679.
261. England, A., and Cohn, E. J. (1935). *J. Am. Chem. Soc.* **57,** 634.
262. Wieland, T. (1943). *Die Chemie* **56,** 213.
263. Leland, J. P., and Foster, G. L. (1932). *J. Biol. Chem.* **95,** 165.
264. Consden, R., Gordon, A. H., and Martin, A J. P. (1944). *Biochem. J.* **38,** 224.
264a. Wieland, T., and Fremerey, H. (1944). *Ber.* **77,** 234.
265. Waldschmidt-Leitz, E., and Turba, F. (1940). *J. prakt. Chem.* [2] **156,** 55 (not seen).
266. Waldschmidt-Leitz, E., Ratzer, J., and Turba, F. (1941). *J. prakt. Chem.* [2] **158,** 72.
267. Potts, A. M., and Gallagher, T. F. (1942). *J. Biol. Chem.* **143,** 561.
268. Consden, R., Gordon, A. H., and Martin, A. J. P. Unpublished.

Para. 5.4

269. *Löddesol, A. (1932). *J. Am. Soc. Agronom.* **24,** 74.
270. *Spiegel-Adolf, M. (1927). Abderhalden's Handbuch der biologischen Arbeitsmethoden III, B, 606. Berlin and Vienna (not seen).
271. *Theorell, H. (1936). *ibid.* V, 10, 1097 (not seen).
271a.*Zhukov, I. I. (1943). *Uspekhi Khim.* **12,** 265.
272. Ikeda, K., and Suzuki, S. (1909). Brit. Pat. 9440/09. (1912) U. S. Pat. 1,015, 891.
273. Foster, G. L., and Schmidt, C. L. A. (1922). *Proc. Soc. Exptl. Biol. Med.* **19,** 348.
274. —— (1923). *J. Biol. Chem.* **56,** 545.
275. Cox, G. J., King, H., and Berg, C. P (1929). *J. Biol. Chem.* **81,** 755.
275a. Gulland, J. M., and Morris, C. J. O. R. (1934). *J. Chem. Soc.* 1644.
276. Albanese, A. A. (1940). *J. Biol. Chem.* **134,** 467.
276a. Theorell, H., and Åkeson, Å. (1942). *Arkiv Kemi, Mineral., Geol.* **16,** No. 8.
277. Gawrilow, N. I., Paradashvili, A. I., Balabouha-Popzova, W. S., Ljapounzowa, S. W. (1938). *Bull. soc. chim.* [5] **5,** 973.
278. Balaboucha-Popsowa, W. S., Gawrilow, N. J., Paradachwili, A. J., and Jakounine, G. F. (1938). *Bull. soc. chim.* [5] **5,** 978.
279. Gawrilow. N. I., and Balabucha-Popzova, W. S. (1934). *Biochem. Z.* **271,** 292.
280. Balabucha-Popzowa, W. S., Gawrilow, N. I., and Rikalewa, A. M. (1936). *Biochem. Z.* **283,** 62.
281. Antonovich, E. G., and Gavrilov, N. I. (1941). *J. Gen. Chem.* (*U.S.S.R.*) **11,** 763.
282. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35,** 1369.
283. —— (1943). *Biochem. J.* **37,** 92.

284. Richardson, G. M. (1934). *Proc. Roy. Soc.* (London) **115B**, 142.
285. Keil, A. W., and Schirck, H. G. (1928). *Z. Biol.* **88**, 153 (through *Chem. Abs.*).
286. Das, N., Ghosh, B. N., and Guha, B. C. (1936). *Z. physiol. Chem.* **238**, 131.
287. Gebauer-Fuelnegg, E., and Kendall, A. I. (1931). *Ber.* **64**, 1067.
288. Freudenberg, K., Westphal, O., Marriott, G., Groenewoud, P., and Molter, H. (1938). *Sitzber. heidelberg. Akad. Wiss.* (*Math. naturw. Klasse*) 8.
289. Kuhn, R., and Desnouelle, P. (1937). *Ber.* **70**, 1907.
289a. Winnick, T. (1944). *J. Biol. Chem.* **152**, 465.
290. Ruppel, W. G. (1920). *Ber. deut. pharm. Ges.* **30**, 314 (through *Chem. Abs.*).
291. Ruppel, W. G., Ornstein, O., Carl, J., and Lasch, G. (1922). *Z. Hyg. Infektionskrankh.* **97**, 188 (through *Chem. Abs.*).
292. Pauli, W. (1922). *Kolloid-Z.* **31**, 252.
293. —— (1924). *Biochem. Z.* **152**, 355.
294. Stern, R. (1923). *Biochem. Z.* **144**, 115.
295. Freundlich, H., and Farmer-Loeb, L. (1924). *Biochem. Z.* **150**, 522.
295a. *Carr, C. W., Gregor, H. P., and Sollner, K. (1945). *J. Gen. Physiol.* **28**, 179.
296. *Watson, P. D. (1934). *Ind. Eng. Chem.* **26**, 640.
297. Cross, R. J. (1935). U. S. Pat. 1,986,920.
298. Freeman, M., Gulland, J. M., and Randall, S. S. (1935). *Biochem. J.* **29**, 2211.
299. Hahn, L., and Tiselius, A. (194). *Biochem. Z.* **314**, 336.
300. Tiselius, A. (194). *Svensk. Kem. Tids.* **53**, 305.
301. Williams, R. J., and Waterman, R. E. (1929). *Proc. Soc. Exptl. Biol. Med.* **27**, 56.
302. Williams, R. J., and Truesdail, J. H. (1931). *J. Am. Chem. Soc.* **53**, 4171.
303. Williams, R. J., Lyman, C. M., Goodyear, G. H., Truesdail, J. H., and Holaday, D. (1933). *J. Am. Chem. Soc.* **55**, 2912.
304. Williams, R. I., and Moser, R. (1934). *J. Am. Chem. Soc.* **56**, 169.
305. Williams, R. J. (1935). *J. Biol. Chem.* **110**, 589.
306. du Vigneaud, V., Irving, G. W., Dyer, H. M., and Sealock, R. R. (1938). *J. Biol. Chem.* **123**, 45.
307. Irving, G. W., and du Vigneaud, V. (1938). *J. Biol. Chem.* **123**, 485.
308. Irving, G. W., Dyer, H. M., and du Vigneaud, V. (1941). *J. Am. Chem. Soc.* **63**, 503.
309. Irving, G. W., and du Vigneaud, V. (1943). *Ann. N. Y. Acad. Sci.* **43**, 273.
310. Cohn, M., Irving, G. W., and du Vigneaud, V. (1941). *J. Biol. Chem.* **137**, 675.
311. Spies, J. R., Bernton, H. S., and Stevens, H. (1941). *J. Am. Chem. Soc.* **63**, 2163.
312. Csonka, F. A. (1941). *Cereal Chem.* **18**, 523.
313. Consden, R., Gordon, A. H., and Martin, A. J. P. (1944). Unpublished.

Para. 5.5

314. *Bailey, K., Chibnall, A. C., Rees, M. W., and Williams, E. F. (1943). *Biochem. J.* **37**, 360.
315. Chibnall, A. C., Rees, M. W., and Williams, E. F. (1943). *Biochem. J.* **37**, 372.
316. Bergmann, M., and Niemann, C. (1937). *J. Biol. Chem.* **122**, 577.
317. Meyer, K. H., Fuld, M., and Klemm, O. (1940). *Helv. Chim. Acta* **23**, 1441.
318. Stein, W. H., Moore, S., and Bergmann, M. (1944). *J. Biol. Chem.* **154**, 191.
319. Fox, S. W. (1936). *Science* **84**, 163.
320. Baptist, N. G., and Robson, W. (1940). *Biochem. J.* **34**, 221.
321. Barnett, H. M. (1933). *J. Biol. Chem.* **100**, 543.
322. Hill, E. M., and Robson, W. (1934). *Biochem. J.* **28**, 1008.
323. Ayre, C. A. (1938). *Biochem. J.* **32**, 1152.

324. Van Slyke, D. D., Hiller, A., and MacFadyen, D. A. (1941). *J. Biol. Chem.* **141,** 681.
325. Van Slyke, D. D., Hiller, A., and Dillon, R. T. (**1942**). *J. Biol. Chem.* **146, 137.**
326. Kiesel, A. (1922). *Z. physiol. Chem.* **118,** 254.
327. Vickery, H. B., and Cook, C. A. (1931). *J. Biol. Chem.* **94, 393.**
328. McCay, C. M., and Schmidt, C. L. A. (1926). *J. Gen. Physiol.* **9, 333.**
329. Kossel, A., and Gross, R. E. (1924). *Z. physiol. Chem.* **135, 167.**
330. Kossel, A., and Staudt, W. (1926). *Z. physiol. Chem.* **156,** 270.
331. Vickery, H. B. (1940). *J. Biol. Chem.* **132,** 325.
332. Vickery, H. B., Smith, E. L., Hubbell, R. B., and Nolan, L. S. (1941). *J. Biol. Chem.* **140,** 613.
333. Beveridge, J. M. R., and Lucas, C. C. (1944). *Biochem. J.* **38,** 88.
333a. Rocha e Silva, M., and Andrade, S. O. (1944). *Rev. brasil. biol.* **4,** 51 (through *Chem. Abs.*).
334. Vickery, H. B. (192). *J. Biol. Chem.* **71,** 303.
335. Vickery, H. B., and Leavenworth, C. S. (1928). *J. Biol. Chem.* **76, 707.**
336. Vickery, H. B., and Leavenworth, C. S. (1928). *J. Biol. Chem.* **79, 377.**
337. Synge, R. L. M. (1939). *Biochem. J.* **33,** 671.
338. Albanese, A. A. (1940). *J. Biol. Chem.* **134,** 467.
339. Kapfhammer, J , and Eck, R. (1927). *Z. physiol. Chem.* **170,** 294.
340. Town, B. W. (1928). *Biochem. J.* **22,** 1083.
341. —— (1936). *Biochem. J.* **30,** 1837.
342. Klabunde, H. K. (1931). *J. Biol. Chem.* **90,** 293.
343. Van Slyke, D. D., Hiller, A., Dillon, R. T., and MacFadyen, D. (1938). *Proc. Soc. Exptl. Biol. Med.* **38,** 548.
344. Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35,** 294.
345. Cox, G. J. and King, H. (1929). *J Biol. Chem.* **84, 533.**
346. Kapfhammer, J., and Spörer, H. (1928). *Z. physiol. Chem.* **173,** 245.
347. Bergmann, M., and Niemann, C. (1937). *J. Biol. Chem.* **118,** 781.
348. Zimmermann, W., and Cuthbertson, D. P. (1932). *Z. physiol. Chem.* **205, 38.**
349. Town, B. W. (1936). *Biochem. J.* **30,** 1833.
350. Block, R J. (1937). *Proc. Soc Exptl. Biol. Med.* **37,** 580.
351. —— (1940). *J. Biol. Chem.* **133,** 67.
351a. Marenzi, A. D., and Villalonga, F. (1943). *Rev. soc. argentina biol.* **19,** 43 (through *Chem. Abs.*).
352. Devine, J. (1941). *Biochem. J.* **35,** 433.
353. Beveridge, J. M. R., and Lucas, C. C. (1944). *Biochem. J.* **38,** 95.
354. Spörer, H., and Kapfhammer, J. (1929). *Z. physiol. Chem.* **187,** 84.
355. Bergmann, M. (1935). *J. Biol. Chem.* **110,** 471.
355a. Maeda, S. (1940). *Bull. Inst. Phys. Chem. Research* (Tokyo) **19,** 261 (through *Chem. Abs*).
355b. Maeda, S., and Maruo, B. (1940). *Bull. Inst. Phys. Chem. Research* (Tokyo) **19, 271** (through *Chem. Abs.*).
356. Zimmermann, W. (1930). *Z. physiol. Chem.* **188,** 180; **189,** 155.
357. Vickery, H. B. (1942) *J. Biol. Chem.* **143,** 77.
358. —— (1942). *J. Biol. Chem.* **144,** 719.
358a. Vickery, H. B., and Winternitz, J. K. (1944). *J. Biol. Chem.* **156,** 211.
358b. Vickery, H. B. (1944). *J. Biol. Chem.* **156,** 283.
359. Schneider, F. (1940). *Collegium* p. 97 (through *Brit. Chem. Abs.*).
360. Zimmermann, W. (1930). *Z. physiol. Chem.* **192,** 124.

361. Ackermann, D. (1934). *Z. physiol. Chem.* **225.** 46.
362. Foreman, F. W. (1914). *Biochem. J.* **8,** 463.
363. Brazier, M. A. B. (1930). *Biochem. J.* **24,** 1188.
364. Damodaran, M. (1931). *Biochem. J.* **25,** 190.
365. Town, B. W. (1941). *Biochem. J.* **35,** 417.
366. Stakheyeva-Kaversneva, E. D. (1940). *Biokhimiya* **5,** 513.
367. Pope, C. G., and Stevens, M. F. (1939). *Biochem. J.* **33,** 1070.
368. Albanese, A. A., and Irby, V. (1944). *J. Biol. Chem.* **153,** 583.
368a. Beveridge, J. M. R., and Lucas, C. C. (1944). *Biochem. J.* **38,** 411.
369. Hlasiwetz, H., and Habermann, J. (1873). *Ann.* **169,** 150.
370. Abderhalden, E., and Fuchs, D. (1908). *Z. physiol. Chem.* **57,** 339.
371. Hopkins, F. G. (1929). *J. Biol. Chem.* **84,** 269
372. Pirie, N. W. (1931). *Biochem. J.* **25,** 614.
373. Vickery, H. B., and White, A. (1933). *J. Biol. Chem.* **99,** 701.
374. Rossouw, S. D., and Wilken-Jorden, T. J. (1935). *Biochem. J.* **29,** 219.
374a. Pirie, N. W. (193). *Biochem. J* **26,** 75.
375. Medes, G., and Padis, K. E. (1936). *Biochem. J.* **30,** 941.
376. Graff, S., Maculla, E., and Graff, A. M. (1937). *J. Biol. Chem.* **121,** 81.
377. Zittle, C. A., and O'Dell, R A. (1941). *J. Biol. Chem.* **139,** 753.
378. Beach, E. F., and Teague, D. M. (1942). *J. Biol. Chem.* **142,** 277.
379. Lucas, C. C., and Beveridge, J. M. R. (1940). *Biochem. J.* **34,** 1356.
380. *Vickery, H. B., and Schmidt, C. L. A. (1931). *Chem. Revs.* **9,** 169.
381. Hanke, M. T. (1925). *J. Biol. Chem.* **66,** 475, 489.
382. Kiesel, A. (1926). *Z. physiol. Chem.* **161,** 147.
383. Vickery, H. B., and Leavenworth, C. S. (1927). *J. Biol. Chem.* **72,** 403
384. —— (1929). *J. Biol. Chem.* **83,** 523.
385. Calvery, H. O. (1929). *J. Biol. Chem.* **83,** 631.
386. Vickery, H. B., and Block R. J. (1930). *J. Biol. Chem.* **86,** 107.
387. —— (1931). *J. Biol. Chem.* **93,** 105.
388. Vickery, H. B., and Shore, A. (1932). *Biochem. J.* **26,** 1101.
389. Vickery, H. B., and White, A. (1933) *J. Biol. Chem.* **103,** 413.
390. Miller, E. J. (1935). *Biochem. J.* **29,** 2344.
391. Block, R. J. (1934). *J. Biol. Chem.* **106,** 457.
392. ——1938. The Determination of the Amino Acids. Burgess, Minneapolis.
393. Mourot, G., and Hoffer, O. (1938). *Bull. soc. chim. biol.* **20,** 274.
394. Tristram, G. R. (1939). *Biochem. J.* **33,** 1271.
395. Dakin, H D. (1942). *J. Biol. Chem.* **146,** 237.
396. Foster, G. L., and Shemin, D. (1938). *Org. Syntheses* **18,** 43.
397. Lang, K. (1933). *Z. physiol. Chem.* **222,** 3.
398. Rosedale, J. L., and da Silva, G. A. (1932). *Biochem. J.* **26,** 369.
399. Pirie, N. W. (19 2). *Biochem. J.* **26,** 12 0.
399a. Toennies, G., and Kolb, J. J. (1938). *J. Biol. Chem.* **126,** 367.
400. Fürth, O., and Minnibeck, H. (1932). *Biochem. Z.* **250,** 18.
401. Bergmann, M., and Stein, W. H. (1939). *J. Biol. Chem.* **128,** 217.
402. Ing, H. R., and Bergmann, M. (1939). *J. Biol. Chem.* **129,** 603.
403. Bergmann, M., and Stein, W. H. (1939). *J. Biol. Chem.* **129,** 609.
404. Doherty, D. G., Stein, W. H., and Bergmann, M. (1940). *J. Biol. Chem.* **135, 487.**
405. Stein, W H., Moore, S., Stamm, G., Chou, C. Y., and Bergmann, M. (1942). *J. Biol. Chem.* **143,** 121.
406. Moore, S., and Stein, W. H. (1943). *J. Biol. Chem.* **150,** 113.

407. *Moore, S., Stein, W. H., and Bergmann, M. (1942). *Chem. Revs.* **30**, 423.
408. *Bergmann, M., and Niemann, C. (1937). *J. Biol. Chem.* **122**, 577.
409. Bergmann, M. (1937). *J. Biol. Chem.* **122**, 569.
410. Bergmann, M., and Fox, S. W. (1935). *J. Biol. Chem.* **109**, 317.

Paras. 5.6 and 5.6.1

411. Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35**, 294.
412. Van Slyke, D. D., Hiller, A., and MacFadyen, D. A. (1941). *J. Biol. Chem.* **141**, 681.
413. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37**, 538.
414. Stein, W. H., Moore, S., and Bergmann, M. (1944). *J. Biol. Chem.* **154**, 191.
415. Van Slyke, D. D. (1923). *J. Biol. Chem.* **83**, 425.
416. Kendrick, A. B., and Hanke, M. E. (1937). *J. Biol. Chem.* **117**, 161.
417. Dunn, M. S., and Porush, I. (1939). *J. Biol. Chem.* **127**, 261.
418. Kendrick, A. B., and Hanke, M. E. (1940). *J. Biol. Chem.* **132**, 739.
419. Van Slyke, D. D., Hiller, A., and Dillon, R. T. (1942). *J. Biol. Chem.* **146**, 142 (footnote).
420. Schmidt, C. L. A. (1929). *J. Biol. Chem.* **82**, 587.
420a. Irving, G. W., Fontaine, T. D., and Samuels, C. S. (1944). *Arch. Biochem.* **4**, 347.
421. Sure, B., and Hart, E. B. (1917). *J. Biol. Chem.* **31**, 527.
422. Dunn, M. S., and Schmidt, C. L. A. (1922). *J. Biol. Chem.* **53**, 401.
423. Hunter, A. (1929). *J. Biol. Chem.* **82**, 731.
424. Lieben, F., and Loo, Y. C. (1942). *J. Biol. Chem.* **145**, 223.
425. Gornall, A. G., and Hunter, A. (1940). *Biochem. J.* **34**, 192.
426. Cannan, R. K., Palmer, A. H., and Kibrick, A. C. (1942). *J. Biol. Chem.* **142**, 803.
427. Carter, H. E., and Dickman, S. R. (1943). *J. Biol. Chem.* **149**, 571.
428. Fraenkel-Conrat, H. (1943). *J. Biol. Chem.* **148**, 453.
428a. Fontaine, T. D., and Irving, G. W. (1944). *Arch. Biochem.* **4**, 455.
429. Fürth, O., and Minnibeck, H. (1932). *Biochem. Z.* **250**, 18.
430. *Olcott, H. S. (1944). *J. Biol. Chem.* **153**, 71.
431. Sandkuhle, J., Kirk, P. L., and Cunningham, B. (1942). *J. Biol. Chem.* **146**, 427.
432. Richardson, G. M. (1934). *Proc. Roy. Soc.* (London) **115B**, 142.
433. Rapoport, S. (1935). *Biochem. Z.* **281**, 30.
434. —— (1937). *Biochem. Z.* **289**, 406.
435. Kendall, A. I., and Friedemann, T. E. (1930). *J. Infectious Diseases* **47**, 171. Cf. Friedemann, T. E., and Kendall, A. I. (1929). *J. Biol. Chem.* **82**, 23.
436. Fürth, O., Scholl, R., and Hermann, H. (1932). *Biochem. Z.* **251**, 404.
437. Hotchkiss, R. D. (1941). *J. Biol. Chem.* **141**, 171.
438. Christensen, H. N., Edwards, R. R., and Piersma, H. D. (1941). *J. Biol. Chem.* **141**, 187.
439. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37**, 86.
440. McChesney, E. W. (1935.) *J. Elisha Mitchell Sci. Soc.* **51**, 147 (through *Chem. Abs.*)
441. Fromageot, C., and Heitz, P. (1939). *Enzymologia* **6**, 258.
441a. Fromageot, C., and Heitz, P. (1938). *Mikrochim. Acta* **3**, 52.
441b. Desnouelle, P. (1938). *Enzymologia* **5**, 37.
441c. Braunshtein, A. E., and Bychkov, S M. (1943). *Biokhimiya* **8**, 234.
441d. Block, R. J., Bolling, D., and Webb, M. (1940). *J. Biol. Chem.* **133**, xiv.
441e. Felix, K., and Zorn, K. (1941). *Z. physiol. Chem.* **263**, 257.
442. Stakheyeva-Kaversneva, E. D. (1940). *Biokhimiya* **5**, 513.

443. Block, R. J., Bolling, D., and Kondritzer, A. A. (1940). *Proc. Soc. Exptl. Biol. Med.* **45**, 289.
444. Block, R. J., and Bolling, D., (1940). The Determination of the Amino Acids (revised edition). Burgess, Minneapolis.
445. Block, R J., and Bolling, D. (1943). *Arch. Biochem.* **3**, 217.
445a. Arhimo, A. A. (1939). *Suomen Kemistilehti* **12 B**, 6.
446. *Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P. (1941). *J. Biol. Chem.* **141**, 627.
447. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35**, 1369.
448. —— (1943). *Biochem. J.* **37**, 92.
449. Winnick, T. (1944). *J. Biol. Chem.* **152**, 465.
450. Stein, W. H., Moore, S., and Bergmann, M. (1944). *J. Biol. Chem.* **154**, 191.
451. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37**, 313.
452. Kibrick, A. C. (1944). *J. Biol. Chem.* **152**, 411.
453. Cannan, R. K. (1944). *J. Biol. Chem.* **152**, 401.
454. Cohen, P. P. (1940). *J. Biol. Chem.* **136**, 565.
455. Neuberger, A., and Sanger, F. (1942). *Biochem. J.* **36**, 662.
455a. *Hamilton, P. B. (1945). *J. Biol. Chem.* **158**, 375, 397.
456. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. (1941). *J. Biol. Chem.* **141**, 671.
456a. Schott, H. F., Rockland, L. B., and Dunn, M. S. (1944). *J. Biol. Chem.* **154**, 397.
457. *MacFadyen, D. A. (1942). *J. Biol. Chem.* **145**, 387.
458. Hamilton, P. B., and Van Slyke, D. D. (1943). *J. Biol. Chem.* **150**, 231.
459. Cramer, F. B., and Winnick, T. (1943). *J. Biol. Chem.* **150**, 259.
460. Van Slyke, D. D., MacFadyen, D. A., and Hamilton, P. B. (1943). *J. Biol. Chem.* **150**, 251.
461. Van Slyke, D. D., and Hamilton, P. B. (1943). *J. Biol. Chem.* **150**, 471.
462. MacFadyen, D. A. (1944). *J. Biol. Chem.* **153**, 507.
463. *Virtanen, A. I., and Laine, T. (1938). *Skand. Arch. Physiol.* **80**, 392.
464. —— (1938). *Nature* **142**, 754.
465. Laine, T. (1938). *Suomen Kemistilehti* **11 B**, 28.
466. Virtanen, A. I., Laine, T., and Toivonen, T. (1940). *Z. physiol. Chem.* **266**, 193. (through *Chem. Abs.*).
466a. MacFadyen, D. A. (1945). *J. Biol. Chem.* **158**, 107.
467. Wieland, T., and Wirth, L. (1943). *Ber.* **76**, 823.
468. *Copley, G. N. (1941). *Analyst* **66**, 492.
469. Cherbuliez, E., and Trusfus, I. (1933). See *Chem. Abs.* **28**, 3092. (1934).
470. Cohen, P. P. (1939). *Biochem. J.* **33**, 551.
471. Herbst, R. M., and Clarke, H. T. (1934). *J. Biol. Chem.* **104**, 769.
471a. Arhimo, A. A., and Laine, T. (1939). *Suomen Kemistilehti* **12 B**, 18.
472. Nicolet, B. H., and Shinn, L. A. (1939). *J. Am. Chem. Soc.* **61**, 1615.
473. Shinn, L. A., and Nicolet, B. H. (1941). *J. Biol. Chem.* **138**, 91.
474. Winnick, T. (1942). *J. Biol. Chem.* **142**, 461.
475. Nicolet, B. H., and Shinn, L. A. (1941). *J. Biol. Chem.* **139**, 687.
476. Boyd, M. J., and Logan, M. A. (1942). *J. Biol. Chem.* **146**, 279.
476a. Neuberger, A. (1944). *Biochem. J.* **38**, 309.
477. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37**, 538.
478. Synge, R. L. M. (1944). *Biochem. J.* **38**, 285.
479. Nicolet, B. H., and Shinn, L. A. (1942). *J. Biol. Chem.* **142**, 139.
480. Brand, E., and Kassell, B. (1942). *J. Biol. Chem.* **145**, 365.

481. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35,** 1369.
482. Nicolet, B. H., and Saidel, L. J. (1941). *J. Biol. Chem.* **139,** 477.
483. Nicolet, B. H., Shinn, L. A., and Saidel, L. J. (1942). *J. Biol. Chem.* **142,** 609.
484. Nicolet, B. H., and Shinn, L. A. (1941). *J. Am. Chem. Soc.* **63,** 1486.
485. Brown, W. L. (1942). *J. Biol. Chem.* **142,** 299.
485a. Beach, E. F., Munks, B., and Robinson, A. (1943). *J. Biol. Chem.* **148,** 431.
486. Block, R. J., and Bolling, D. (1939). *J. Biol. Chem.* **130,** 365; cf. *Proc. Soc. Exptl. Biol. Med.* **40,** 710.
487. Borchers, R., Totter, J. R., and Berg, C. P. (1942). *J. Biol. Chem.* **142,** 697.
487a. Tsuverkalov, D. A. (1944). *Biokhimiya* **9,** 101 (through *Chem. Abs.*).

Para. 5.6.2

488. *Hunter, A., and Dauphinee, J. A. (19?0). *J. Biol. Chem.* **85,** 627.
489. *Hegsted, D. M., and Wardwell, E. D. (1944). *J. Biol. Chem.* **153,** 167.
490. Ryan, F. J., and Brand, E. (1944). *J. Biol. Chem.* **154,** 161.
490a. Fox, S. W., Fling, M., and Bollenback, G. N. (1944). *J. Biol. Chem.* **155,** 465.
491. Woods, D. D., and Trim, A. R. (1942). *Biochem. J.* **36,** 501.
492. *Gale, E. F. (1943). *Bact. Revs.* **7,** 139.
493. Hunter, A., and Pettigrew, J. B. (1937). *Enzymologia* **1,** 341.
494. Graff, S., Maculla, E., and Graff, A. M. (1937). *J. Biol. Chem.* **121,** 71.
495. Scull, C. W., and Rose, W. C. (1930). *J. Biol. Chem.* **89,** 109.
496. Mourot, G., and Hoffer, O. (1938). *Bull. soc. chim. biol.* **20,** 274.
497. Kiech, V. C., Luck, J. M., and Smith, A. E. (193). *J. Biol. Chem.* **90,** 677.
497a. Archibald, R. M., and Hamilton, P. B. (1943). *J. Biol. Chem.* **150,** 155.
498. Vickery, H. B. (1940). *J. Biol. Chem.* **132,** 325.
499. *Kraus-Ragins, I. (1938). *J. Biol. Chem.* **123,** 761.
500. *Hunter, A., and Woodward, H. E. (1941). *Biochem. J.* **35,** 1298.
501. Kitagawa, M., and Eguchi, S. (1938) *J. Agr. Chem. Soc. Japan* **14,** 525 (through *Chem. Abs.*).
502. Damodaran, M., and Narayanan, K. G. A. (1940). *Biochem. J.* **34,** 1449.
503. Kitagawa, M. (1939). *J. Agr. Chem. Soc. Japan* **15,** 267 (through *Chem. Abs.*).
504. Kitagawa, M., and Watanabe, T. (1938). *J. Agr. Chem. Soc. Japan* **14,** 779 (through *Chem. Abs.*).
504a. Hunter, A., and Downs, C. E. (1945). *J. Biol. Chem.* **157,** 427.
505. Gale, E. F., and Epps, H. M. R. (1943). *Nature* **152,** 327.
505a. Gale, E. F., and Epps, H. M. R. (1944). *Biochem. J.* **38,** 232.
506. Neuberger, A., and Sanger, F. (1944). *Biochem. J.* **38,** 119.
506a. Zittle, C. A., and Eldred, N. R. (1944). *J. Biol. Chem.* **156,** 401.
507. Gale, E. F. (1940). *Biochem. J.* **34,** 392, 846, 853; (1941). *Ibid.* **35,** 66.
507a. Epps, H. M. R. (1944). *Biochem. J.* **38,** 242.
507b. Gale, E. F., Epps. H. M. R. (1944). *Biochem. J.* **38,** 250.
508. Krebs, H. A. (1935). *Biochem. J.* **29,** 1620.
509. Lipmann, F., Behrens, O. K., Kabat, E. A., and Burk, D. (1940). *Science* **91,** 21.
510. Kögl, F., Herken, H., and Erxleben, H. (1940). *Z. physiol. Chem.* **264,** 220 (through *Chem. Abs.*).
511. Lipmann, F., Hotchkiss, R. D., and Dubos, R. J. (1941). *J. Biol. Chem.* **141,** 163.
512. Herken, H., and Erxleben, H. (1940). *Z. physiol. Chem.* **264,** 251. (1941). *Ibid.* **269,** 47, 240 (through *Chem. Abs.*).
513. Karrer, P., and Frank, H. (1940). *Helv. Chim. Acta* **23,** 948.
514. Karrer, P., Koenig. H., and Legler, R. (1941). *Helv. Chim. Ac·a* **24,** 127, 861.

515. Karrer, P., Koenig, H., and Appenzeller, R. (1942). *Helv. Chim. Acta* **25,** 911.
516. Karrer, P., and Appenzeller, R. (1942). *Helv. Chim. Acta* **25,** 595, 1149.
517. Krebs, H. A. (1939). *Enzymologia* **7,** 53.
518. Klein, J. R., and Handler, P. (1941). *J. Biol. Chem.* **139,** 103.
519. Felix, K., and Zorn, K. (1939). *Z. physiol. Chem.* **258,** 16.
520. Edlbacher, S., and Bauer, H. (1941). *Z. physiol. Chem.* **270,** 176 (through *Chem. Abs.*).
521. Holtz, P., Büchsel, H., and Strübing, C. (1942). *Z. physiol. Chem.* **272,** 201 (through *Chem. Abs.*).
522. Horowitz, N. H. (1944). *J. Biol. Chem.* **154,** 141.
523. Braunshtein, A. E., and Bychkov, S. M. (1940). *Biokhimiya* **5,** 261; (1939) *Nature* **144,** 751.
524. Cedrangolo, F., and Carandante, G. (1943). See *Chem. Abs.* **37,** 5424.
525. Green, D. E., Nocito, V., and Ratner, S. (1943). *J. Biol. Chem.* **148,** 461.
526. Stumpf, P. K., and Green, D. E. (1944). *J. Biol. Chem.* **153,** 387.
526a. Ratner, S., Nocito, V., and Green, D. E. (1944). *J. Biol. Chem.* **152,** 119.
526b. Blanchard, M., Green, D. E., Nocito, V., and Ratner, S. (1944). *J. Biol. Chem.* **155,** 421.
526c. Green, D. E., Moore, D. H., Nocito, V., and Ratner, S. (1944). *J. Biol. Chem.* **156,** 383.
526d. Edlbacher, S., and Grauer, H. (1944). *Helv. Chim. Acta* **27,** 928.

Para. 5.6.3

527. *Calvery, H. O. (1938). The Chemistry of the Amino Acids and Proteins (ed. Schmidt, C. L. A.), 189. Thomas, Springfield, Ill.
528. *Van Slyke, D. D., and Kirk, E. (1933). *J. Biol. Chem.* **102,** 651.
529. Richardson, G. M. (1934). *Proc. Roy. Soc* (London) **115 B,** 121, 142, 170.
530. Neuberger, A. (1934) *Proc. Roy. Soc.* (London) **115 B,** 180.
531. Jukes, T. H., and Schmidt, C. L. A. (1934). *J. Biol. Chem.* **105,** 359.
532. Ogston, A. G., and Brown, J. F. (1935). *Trans. Faraday Soc.* **31,** 574.
533. Harris, L. J. (1924). *Proc. Roy. Soc.* (London) **95 B,** 440, 500.
534. Willstätter, R., and Waldschmidt-Leitz, E. (1921). *Ber.* **54,** 2988.
535. Grassmann, W., and Heyde, W. (1929). *Z. physiol. Chem.* **183,** 32.
536. Balson, E. W., Earwicker, G. A., and Lawson, A. (1935). *Biochem. J.* **29,** 2700.
537. Broude, L. M., and Kokovikhina, K. I. (1940). *Biokhimiya* **5,** 217.
538. Linderstrom-Lang, K. (1928). *Z. physiol. Chem.* **173,** 32.
539. Zirm, K. L., and Benedict, J. (1931). *Biochem. Z.* **243,** 312.
539a. Rask, O. S., and Eckles, N. E. (1941). *Am. J. Hyg.* **33 A,** 86.
540. Popovici, N., and Radulescu, A. (1938). *Bull. soc. chim. biol.* **20,** 73.
540a. Loiseleur, J. (1943). *Bull. soc. chim. biol.* **25,** 351.
541. Harris, L. J. (1924). *Proc. Roy. Soc.* London) **104 B,** 412.
542. Harris, L. J., and Birch, T. W. (1930). *Biochem. J.* **24,** 1080.
543. Levy, M. (1933). *J. Biol. Chem.* **99,** 767.
544. —— (1934). *J. Biol. Chem.* **105,** 157.
545. —— (1935). *J. Biol. Chem.* **109,** 365.
546. Levy, M., and Silberman, D. E. (1937). *J. Biol. Chem.* **118,** 723.
547. Tomiyama, T. (1935). *J. Biol. Chem.* **111,** 51.
548. Balson, E. W., and Lawson, A. (1936). *Biochem. J.* **30,** 1257.
549. Wadsworth, A., and Pangborn, M. C. (1936). *J. Biol. Chem.* **116,** 423.
550. Ratner, S., and Clarke, H. T. (1937). *J. Am. Chem. Soc.* **59.** 200.

551. Dunn, M. S., and Loshakoff, A. (1936). *J. Biol. Chem.* **113**, 359.
552. Dunn, M. S., and Weiner, J. G. (1937). *J. Biol. Chem.* **117**, 381.
553. Janke, A., and Mikschik, E. (1939). *Mikrochemie* **27**, 176 (through *Chem. Abs.*).
553a. Sisco, R. C., Cunningham, B., and Kirk, P. L. (1941). *J. Biol. Chem.* **139**, 1.
554. Conant, J. B., and Fieser, L. F. (1923). *J. Am. Chem. Soc.* **45**, 2194.
555. Conant, J. B., Small, L. F., and Taylor, B. S. (1925). *J. Am. Chem. Soc.* **47**, 1959.
556. Conant, J. B., and Hall, N. F. (1927). *J. Am. Chem. Soc.* **49**, 3047, 3062.
557. Hall, N. F., and Werner, T. H. (1928). *J. Am. Chem. Soc.* **50**, 2367.
558. Conant, J. B., and Werner, T. H. (1930). *J. Am. Chem. Soc.* **52**, 4436.
559. Hall, N. F. (1930). *J. Am. Chem. Soc.* **52**, 5115.
560. Harris, L. J. (1935). *Biochem. J.* **29**, 2820.
561. Nadeau, G. F., and Branchen, L. E. (1935). *J. Am. Chem. Soc.* **57**, 1363.
562. Blumrich, K., and Bandel, G. (1941). *Angew. Chem.* **54**, 374.
563. Toennies, G., and Callan, T. P. (1938). *J. Biol. Chem.* **125**, 259.
564. Kolb, J. J., and Toennies, G. (1942). *J. Biol. Chem.* **144**, 193.
565. Sakami, W., and Toennies, G. (1942). *J. Biol. Chem.* **144**, 203.
566. Toennies, G., and Kolb, J. J. (1942). *J. Biol. Chem.* **144**, 219.
567. Bancroft, W. D., and Barnett, C. E. (1930). *J. Phys. Chem.* **34**, 449, 753, 1217, 1930, 2433.
568. Czarnetzky, E. J., and Schmidt, C. L. A. (1932). *Biol. Chem.* **97**, 333.
569. —— (1934). *J. Biol. Chem.* **105**, 301.
570. Belden, B. C. (1931). *J. Phys. Chem.* **35**, 2164.
571. Kober, P. A., and Sugiura, K. (1913). *J. Am. Chem. Soc.* **35**, 1546.
572. —— (1912). *J. Biol. Chem.* **13**, 1.
573. —— (1912). *Am. Chem. J.* **48**, 383.
574. Kober, P. A., and Haw, A. B. (1916). *J. Am. Chem. Soc.* **38**, 457.
575. Kober, P. A. (1917). *J. Ind. Eng. Chem.* **9**, 501.
576. Utkin, L. (1933). *Biochem. Z.* **267**, 69.
577. Pope, C. G., and Stevens, M. F. (1939). *Biochem. J.* **33**, 1070.
578. Albanese, A. A., and Irby, V. (1944). *J. Biol. Chem.* **153**, 583.
579. Borsook, H., and Thimann, K. V. (1932). *J. Biol. Chem.* **98**, 671.

Para. 5.6.4

580. Folin, O. (1922). *J. Biol. Chem.* **51**, 377, 393.
581. *Van Slyke, D. D., and Kirk, E. (1933). *J. Biol. Chem.* **102**, 651.
582. *Frame, E. G., Russell, J. A., and Wilhelmi, A. E. (1943). *J. Biol. Chem.* **149**, 255.
582a. Russell, J. A. (1944). *J. Biol. Chem.* **156**, 467.
582b. Krauel, K. K. (1944). *J. Lab. Clin. Med.* **29**, 222.
583. Florkin, M. (1937). *Arch. intern. physiol.* **44**, 551 (through *Chem. Abs.*).
584. Ley, H., and Arends, B. (1930). *Z. physiol. Chem.* **192**, 131.
585. Lieben, F., and Edel, E. (1932). *Biochem. Z.* **244**, 403.
586. Edlbacher, S., and Litvan, F. (1940). *Z. physiol. Chem.* **265**, 241 (through *Chem. Abs.*).
587. Karrer, P., and Keller, R. (1943). *Helv. Chim. Acta* **26**, 50.
588. Vanags, G. (1941). *Z. anal. Chem.* **122**, 119 (through *Chem. Abs.*).
589. Sakaguchi, S. (1925). *J. Biochem.* (Japan) **5**, 13, 25, 133.
590. Poller, K. (1926). *Ber.* **59**, 1927.
591. Weber, C. J. (1930). *J. Biol. Chem.* **86**, 217.
592. —— (1930). *J. Biol. Chem.* **88**, 353.
593. Jorpes, E., and Thoren, S. (1932). *Biochem. J.* **26**, 1504.

594. Fisher, R. B., and Wilhelmi, A. E. (1937). *Biochem. J.* **31**, 1136.
595. —— (1938). *Biochem. J.* **32**, 606.
596. Jean, G. (1934). *Bull. soc. chim. biol.* **16**, 307.
597. Thomas, L. E., Ingalls, J. K., and Luck, J. M. (1939). *J. Biol. Chem.* **129**, 263.
598. Brand, E., and Kassell, B. (1942). *J. Biol. Chem.* **145**, 359, 365.
599. Dubnoff, J. W. (1941). *J. Biol. Chem.* **141**, 711.
600. Macpherson, H. T. (1942). *Biochem. J.* **36**, 59.
601. Devine, J. (1941). *Biochem. J.* **35**, 433.
601a. Dumazert, C., and Poggi, R. (1939). *Bull. soc. chim. biol.* **21**, 1381.
602. Weber, C. J. (1935). *J. Biol. Chem.* **109**, xcvi.
603. Bodansky, M. (1936). *J. Biol. Chem.* **115**, 641.
604. Davenport, H. W., and Fisher, R. B. (1938). *Biochem. J.* **32**, 602.
605. Dubnoff, J. W., and Borsook, H. (1941). *J. Biol. Chem.* **138**, 381.
605a. Sims, E. A. H. (1945). *J. Biol. Chem.* **158**, 239.
606. Lang, K. (1932). *Z. physiol. Chem.* **208**, 273.
607. *Koessler, K. K., and Hanke, M. T. (1919). *J. Biol. Chem.* **39**, 497.
608. Hanke, M. T., and Koessler, K. K. (1920). *J. Biol. Chem.* **43**, 527.
609. *Jorpes, E. (1932). *Biochem. J.* **26**, 1507.
610. *Lang, K. (1933). *Z. physiol. Chem.* **222**, 3.
611. Cavett, J. W. (1932). *J. Biol. Chem.* **95**, 335.
612. Meschkowa, P. P. (1936). *Z. physiol. Chem.* **240**, 199.
613. Armstrong, A. R., and Walker, E. (1932). *Biochem. J.* **26**, 143.
614. Hanke, M. T. (1925). *J. Biol. Chem.* **66**, 475, 489.
615. Racker, E. (1940). *Biochem. J.* **34**, 89.
616. Kapeller-Adler, R. (1933). *Biochem. Z.* **264**, 131.
617. —— (1934). *Biochem. Z.* **271**, 206.
618. Földes, F. (1935). *Biochem. Z.* **283**, 199. Cf. *ibid.* **285**, 123, 294, 296.
619. Langley, W. D. (1941). *J. Biol. Chem.* **137**, 255.
620. *Tschopp, W., and Tschopp, H. (1938). *Biochem. Z.* **298**, 206.
621. Conrad, R. M., and Berg, C. P. (1937). *J. Biol. Chem.* **117**, 351.
622. Woolley, D. W., and Peterson, W. H. (1937). *J. Biol. Chem.* **122**, 207.
623. Block, R. J. (1938). The Determination of Amino Acids. Burgess, Minneapolis.
624. Plimmer, R. H. A., and Phillips, H. (1924). *Biochem. J.* **18**, 312.
625. DaSilva, G. A. (1931). *Biochem. J.* **25**, 1634.
626. Rosedale, J. L., and DaSilva, G. A. (1932). *Biochem. J.* **26**, 369.
627. Calvery, H. O. (1929). *J. Biol. Chem.* **83**, 631.
628. Plimmer, R. H. A., and Lowndes, J. (1938). *Compt. rend. trav. lab. Carlsberg.* (*Sér. chim.*) **22**, 434.
629. Zimmermann, W. (1930). *Z. physiol. Chem.* **189**, 4.
630. Abderhalden, E., and Neumann, A. (1936). *Z. physiol. Chem.* **238**, 177.
631. Klein, G., and Linser, H. (1932). *Z. physiol. Chem.* **205**, 251.
632. Patton, A. R. (1934). *J. Biol. Chem.* **108**, 267.
632a. Brecht, K., and Grundmann, G. (1939). *Biochem. Z.* **302**, 42.
633. Christensen, H. N., Edwards, R. R., and Piersma, H. D. (1941). *J. Biol. Chem.* **141**, 187.
634. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37**, 86.
635. Lang, K. (1933). *Z. physiol. Chem.* **219**, 148.
636. Waldschmidt-Leitz, E., and Akabori, S. (1934). *Z. physiol. Chem.* **224**, 187.
637. Morse, W. (1933). *J. Biol. Chem.* **100**, 373.
638. MacFarlane, W. D., and Guest, G. H. (1939). *Can J. Research* **17 B**, 139.

639. Guest, G. H. (1939). *Can. J. Research,* **17 B,** 143.
639a. Engeland, R., and Bastian, A. (1937). *Bull. soc. chim. biol.* **19,** 1126.
640. *Holiday, E. R. (1936). *Biochem. J.* **30,** 1705, 1709.
641. Crammer, J. L., and Neuberger, A. (1943). *Biochem. J.* **37,** 302.
642   Darby, H. H. (1941). *J. Biol. Chem.* **139,** 721.
643. Ross, W. F. (1934). *J. Biol. Chem.* **104,** 531.
644. Feraud, K., Dunn, M. S., and Kaplan, J. (1935). *J. Biol. Chem.* **112,** 323.
645. Folin, O., and Marenzi, A. D. (1929). *J. Biol. Chem.* **83,** 89.
646. Folin, O., and Ciocalteu, V. (1927). *J. Biol. Chem.* **73,** 627.
647. Schild, E., and Enders, C. (1936). *Biochem. Z.* **286,** 220.
648. Fujiwara, H., and Kataoka, E. (1933). *Z. physiol. Chem.* **216,** 133.
649. Lugg, J. W. H. (1937). *Biochem. J.* **31,** 1422.
650. —— (1938). *Biochem. J.* **32,** 775.
651. Arnow, L. E. (1937). *J. Biol. Chem.* **118,** 531.
652. Bernhart, F. W. (1938). *J. Biol. Chem.* **123,** x.
652a. Rossi, A., and Vescia, A. (1942). *Arch. sci. biol.* (Italy) **28,** 207 (through *Chem. Abs.*).
653. Balint, P. (1938). *Biochem. Z.* **299,** 133.
654. Brand, E., and Kassell, B. (1939). *J. Biol. Chem.* **131,** 489.
655. v. Deseö, D. (1934). *Biochem. Z.* **271,** 142.
656. Zuwerkalow, D. (1927). *Z. physiol. Chem.* **163,** 185.
657. Calvery, H. O., Block, W. D., and Schock, E. D. (1936). *J. Biol. Chem.* **113,** 21.
658. Gerngross, O., Voss, K., and Herfeld, H. (1933). *Ber.* **66,** 435.
658a. Thomas, L. E. (1944). *Arch. Biochem.* **5,** 175.
659. Kapeller-Adler, R. (1932). *Biochem. Z.* **252,** 185.
660. Block, R. J., and Bolling, D. (1939). *J. Biol. Chem.* **129,** 1.
661. Jervis, G. A., Block, R. J., Bolling, D., and Kanze, E. (1940). *J. Biol. Chem.* **134,** 105.
661a. Block, R. J., Jervis, G. A., Bolling, D., and Webb, M. (1940). *J. Biol. Chem.* **134,** 567.
662. Macara, T. J. R., and Plimmer, R. H. A. (1940). *Biochem. J.* **34,** 1431.
663. Baptist, N. G., and Robson, W. (1940). *Biochem. J.* **34,** 221.
664. Arnow, L. E., Burns, J., and Bernhart, F. W. (1939). *Proc. Soc. Exptl. Biol. Med.* **41,** 499.
665. Knight, C. A., and Stanley, W. M. (1941). *J. Biol. Chem.* **141,** 39.
666. Block, R. J. (1937). *J. Biol. Chem.* **128,** 181.
666a. Brown, W. L. (1944). *J. Biol. Chem.* **155,** 277.
666b. Albanese, A. A. (1944). *J. Biol. Chem.* **155,** 291.
666c. Hess, W. C., and Sullivan, M. X. (1944). *Arch. Biochem.* **5,** 165.
667. Leland, J. P., and Foster, G. L. (1932). *J. Biol. Chem.* **95,** 165.
668. May, C. E., and Rose, E. R. (1922). *J. Biol. Chem.* **54,** 213.
669. Holm, G. E., and Greenbank, G. R. (1923). *J. Am. Chem. Soc.* **45,** 1788.
670. Sullivan, M. X., Milone, H. S., and Everitt, E. L. (1938). *J. Biol. Chem.* **125,** 471.
671. Bates, R. W. (1937). *J. Biol. Chem.* **119,** vii.
672. Komm, E., and Pöhringer, E. (1922). *Z. physiol. Chem.* **124,** 287.
672a. Komm, E. (1924). *Z. physiol. Chem.* **140,** 74; (1926); **156,** 35, 161, 202.
673. Boyd, W. J. (1929). *Biochem. J.* **23,** 78.
674. Tomiyama, T., and Shigematsu, S. (1934). *Proc. Soc. Exptl. Biol. Med.* **32,** 446.
675. Shaw, J. L. D., and MacFarlane, W. D. (1940). *J. Biol. Chem.* **132,** 387.
675a. Horn, M. J., and Jones, D. B. (1945). *J. Biol. Chem.* **157,** 153.

676. Ragins, I. K. (1928). *J. Biol. Chem.* **80**, 543, 551.
677. Winkler, S. (1934). *Z. physiol. Chem.* **228**, 50.
678. Shaw, J. L. D., and MacFarlane, W. D. (1938). *Can. J. Research* **16 B**, 361.
679. Brown, W. L. (1944). *J. Biol. Chem.* **154**, 57.
680. Synge, R. L. M. (1944). *Biochem. J.* **38**, 285.
680a.*Sullivan, M. X., and Hess, W. C. (1944). *J. Biol. Chem.* **155**, 441.
681. Albanese, A. A., and Frankston, J. E. (1942). *J. Biol. Chem.* **144**, 563.
681a. Albanese, A. A., and Frankston, J. E. (1945). *J. Biol. Chem.* **157**, 59.
682. Eckert, H. W. (1943). *J. Biol. Chem.* **148**, 2?5.
682a. Kratzer, F. H. (1944). *J. Biol. Chem.* **156**, 507.

Para. 5.7

683. du Vigneaud, V., Miller, G. L., and Rodden, C. J. (1939). *J. Biol. Chem.* **131**, 631.
684. Kassell, B., and Brand, E. (1936). *Proc. Soc. Exptl. Biol. Med.* **35**, 444.
685. Tropp, C. (1938). *Klin. Wochschr.* **17**, 465.
686. Sullivan, M. X., Hess, W. C., and Smith, E. R. (1939). *J. Biol. Chem.* **130**, 741.
687. Baernstein, H. D. (1936). *J. Biol. Chem.* **115**, 33.
688. Vickery, H. B., and White, A. (1933). *J. Biol. Chem.* **99**, 701.
689. Lugg, J. W. H. (1932). *Biochem. J.* **26**, 2144, 2160.
690. Sullivan, M. X. (1926). *U. S. Pub. Health Repts.* **41**, 1030.
      (1929). *U. S. Pub. Health Repts.* **44**, 1421, 1599.
      (1931). *U. S. Pub. Health Repts.* **46**, 390.
      (1929). *U. S. Pub. Health Suppl.* **78**.
691. Lugg, J. W. H. (1933). *Biochem. J.* **27**, 668.
692. Prunty, F. T. G. (1933). *Biochem. J.* **27**, 387.
693. Bushill, J. H., Lampitt, L. H., and Baker, L. C. (1934). *Biochem. J.* **28**, 1293.
694. Hess, W. C., and Sullivan, M. X. (1935). *J. Biol. Chem.* **108**, 195.
695. Medes, G., and Padis, K. E. (1936). *Biochem. J.* **30**, 941.
696. Sullivan, M. X., and Hess, W. C. (1936). *J. Biol. Chem.* **116**, 221.
697. Brand, E., Harris, M. M., and Biloon, S. (1930). *J. Biol. Chem.* **86**, 315.
698. Sullivan, M. X., and Hess, W. C. (1937). *J. Biol. Chem.* **117**, 423.
699. Andrews, J. C., and Andrews, K. C. (1936). *Am. J. Med. Sci.* **191**, 594.
700. Sullivan, M. X., Howard, H. W., and Hess, W. C. (1937). *J. Biol. Chem.* **119**, 721.
701. Kassell, B., and Brand, E. (1938). *J. Biol. Chem.* **125**, 435.
702. Brand, E., Cahill, G. F., and Kassell, B. (1940). *J. Biol. Chem.* **133**, 431.
703. Zittle, C. A., and O'Dell, R. A. (1941). *J. Biol. Chem.* **139**, 753.
704. Sullivan, M. X., and Hess, W. C. (1937). *J. Biol. Chem.* **120**, 537.
705. White, J. (1934). *J. Biol. Chem.* **106**, 141.
706. Sullivan, M. X., Hess, W. C., and Howard, H. W. (1942). *J. Biol. Chem.* **145**, 621.
707. Hess, W. C. (1934). *J. Biol. Chem.* **105**, xxxix.
708. Hess. W. C., and Sullivan, M. X. (1943). *J. Biol. Chem.* **151**, 635.
709. Sullivan, M. X., and Hess, W. C. (1937). *J. Biol. Chem.* **121**, 323.
710. —— (1937). *J. Biol. Chem.* **122**, 11.
711. Lee, H. J. (1936). *Australian J. Exptl. Biol. Med. Sci.* **13**, 220.
711a. Neubeck, C. E., and Smythe, C. V. (1944). *Arch. Biochem.* **4**, 435.
712. Rossouw, S. D., and Wilken-Jorden, T. J. (1935). *Biochem. J.* **29**, 219.
712a. Csonka, F. A., Lichtenstein, H., and Denton, C. A. (1944). *J. Biol. Chem.* **156**, 571.
713. Sullivan, M. X., and Hess, W. C. (193). *J. Biol. Chem.* **128**, 93.
714. Jones, D. B., and Gersdorff, C. E. F. (1934). *J. Biol. Chem.* **104**, 99.
715. Lugg, J. W. H. (1932). *Biochem. J.* **26**, 2144, 2160.

716. Folin, O., and Looney, J. M. (1922). *J. Biol. Chem.* **51,** 427.
717. Folin, O., and Trimble, H. (1924). *J. Biol. Chem.* **60,** 473.
718. Folin, O., and Marenzi, A. D. (1929). *J. Biol. Chem.* **83,** 103.
719. Rimington, C. (1930). *Biochem. J.* **24,** 1114.
720. Tompsett, S. L. (1931). *Biochem. J.* **25,** 2014.
721. Jones, D. B., and Gersdorff, C. E. F. (1933). *J. Biol. Chem.* **101,** 657.
722. Shinohara, K. (1935). *J. Biol. Chem.* **109,** 665; **110,** 263.
723. —— (1935). *J. Biol. Chem.* **112,** 671, 683.
724. Shinohara, K., and Padis, K. E. (1935). *J. Biol. Chem.* **112,** 697, 709.
725. Shinohara, K. (1937). *J. Biol. Chem.* **120,** 743.
726. Schöberl, A., and Rambacher, P. (1938). *Biochem. Z.* **295,** 377.
727. Schöberl, A., and Ludwig, E. (1937). *Ber.* **70,** 1422.
728. Calvery, H. O., Block, W. D., and Schock, E. D. (1936). *J. Biol. Chem.* **113,** 21.
729. Kassell, B., and Brand, E. (1938). *J. Biol. Chem.* **125,** 115, 131.
730. Okuda, Y. (1919). *J. Coll. Agr. Imp. Univ. Tokyo* **7,** 69 (through *Chem. Abs.*).
731. —— (1923). *J. Sci. Agr. Soc.* (Japan) **253,** 1 (through *Chem. Abs.*).
732. —— (1924). *J. Chem. Soc. Japan* **45,** 1 (through *Chem. Abs.*).
733. —— (1925). *J. Biochem.* (Japan) **5,** 201, 217.
734. Teruuchi, Y., and Okabe, L. (1928). *J. Biochem.* (Japan) **8,** 459.
734a. Sato, M., Hirano, T., and Kan, T. (1939). *J. Agr. Chem. Soc. Japan* **15,** 783 (through *Chem. Abs.*).
735. Baernstein, H. D. (1930). *J. Biol. Chem.* **89,** 125.
736. Hess, W. C. (1933). *J. Biol. Chem.* **103,** 449.
737. Lucas, C. C., and King, E. J. (1932). *Biochem. J.* **26,** 2076.
738. Lavine, T. F. (1935). *J. Biol. Chem.* **109,** 141.
739. Virtue, R. W., and Lewis, H. B. (1934). *J. Biol. Chem.* **104,** 415.
740. Tunnicliffe, H. E. (1925). *Biochem. J.* **19,** 194.
741. Baernstein, H. D. (1936). *J. Biol. Chem.* **115,** 33.
742. Toennies, G. (1937). *J. Biol. Chem.* **122,** 27.
743. Kuhn, R., Birkhofer, L., and Quackenbush, F. W. (1939). *Ber.* **72,** 407.
744. Brdička, R. (1933). *Collect. Czechoslov. Chem. Commun.* **5,** 112, 148, 238.
745. —— (1934). *Biochem. Z.* **272,** 104. *Mikrochem.* **15,** 167 (through *Chem. Abs.*); (1938). *J. Chim. Phys.* **35,** 89.
746. Sladek, J., and Lipschütz, M. (1934). *Collect. Czechoslov. Chem. Commun.* **6,** 487; *Bull. intern. acad. Prague,* December.
747. Rosenthal, H. G. (1937). *Mikrochem.* **22,** 233.
748. Smith, E. R., and Rodden, C. J. (1939). *J. Research Natl. Bur. Standards* **22,** 669.
749. Stern, A., Beach, E. F., and Macy, I. G. (1939). *J. Biol. Chem.* **130,** 733.
750. Stern, A., and Beach, E. F. (1940). *Proc. Soc. Exptl. Biol. Med.* **43,** 104.
751. Vassel, B. (1941). *J. Biol. Chem.* **140,** 323.
751a. Fujita, A., and Numata, I. (1939). *Biochem. Z.* **300,** 264.
752. Mecham, D. K. (1943). *J. Biol. Chem.* **151,** 643.
753. Baernstein, H. D. (1932). *J. Biol. Chem.* **97,** 663.
754. —— (1934). *J. Biol. Chem.* **106,** 451.
755. —— (1936). *J. Biol. Chem.* **115,** 25, 33.
756. Riegel, B., and du Vigneaud, V. (1935). *J. Biol. Chem.* **112,** 149.
757. Kassell, B., and Brand, E. (1938). *J. Biol. Chem.* **125,** 145.
757a. Åkeson, Å. (1942). *Acta Physiol. Scand.* **4,** 362.
758. Beach, E. F., and Teague, D. M. (1942). *J. Biol. Chem.* **142,** 277.
759. Lavine, T. F. (1943). *J. Biol. Chem.* **151,** 281.

760. Kolb, J. J., and Toennies, G. (1940). *Ind. Eng. Chem., Anal. Ed.* **12**, 723.
760a. Albanese, A. A., Frankston, J. E., and Irby, V. (1944). *J. Biol. Chem.* **156**, 293.
761. Kolb, J. J., and Toennies, G. (1939). *J. Biol. Chem.* **131**, 401.
762. Sofin, L. H., Rosenblum, H., and Shultz, R. C. (1943). *J. Biol. Chem.* **147**, 557.
763. McCarthy, T. E., and Sullivan, M. X. (1941). *J. Biol. Chem.* **141**, 871.
764. Sullivan, M. X., and McCarthy, T. E. (1940). *J. Biol. Chem.* **133**, c.
765. Hess, W. C., and Sullivan, M. X. (1943). *J. Biol. Chem.* **151**, 635.
765a. White, W., and Koch, F. C. (1945). *J. Biol. Chem.* **158**, 535.
766. Hess, W. C., and Sullivan, M. X. (1942). *J. Biol. Chem.* **146**, 15.
767. Lindley, H., and Phillips, H. (1945). *Biochem. J.*, **39**, 17.
767a. Schöberl, A. (1943). *Ber.* **76**, 970.
768. *Bailey, K. (1937). *Biochem. J.* **31**, 1396. 1406.
769. Lugg, J. W. H. (1938). *Biochem. J.* **32**, 2114, 2123.
770. Lugg, J. W. H. (1939). "Protein Metabolism in the Plant" (A. C. Chibnall). Yale Univ. Press. Appendix I (not seen).
771. Best, R. J., and Lugg, J. W. H. (1944). *Australian J. Exptl. Biol. Med. Sci.* **22**, 247.

The Microbiological Assay of Amino Acids

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CONTENTS

A. INTRODUCTION

Since the discovery of "bios" by Wildiers (1901), microörganisms have been used to determine the presence of substances which increase the rate or extent of their growth, and to guide efforts to purify and isolate these substances. Williams (1919) observed the great similarity in properties of "bios" and "water-soluble vitamine" required by animals, and suggested

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the use of yeast for the determination of the latter substance. Subsequent developments, in both animal and yeast nutrition, showed the complex nature of both "bios" and "vitamine." The use of microörganisms for the quantitative determination of individual nutrilites had to await further elucidation of their nutritive requirements. How these requirements were determined, and the amazing similarity which then became evident between the nutritive requirements of microörganisms and those of experimental animals, is a familiar story which has been emphasized elsewhere (Peterson, 1941; Williams, 1941), and which lies outside the scope of this article.

Because of this similarity in nutritive requirements it is now possible to use microörganisms to determine quantitatively many of those substances which are now known to be essential constituents of all living tissues. Such quantitative methods are commonly referred to as "microbiological assay methods."

One of the first microbiological assay methods to achieve reasonable specificity was that devised by Schopfer (1935) for vitamin B₁, using the mold *Phycomyces blakesleeanus*. Despite continued study and modification (Meiklejohn, 1937; Burkholder and McVeigh, 1940; Hamner, *et al.*, 1943), the method has not been widely used.

Development of a microbiological method for determining riboflavin (Snell and Strong, 1939) was followed by its rapid acceptance and widespread use. It was with this method that use of the lactic acid bacteria for such determinations was introduced. As knowledge of the nutritional requirements of this diverse group of organisms increased, it was applied to the determination of additional vitamins. With the identification of the vitamin requirements, it became possible to examine the nitrogen requirements of the group. It was found that a variety of amino acids was also required by various members of the group. Further investigation showed that this requirement, in many instances, could be made the basis for quantitative methods for determining these amino acids.

To summarize and examine these methods, and to present available data to contribute to their further development, is the object of this review.

B. NUTRITIVE REQUIREMENTS OF MICROÖRGANISMS USED FOR DETERMINATION OF AMINO ACIDS

An ideal medium for use in the microbiological assay of any essential nutrilite would be one which contained every other substance either essential for growth or stimulatory to the growth of the assay organism, but was completely free of the nutrilite to be determined. This ideal is approached only more or less closely by assay methods now in use. Intimate knowledge of the nutritional requirements of the test organism is thus fundamental to the development of an assay method; increased understanding of the

metabolic requirements of the organism underlies all future improvement in these methods. A short summary of the nutritional requirements of organisms so far recommended for amino acid assay is included below.

I. LACTIC ACID BACTERIA

1. *General Requirements*

Like all other living organisms, lactic acid bacteria require for growth mineral salts, and a utilizable source of energy and of nitrogen. They also require an assortment of "growth factors" or vitamins. In the complexity of its requirements, this group of microörganisms equals or surpasses all others which have been studied to date. A summary of the general requirements for rapid growth under laboratory conditions is given in Table I.

TABLE I

Requirements of Lactic Acid Bacteria for Rapid Growth
on Laboratory Media

Energy source: Carbohydrate (glucose).
Nitrogen source: Amino acids (casein hydrolyzate and tryptophan).
Accessory substances: Vitamins and growth factors.
Mineral salts.
Buffer.

For their energy supply, lactic acid bacteria are dependent upon the presence of fermentable carbohydrate. Glucose has been universally used in assay media for this purpose. In their action upon glucose the organisms fall into two large groups, homofermentative and heterofermentative (Orla-Jensen, 1919). The homofermentative group, which includes such commonly used organisms as *Lactobacillus casei*, *Lactobacillus arabinosus*, *Lactobacillus pentosus*, *Lactobacillus delbrueckii*, and *Streptococcus fecalis*, converts the fermented glucose almost quantitatively to lactic acid according to the equation:

$$\text{Glucose} \rightarrow 2 \text{ Lactic acid}$$
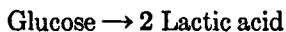
The heterofermentative group includes organisms such as *Leuconostoc mesenteroides* and *Lactobacillus fermentum*, which produce from glucose lactic acid plus other products, such as ethanol and carbon dioxide (cf. also Pederson, 1936; Sherman, 1937). The optimum pH for growth of most of these organisms lies between 6 and 7; but growth will continue until pH 4.0 or less is reached. To prevent the lactic acid produced from shifting the pH to levels which prevent growth, the buffer capacity of the medium must be increased. Acetate was found to be especially suitable for this purpose (Snell, Strong, and Peterson, 1937) and has been widely used.

No extended study of the mineral requirements of organisms of the lactic group has been made. The mineral salt mixture most commonly used is that recommended by Speakman (1923) for culture of certain spore-forming soil anaerobes, and was first used with lactic acid bacteria by Snell, Tatum, and Peterson (1937). It differs from many salt mixtures used for other organisms in its content of manganese, which is required in relatively high concentration by some lactic acid bacteria (Möller, 1939; Woolley, 1941). For some of these organisms, however, variation in the salt mixture improves growth. Luckey, *et al.* (1944) showed, for example, that additional potassium salts greatly improved growth of *Streptococcus fecalis;* and modification of the mineral mixture for some other organisms might be helpful.

2. *Vitamin and Growth Factor Requirements*

Included in this category are substances other than mineral salts and amino acids which have been found necessary for growth, or which greatly stimulate growth, of one or more of these organisms. The list (Table II) is not complete, since all the materials which *stimulate* growth of these organisms are not yet known. Omitted also are substances, such as fatty acids, which stimulate growth under some conditions but which are not ordinarily added to media intended for assay purposes.

Among individual species and strains of the lactic acid bacteria, cultures can be found which require various combinations of the growth factors listed. Where an individual nutrilite is not *essential* for growth of a given organism, it often aids early growth. Thus *L. arabinosus* grows well without vitamin B$_6$, but its development is greatly speeded by pyridoxin (Snell and Wright, 1941), pyridoxamine, or pyridoxal (Snell and Rannefeld, 1945). Any synthetic medium intended for use with a number of different species of these organisms should, therefore, contain a complete quota of these growth factors or their physiological equivalents.

3. *Amino Acid Requirements*

In early studies with lactic acid bacteria, amino acid requirements (and many vitamin requirements) were supplied by peptone. As the vitamin requirements were elucidated it became clear that the favorable effect of peptones was at least partly due to the amino acids furnished by them. Thus, tryptophan was found necessary for growth when acid-hydrolyzed peptone was used as nitrogen source for *L. casei* (Snell, Strong, and Peterson 1937). Möller (1939) grew *Lactobacillus plantarum* in a medium which contained only amino acids in place of crude peptones or protein hydrolyzates. He found tryptophan, glutamic acid, leucine, aspartic acid, and valine to be essential for growth of this organism, while other amino acids improved growth. Wood, Geiger, and Werkman (1940) showed that certain hetero-

TABLE II

*Vitamins and Growth Factors Essential for or Stimulatory
to Growth of Various Lactic Acid Bacteria*

Growth factor	Typical organism responding	Reference
Riboflavin	*Lactobacillus casei*	Snell, Strong (1939b)
Pantothenic acid	*Lactobacillus casei*	Snell, Strong, Peterson (1938)
Nicotinic acid	*Lactobacillus arabinosus*	Snell, Strong, Peterson (1938)
		Snell, Wright (1941)
Biotin	*Lactobacillus plantarum*	Möller (1939)
p-Aminobenzoic acid	*Lactobacillus arabinosus*	Isbell (1942)
		Lewis (1942)
Vitamin B₆*	*Lactobacillus plantarum*	Möller (1939)
Thiamine	*Streptococcus salivarius*	Niven, Smiley (1943)
	Lactobacillus fermentum	Sarett, Cheldelin (1944)
Folic acid (Eluate	*Lactobacillus casei*	Snell, Peterson (1939, 1940)
factor, vitamin B₆)	*Streptococcus fecalis*	Mitchell, Snell, Williams (1941)
Inositol†	*Streptococcus hemolyticus*	McIlWain (1940)
Adenine‡	*Lactobacillus plantarum*	Möller (1939)
	Lactobacillus arabinosus	Snell, Mitchell (1941)
Guanine‡	*Lactobacillus arabinosus*	Snell, Mitchell (1941)
	Leuconostoc mesenteroides	
Xanthine‡	*Leuconostoc mesenteroides*	Snell, Mitchell (1941)
Uracil	*Lactobacillus arabinosus*	Snell, Mitchell (1941)
Thymine	*Streptococcus fecalis*	Snell, Mitchell (1941)
	Lactobacillus casei	Stokstad (1941)

$Vitamin\ B_6^*$ marker corresponds to the italicized row above.

* The growth-promoting effect of vitamin B_6, originally discovered with pyridoxin, is more effectively demonstrated with pyridoxal or pyridoxamine, which far surpass pyridoxin in activity for these organisms (Snell, 1944; Snell and Rannefeld, 1945).

† *Streptococcus hemolyticus* is not ordinarily classified with the lactic acid bacteria because of its pathogenic character; inositol stimulated its growth, but the effect was non-specific.

‡ For most organisms, adenine, guanine, and xanthine are interchangeable, but various organisms differ in the ease with which they utilize the individual compounds.

¶ The effect of thymine is demonstrable only in a folic acid-free medium. Thymine plus purine bases replace folic acid more or less completely for certain organisms (Mitchell and Snell, 1941; Stokstad, 1941; Stokes, 1944).

fermentative lactic acid bacteria (including *Lactobacillus mannitopoeus* and *Lactobacillus lycopersici*) required a variety of amino acids for growth and suggested use of these organisms for the quantitative determination of threonine. At this time, however, lack of knowledge concerning the growth factor requirements of these organisms prevented realization of a full and specific response to any amino acid. Later, Snell and Wright (1941) presented a medium for the determination of nicotinic acid which contained all the growth factors necessary for the test organism, *L. arabinosus*, and in which amino acids were supplied as a hydrolyzate of vitamin-free casein plus tryptophan and cystine. It was stated at that time that the test organism would grow luxuriantly on continued subculture when the hydro-

lyzed casein of the medium was replaced by a mixture of amino acids.[1] Further addition to this medium of folic acid, purified (Mitchell, Snell, and Williams, 1941, 1944; Hutchings, Bohonos, and Peterson, 1941) since that time, permits the growth of additional organisms which require this substance (cf. Landy and Dicken, 1942).

When the hydrolyzed casein contained in media such as these is replaced by mixtures of amino acids, the media become essentially identical with those so far recommended for amino acid assay. The nutritional requirements of various organisms for individual amino acids can then be determined by omitting each acid in turn from a mixture which supports growth and noting the effect on growth or acid production. With any given organism, the amino acids can then be classified into three groups: (1) essential, in the absence of which no growth or acid production occurs; (2) accessory or auxiliary, in the absence of which maximal growth will not occur, but significant growth and acid production do occur; and (3) non-essential, the absence of which does not appear to affect growth adversely.

A number of investigators have independently determined requirements of *L. arabinosus* for amino acids. Results are given in Table III.

Despite use of methods which differ in detail for determining these requirements, essential agreement is evident concerning the effect of amino acids on this organism. All agree on the essential nature of glutamic acid, leucine, isoleucine, valine, cystine, and tryptophan, and on the nonessential nature of glycine, hydroxyproline, norleucine, and norvaline. With phenylalanine, tyrosine, threonine, arginine, and lysine, there is disagreement as to whether each individual acid is essential or accessory; all agree, however, that these substances are required for maximal growth of the test organism. Some authors find alanine, aspartic acid, histidine, proline, and serine to be accessory amino acids; others find them nonessential.

A number of different reasons for this failure to agree in detail have become evident. A proven source of error lies in the impurity of some commercial sources of amino acids. Thus, McMahan and Snell (1944) found isoleucine to be non-essential for various organisms tested, including *L. arabinosus*. Other workers, using different sources of amino acids, found isoleucine essential, and noted that different results were obtained with different sources of amino acids. Hegsted and Wardwell (1944) tested seven samples of *dl*-leucine, and found only two which contained no isoleucine. The others contained from less than 1 per cent to almost 20

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[1] It was later found that *p*-aminobenzoic acid, though not added to this medium, was required by the test organism (Isbell, 1942). The very minute amount necessary is present as a contaminant in other constituents of the medium unless extreme care is taken in their preparation (Lewis, 1942).

TABLE III

Amino Acid Requirements of Lactobacillus arabinosus*

Glutamic acid	Leucine	Isoleucine	Valine	Cystine	Methionine	Tryptophan	Phenylalanine	Tyrosine	Threonine	Arginine	Lysine	Histidine	Serine	Proline	Aspartic acid	Alanine	Hydroxyproline	Glycine	Norvaline	Norleucine	Reference
+	+	+	+	+	+	+	+	+	+	±	±	−	±	±	±	±	−	−			(a) Shankman (1943)
+	+	+	+	+	+	+	±	±	+	±	±										(b) Shankman (1943)
+	+	+	+	+	±	+	+	±	+	±	+	±	±	±	±	±	−	−	−	−	Kuiken, et al. (1943)†
+	+	+	+	+	+	+	+	+	±	+	±	−	−	−	−	±	−	−			Hegsted (1944)
+	+	+	+	+	±	+	±	±	±	±	+	±	−	−	−	±	−	−	−	−	McMahan, Snell (1944)‡

● + =essential; ± =accessory; − =non-essential.
† α-Aminoisobutyric and α-amino-n-butyric acids were also tested and found non-essential.
‡ Also unpublished data of the author.

per cent of this amino acid, as determined with *L. arabinosus*. Isoleucine was isolated from the latter sample, definitely proving its presence. Similarly, a commercial sample of $l(+)$ valine was found (Guirard and Snell, unpublished) to contain only 78 per cent of $l(+)$ valine, when assayed against a pure sample. The nature of the impurities present was not determined. It had been previously supposed that the synthetic amino acids could be considered biologically pure and that the isolated or natural amino acids were more likely to be impure. Now samples from both sources are suspect.

It is obvious that only slight impurities in other amino acids present in the medium would suffice to throw an amino acid from the "essential" category into the "accessory" or even into the "non-essential" category. Such impurities are a likely explanation for some of the conflicting results noted above. Another source of confusion is that the amino acid requirements may vary depending upon the basal medium with which they were determined. Thus, Shankman (1943) found both phenylalanine and tyrosine to be essential for growth of *L. arabinosus* upon one mixture of amino acids (cf. *a*, Table III); when the basal mixture was varied, chiefly by adding increased amounts of each amino acid, tyrosine and phenylalanine were no longer essential, but only accessory (*b*, Table III). Similarly, Hegsted (1944) found only ten amino acids essential when removed individually from a medium which contained each of nineteen amino acids. When he attempted to culture the organism on a mixture containing only these ten amino acids, growth failed. Addition of aspartic acid, previously found accessory or non-essential for this organism, was now essential for growth to occur; further addition of threonine and lysine gave maximum growth. Thus, the apparent amino acid requirement of an organism may differ with the composition of the medium.[1]

A further source of possible variation is the test organism itself. It has proved possible to "train" some microörganisms which require amino acids for growth on initial culture to dispense with them entirely. The literature on this subject has been reviewed by Knight (1936; cf. also Gladstone, 1937, and Burrows, 1939). Ryan and Brand (1944) have shown that cultures of a *Neurospora* mutant which ordinarily require leucine for

[1] A striking example of variation in amino acid requirements with the composition of the medium is afforded by the recent work of Stokes and Gunness (Science 101, 43 (1945)) who found that replacement of the pyridoxin in a medium by an equal amount of pyridoxamine eliminated the lysine, threonine, and alanine requirements of *L. casei*, *L. arabinosus*, and *L. delbrueckii*. This may explain the disagreement among various workers concerning the essential nature of lysine and threonine for *L. arabinosus* (cf. Tables III and IV), since pyridoxin is partially converted to pyridoxamin (or a substance of similar activity) by autoclaving with amino acids (Snell and Rannefeld, 1945).

TABLE IV

*Comparative Amino Acid Requirements of Various Lactic Acid Bacteria**

Organism	Aspartic acid	Glutamic acid	Arginine	Histidine	Lysine	Alanine	Cystine	Glycine	Isoleucine	Leucine	Methionine	Phenylalanine	Proline	Serine	Threonine	Tryptophan	Tyrosine	Valine
L. arabinosus 17-5†	±	+	±	−	±	−	+	−	+	+	±	±	−	−	±	+	±	+
L. casei‡	+	+	+	±	±	±	+	·−	±	+	±	+	−	+	±	+	+	+
L. pentosus 124-2§		+		−	−	−			−	+				−		+		
S. fecalis R‖	+	+	+	−	+	+	±	+	+	+	−	−	−	+	+	+	±	−
Leuc. mesenteroides P-60¶	+	+	+	+	+	±	+	+	+	+	+	+	+	+	+	+	+	+
S. lactis L103♯	−	−	+	±	±	−	−	−	+	+	+	±	−	±	−	−	−	+

* + = essential; ± = accessory; − = non-essential. Norleucine, norvaline, and hydroxyproline were non-essential for all organisms. Glutamine is more effective than glutamic acid for some organisms which require the latter.
† From cumulative data of Shankman; Hegsted; Kuiken, et al.; McMahan and Snell, cf. Table III.
‡ Hutchings and Peterson (1943).
§ McMahan and Snell, unpublished.
‖ Snell and Guirard (1943).
¶ Dunn, et al. (1944c); Hac and Snell (1945).
♯ Niven (1944). Asparagine and/or glutamine are essential for growth; both were added to the medium which probably explains lack of requirement for glutamic or aspartic acid.

growth will occasionally develop the power to synthesize it, and thenceforth grow in its absence. Although confirmed adaptations of this nature have not been reported with lactic acid bacteria, their possible occurrence should be borne in mind, and remains as a possible explanation for some conflicting findings.

It may be expected that *L. arabinosus* will provide a convenient tool for the determination of each of those amino acids which all workers agree are required by it. Indeed, several such methods have been presented. *L. arabinosus*, however, is only one of a great number of species and strains of lactic acid bacteria which can be used in this manner. If the requirements of these other organisms differ, the number of amino acids which might be determined in this fashion would be increased, and the utility of the approach correspondingly broadened. Data available on this subject are given in Table IV.

That the various organisms differ rather markedly in their amino acid requirements is apparent. Thus, for *L. casei*, tyrosine and phenylalanine were reported as essential; they are variously regarded as essential or accessory for *L. arabinosus*. *L. casei* also requires arginine, which is merely auxiliary for *L. arabinosus*, and serine, which is non-essential for *L. arabinosus*. *Streptococcus fecalis*, *Streptococcus lactis* and *Leuconostoc mesenteroides* differ considerably from other organisms studied in their requirements. The latter organism is especially exacting in its requirements, and should prove of great value as an assay organism for amino acids. Of the amino acids which are accessory or non-essential for *L. arabinosus* and *L. casei*, one or the other of these three organisms requires threonine, lysine, histidine, serine, proline, alanine, glycine, and aspartic acid. Thus, of the twenty-one or so commonly occurring amino acids, the only ones not *so far* known to be required for one or another organism of this group are hydroxyproline, norleucine, and norvaline. The requirements of only five species of a very large group of organisms have been determined; it is not impossible that further investigation will reveal organisms which require these and additional amino acids. If, as has proved possible in cases so far investigated, these nutritive requirements can be made the basis for quantitative methods for the determination of the amino acids, a tool of immense potentialities for enlarging our knowledge concerning the natural occurrence of amino acids and the composition of proteins is at hand.

II. OTHER ORGANISMS

Only two organisms outside the group of lactic acid bacteria have been suggested for amino acid assay. These are mutant cultures of *Neurospora crassa* (Beadle and Tatum, 1941; Tatum and Beadle, 1942) and of *Escherichia coli* (Lampen, Jones, and Roepke, 1944). The growth requirements

of both organisms are very simple. Parent cultures of *N. crassa* grow on synthetic media containing inorganic salts, an inorganic nitrogen source, a suitable source of organic carbon (*e.g.*, sucrose) and biotin (Ryan, Beadle, and Tatum, 1943). The X-ray induced mutants require in general that only one specific nutrilite (*e.g.*, leucine, Regnery, 1944) be added to this minimal medium for growth to occur. *E. coli* will also grow on media which contain only inorganic salts, an inorganic or organic nitrogen source, and a suitable source of organic carbon (*e.g.*, glucose; Lampen, *et al.*, 1944). The mutant strains require a specific amino acid in addition to the synthetic medium.

Although only lactic acid bacteria, and mutant strains of *Neurospora* and *E. coli* have been suggested as assay agents for amino acids, many other microörganisms are known to require amino acids for growth, or to be markedly stimulated by them. There is no apparent reason why any such organism which has a specific nutritive requirement for a given amino acid can not be used for its determination if proper growth conditions are developed.[1]

C. Assays for Amino Acids

I. Measurement of Response to the Amino Acid

1. *Comparative Growth Rate vs. Extent of Total Growth*

In any microbiological assay, the sample is run at a series of concentration levels in parallel with the pure substance to be determined; the concentration of this substance present in the sample is then determined by comparing the effect of sample and standard on growth at the various concentration levels. This comparison can be carried out before or after growth has stopped. In the former case, one measures comparative rates of growth; in the latter case, the total growth permitted by a given concentration of the essential substance is measured. All of the substances which, though not essential for growth, stimulate the rate of growth are not yet known and cannot be added to the basal media. In most cases, the extent of total growth is not affected by such substances; since the organism slowly synthesizes them for itself, their absence does not affect the total yield of cells. In general, therefore, assays based upon extent of total growth (terminal growth), as determined following prolonged incubation periods, are likely to be more reliable than those in which results are obtained before growth has proceeded to completion (that is, after short incubation periods). Sometimes organisms are used for assay which do not require the material to be determined, but whose growth rates are

[1] Since this article was written, Woolley and Sebrell (*J. Biol. Chem.* **157**, 141 (1945)) have shown that tryptophan can be estimated successfully with *Ebertiella typhosa*.

markedly stimulated by it. In such cases, one is limited to the determination of comparative growth rates. In all amino acid assays so far suggested, measurement of total growth has been used.

2. *Measurement of Growth*

The method used to determine growth depends upon the organism used for assay. With the lactic acid bacteria, two methods have been commonly used. One is based upon comparison of the number of cells present, by quantitative determinations of turbidities with a suitable instrument, such as a photoelectric colorimeter. When this method is used, one must be certain that the organisms are uniformly suspended by shaking before measurement, and that no air bubbles are present. The measurements must be made at a wave length which will avoid interference by any colored materials added with the sample. Samples used must be optically clear, and turbidity not due to cells must not develop during incubation. Some organisms (*e.g. L. delbrueckii* 3) clump during growth, and it is difficult to suspend them uniformly for turbidimetric measurement.

The second method used to evaluate growth with these organisms involves titration of acid produced as a by-product of growth. Rates of growth and acid production do not run exactly parallel courses. In general, growth (as measured turbidimetrically) will have reached a maximum considerably before maximum acid production has occurred. The amount of acid eventually produced is, however, closely related to the total number of cells present. Some of the precautions necessary when turbidimetric measurement is used are unnecessary if one titrates acid. Color of the sample, for example, is of little consequence, as is development of turbidity during incubation or clumping of the organism. This procedure has been widely used. Although the impression has gained ground that titrimetric measurement gives more accurate results than the turbidimetric method, no data confirming this impression have been published. In an investigation of this point relative to valine assay (McMahan and Snell, 1944), pantothenic acid assay (Thompson, *et al.*, 1944), and riboflavin assay (Emmett, *et al.*, 1941), no differences in results were obtained by the two methods. With some of the heterofermentative lactic acid bacteria, and with *E. coli*, which produce smaller amounts of acid, turbidimetric measurement of growth is usually used.

With the fungus *Neurospora* neither of these methods can be used. When rate measurements are desired, the fungus is grown in special tubes on semisolid media. The progression of the mycelial frontier of the mold along the surface of the culture medium is linear with time for any given concentration of nutrilite, but increases to a maximum with increasing concentration of the nutrilite. Rate of progression can be readily measured

and compared at either one or several time intervals (Ryan, Beadle, and Tatum, 1943). A variation of this procedure involving measurement of the diameter of mycelial growth in Petri plates has been described by Thompson, Isbell, and Mitchell (1943). For assay of leucine, however, the determination of total growth was found to be preferable to rate measurements. The organism is cultured in a liquid medium containing increasing concentrations of leucine. After growth has proceeded to completion, the mycelial mat is filtered out, washed, dried, and weighed (Ryan and Brand, 1944).

II. CRITERIA FOR ESTABLISHING RELIABILITY IN AN ASSAY

1. *Agreement with Other Methods*

Where reliable and accepted methods are available for the determination of an amino acid, it is easy to establish the reliability of a proposed microbiological method. If the proposed method yields values which consistently check those obtained by the proven procedures, it can also be accepted as reliable. Thus the microbiological method for arginine (McMahan and Snell, 1944), utilizing *L. casei*, and the microbiological method for leucine, utilizing a mutant strain of *N. crassa* (Ryan and Brand, 1944), can be accepted as yielding reliable values on these grounds. The former yields values which check the procedure of Vickery (1940), involving isolation of arginine as its flavianate; the latter yields values which check those obtained by the solubility product method (Moore and Stein, 1943) and by isotope dilution.

For some amino acids, however, reliable methods either are not available,[1] or values obtained by their use are so few as to be of little value in determining reliability of a new procedure. A number of other criteria for accuracy are available, none of which alone is conclusive, but which strongly indicate reliability when taken together. These are listed below.

2. *Agreement of Values Calculated from Various Assay Levels*

In running an assay, a standard curve is obtained in which growth response to the standard substance is plotted against its concentration. The sample is also assayed at several concentration levels, calculated to yield an amount of growth which falls upon the standard curve. The per cent of the amino acid present is then calculated from interpolation on the standard curve. The per cent thus found should be constant regardless of the portion of the standard curve from which it was calculated;

---

[1] The isotope dilution method (Rittenberg and Foster, 1943; Graff, Rittenberg, and Foster, 1940), theoretically applicable to any amino acid, is of course an exception. Figures obtained by its use are not yet generally available, and the equipment required precludes its use by most investigators.

that is, there should be no consistent drift up or down in assay values as the amount of sample added to the medium is increased. Such constancy of assay values with increasing concentration of sample indicates that the test organism responds to the sample in exactly the same fashion as it responds to the pure amino acid used in establishing the standard curve. Presumably it does so because that amino acid is the sole substance in the sample which is effective in influencing growth. This behavior is illustrated in Table V. There is no drift in values either up or down as the amount of sample is increased.

TABLE V
Constancy of Assay for Valine at Ascending Levels of Sample*

Casein Hydrolyzate Added	Valine Indicated From Standard Curve	Valine Present In Casein
		per cent
γ	γ	
40	2.75	6.9
60	3.88	6.5
80	5.20	6.5
100	6.82	6.9
120	7.88	6.5
140	9.10	6.5
160	10.4	6.5
		Average: 6.6

* Assay organism: *L. casei* (McMahan and Snell, 1944).

3. *Consistent Values on Repeated Assay*

The assay must yield consistent results for a single sample on repeated assay. This demonstrates that regardless of variable conditions obtaining upon different days — variations in inoculum, etc. — the organism consistently behaves toward the sample in the same manner as it does toward the standard substance. Such behavior is illustrated in Table VI.

TABLE VI
Constancy of Results on Repeated Assay: Effect of Altered Conditions*

Assay No.	Valine Present in Casein	
	per cent	
1	6.72	
2	6.66	Turbidimetric: 6.69
3	6.90	Acidimetric: 6.63
4	6.46	Incubated 32° C. 6.58
5	6.80	Incubated 37° C. 6.60
6	6.62	

Average: 6.69 ± 0.23

* Assay organism: *L. casei* (McMahan and Snell, 1944).

This test can be made more rigorous by purposefully changing conditions of assay, to determine if the response to the sample changes in the same manner as the response to the standard. If so, the same assay value will be obtained as under the standard conditions. Thus, in valine assay with *L. casei* (Table VI), exactly the same result was obtained when the test cultures were grown for three days at 32° or at 37°, or whether one determined response to the amino acid by turbidimetric or acidimetric means.

4. *Recovery Experiments*

When a known amount of the amino acid being determined is added to the sample, it must be quantitatively recovered. High or low recoveries indicate stimulation or suppression of response to the amino acid by other substances present in the sample. In either case, the assay value is suspect. In a refinement of this test, Dunn, *et al.* (1944a) simulate, from the best available data, the composition of the sample by mixtures of known substances, then determine if the known amount of the amino acid present in this "artificial sample" can be quantitatively determined. Examples of some recoveries obtained in valine assay are given in Table VII.

TABLE VII

Recovery Experiments in Valine Assay: Comparison of Test Organisms

Organism	Valine Found		Recovery
	Casein	Casein +6% Valine	
	per cent	*per cent*	*per cent*
*L. casei**	6.72	12.92	103
	6.80	12.79	100
*L. arabinosus**	6.86	12.88	100
L. pentosus†	6.82	12.81	100
S. fecalis‡	6.95	12.93	100
	6.70	—	—

* McMahan and Snell (1944).
† McMahan and Snell (unpublished data).
‡ Guirard and Snell (unpublished data).

5. *Agreement between Different Assay Organisms*

If two or more organisms which vary in other nutritive requirements yield the same assay value on a sample, the probability that the assay value is correct is greatly increased; for it is improbable that two or more different organisms would respond to interfering materials in exactly the same manner and to exactly the same extent. Such agreement is evident in valine assay with *L. arabinosus, L. casei, L. pentosus,* and *S. fecalis* (Table VII); all four organisms give quantitative recovery of added valine. Similarly, values for leucine obtained with *L. arabinosus* (Kuiken, *et al.*,

1943) check rather well those obtained with *Neurospora* (Ryan and Brand, 1944). Such agreement between two such diverse organisms would strongly indicate reliability of the values obtained by both methods even in the absence of the rigorous proof for reliability of their method furnished by Ryan and Brand.

6. *Specificity Studies*

The specificity of response to an amino acid should always be investigated preliminary to use of a medium or organism for assay. Obviously, if the organism responds to substances other than the pure amino acid which occur in the samples under investigation, steps must be taken to correct such lack of specificity. In general, the lactic acid bacteria have proved very specific in their response when complete media are used. Details of such studies will be given below when the individual amino acids are considered.

The problem of devising media upon which the requirement for an amino acid can be demonstrated, and with which the amount present in a pure solution of the amino acid can be determined, is relatively easy. It is not certain, however, that such a medium will permit quantitative and specific estimation of the amino acid in a protein hydrolyzate or other natural sample. As indicated previously, the apparent requirement for an amino acid can vary with the composition of the basal medium. As a general rule, therefore, the assay medium used should be so complete that additions of the test sample will not significantly change its quantitative or qualitative composition, except with respect to the concentration of the substance to be studied. Effect of failure to observe this rule is strikingly shown in data of Table VIII.

TABLE VIII

Non-Specificity of Response of L. arabinosus to Glutamic Acid in Medium Lacking Certain "Non-Essential" Amino Acids

Hydrolyzate	Glutamic Acid Found				
	Microbiological Method*				Chemical Methods
	Medium A†		Medium B‡		
	Assay per cent	*Recovery per cent*	*Assay per cent*	*Recovery per cent*	
Gliadin	67.9	124	45.7	100	45.7 (Olcott, 1944)
Casein	35.2	112	20.4	101	22.0 (Olcott, 1944; Bailey, et al., 1943)
Gelatin	16.7	123	10.5	103	12.0 (Olcott, 1944)

* Data taken from paper of Lewis and Olcott (1945). Dunn, *et al.* (1944a) have independently made similar observations.

† Amino acid mixture "b" of Shankman (1943), which contains no serine, proline, hydroxyproline, glycine, or tyrosine.

‡ Same as medium A, but supplemented with serine, proline, hydroxyproline, and tyrosine.

A medium which supported good growth of *L. arabinosus* when glutamic acid was added, but which contained no serine, proline, glycine, hydroxy-proline, or tyrosine, was used for the determination of glutamic acid. Such a medium permits quantitative recoveries of pure glutamic acid, or of the glutamic acid present in a simple mixture of amino acids (Shankman, Dunn, and Rubin, 1943). When the medium was used with *L. arabinosus* for assay of protein hydrolyzates, however, the values obtained for glutamic acid were much too high, and recovery of added glutamic acid was likewise high. When the missing amino acids were added to the medium, values obtained for glutamic acid were lowered to fair agreement with chemical methods, and added glutamic acid was quantitatively recovered (Lewis and Olcott, 1945; Dunn *et al.*, 1944a). The response to glutamic acid was non-specific in the absence of the missing amino acids, which themselves were either completely non-essential or accessory (cf. Table III). The value of recovery experiments for detecting this type of behavior is well illustrated by this case.

Another argument for the use of complete amino acid mixtures lies in reported antagonistic effects between certain amino acids. Thus, a toxic effect of glycine (and to a lesser extent of serine and threonine) on growth of *S. fecalis* is eliminated if adequate quantities of vitamin B6 or of alanine, or both, are present in the medium (Snell and Guirard, 1943). With *Proteus morganii*, Porter and Meyers (1944) observed that addition of norvaline, norleucine, or allothreonine inhibited growth on an otherwise adequate medium (cf. also Pelczar and Porter, 1943). In the presence of several other amino acids, however, this inhibition was not apparent. Similarly, Hutchings and Peterson (1943) observed that inhibitory effects of histidine, lysine, and isoleucine which were evident with *L. casei* under some conditions were generally counteracted by alanine, threonine, and other amino acids. Thus, presence of a complete quota of amino acids in adequate quantities in basal media used for assay appears highly desirable.

III. INDIVIDUAL ASSAY METHODS

1. *Methods Using Lactic Acid Bacteria: Assay Media and Methodology*

The composition of assay media suggested for use in assaying for amino acids with lactic acid bacteria is compared in Table IX. In general, the media are very similar to each other and to those used earlier for assay of vitamins. The chief differences are in the number and amount of amino acids used to replace the casein hydrolyzate of earlier media, and in the concentrations of vitamins included. The possibility for variation of concentrations of individual constituents in media of such complexity is almost infinite. In accord with the views on specificity expressed above, there has

TABLE IX

Suggested Media for Use in Determining Amino Acids with Lactic Acid Bacteria

Substance	Concentration Per 10 cc. of Final Medium[e]								
	Greene, Black (1943, 1944)*	Shankman (1943)†	Kuiken, et al. (1943)‡	McMahan, Snell (1944)‖	Hegsted (1944)¶	Baumgarten, et al. (1944)§	Dunn, et al. (1944a)#	Lewis, Olcott (1945)**	Schweigert, et al. (1944)††
Glucose, mg.	100	100	200	100	175	100	100	100	200
Sodium acetate, mg.	60	60	72.5	60	87.5	60	60	60	200
KH_2PO_4, mg.	5	5	5	10	5	5	5	5	5
K_2HPO_4, mg	5	5	5	10	5	5	5	5	5
$MgSO_4.7H_2O$ mg.	2	2	2	2	2	2	2	2	2
$FeSO_4.7H_2O$, mg.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
$MnSO_4.4H_2O$, mg.	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
NaCl, mg.[a]	0.1	0.1	0.1	0.1	0.1	0.1		100	0.1
$(NH_4)_2SO_4$, mg.				30	30				
Thiamine hydrochloride, γ	1.0	1.0	1.0	1.0	4.0	10	10	5.0	2
Pyridoxin hydrochloride, γ	1.0	1.0	1.0	3.0	4.0	2.0	16	5.0	2
Calcium pantothenate, γ	1.0	1.0	1.0	1.0	4.0	2.0	10	1.0	2
Riboflavin, γ	2.0	2.0	2.0	1.0	4.0	2.0	20	2.0	4
Nicotinic acid, γ	2.0	2.0	4.0	1.0	4.0	4.0	20	5.0	2
Biotin, γ	0.004	0.004	0.004	0.002	0.004	0.05	0.05	0.1	1
p-Aminobenzoic acid, γ	1.0	0.1	0.005	3.0	4.0	2.0	1.0	0.1	0.002
Folic acid, γ[b]				0.1			0.11	0.04	
Choline chloride, γ				25					
Inositol, γ				25					
Adenine, mg.	0.1	0.1	0.05	0.1	0.04	0.5	1.0	0.1	0.1
Guanine, mg.	0.1	0.1	0.05	0.1	0.04	0.5	1.0	0.1	0.1
Uracil, mg.	0.1	0.1	0.05	0.1	0.04	0.5	1.0	0.1	0.1
Xanthine, mg.				0.1	0.04				
Aspartic acid mg.		1.6	4.0	4.0	0.83	1.0	‡		4.0††
Glutamic acid mg.		2.0	4.0	10.0	2.5	2.0	‡		4.0
Arginine monohydrochloride, mg.		0.1	2.0	1.0	0.83	2.0	0.50		0.5
Histidine monohydrochloride, mg.			2.0	1.0	0.83	2.0	0.50		0.5
Lysine monohydrochloride, mg.		0.4	2.0	1.0	0.42	1.0	1.0		2.0
Alanine, mg.		1.0	1.0	1.0	0.42	1.0	1.0		1.0
Cystine, mg.	1.0	0.10	2.0	1.0	0.83	2.0	1.0	1.0	1.0
Glycine, mg.				1.0		2.0	1.0		
Hydroxyproline, mg.				1.0	0.83	2.0	1.0		
Isoleucine, mg.		0.50	1.0	1.0	0.42	1.0	1.0		1.0
Leucine, mg.		0.50	1.0	1.0	0.83	2.0	2.0		1.0
Methionine, mg.		0.10	1.0	1.0	0.42	1.0	0.50		0.5
Norleucine, mg.				1.0	0.42	1.0	0.5		
Norvaline, mg.				1.0					
Phenylalanine, mg.		0.10	2.0	1.0	0.42	1.0	0.50		0.5
Proline, mg.			2.0	1.0	0.83	2.0	1.0		
Serine, mg.			1.0	1.0	0.42	1.0	0.25		
Threonine, mg.		0.50	1.0	1.0	0.42	1.0	1.0		1.0
Tryptophan, mg.	●	0.10	2.0	1.0	0.83	2.0	0.50	1.0	0.4
Tyrosine, mg.		0.10	1.0	1.0	0.83	2.0	0.20		0.4
Valine, mg.		0.50	1.0	1.0	0.42	1.0	1.0		1.0
Hydrolyzed casein, mg.	50							50**	

* Recommended for assay of tryptophan with *L. arabinosus*. Tryptophan is omitted when medium is used for this purpose. The medium is the same as that recommended by Snell and Wright (1941) for nicotinic acid assay, except for addition of p-aminobenzoic acid and nicotinic acid, and omission of tryptophan.

† Media similar to this have been used with *L. arabinosus* for assay of some amino acids, e.g., valine and leucine, by omitting the proper one from the medium (cf. Schweigert, et al., 1944).

‡ Recommended for assay of leucine, isoleucine, or valine with *L. arabinosus* by omitting the proper amino acid. The authors also add a tomato juice eluate factor at a level of 1 mg. per 10 cc. of medium. Some evidence indicates this may be replaced by a folic acid concentrate (cf. Baumgarten, et al.), although the active principle does not appear to be folic acid.

‖ Recommended for assay of arginine and valine with either *L. casei* or *L. arabinosus*, but devised to support growth of all lactic acid bacteria whose nutritional requirements are known, and thus to be generally applicable as a base medium for assay of other amino acids by omitting proper amino acid from the medium.

¶ Used as base for determining amino acid requirements of *L. arabinosus*, and used for tentative determination of leucine, valine, and phenylalanine.

§ Devised as a complete medium allowing maximum growth of *L. arabinosus* and *L. casei*, for later use in determining amino acids.

Devised for determination of glutamic acid with *L. arabinosus*. 4.0 mg. asparagine is added per 10 cc. in place of aspartic acid.

been a tendency for later investigators to employ each essential constituent at higher levels than previously used, and to include constituents which were previously omitted because they were thought to be without effect on growth. Both McMahan and Snell, and Hegsted independently included ammonium sulfate in their media. This was done because ammonium salts arise during hydrolysis of proteins, and may influence growth under some conditions (cf. glutamic acid assay, below). The most flexible and commonly used method for producing a medium lacking a single amino acid is to employ a mixture of pure amino acids as the nitrogen source, and omit the one to be determined. If large numbers of routine assays are made, this is a fairly expensive method. An alternate approach is to remove quantitatively the single amino acid to be determined from the hydrolyzate of a complete protein, then use this hydrolyzate as the nitrogen source. Simple acid hydrolysis accomplishes this for tryptophan (Greene and Black, 1944). Lewis and Olcott (1945) have devised a method for selective removal of glutamic acid from protein hydrolyzates by its conversion to pyrrolidone carboxylic acid and subsequent extraction with ethyl acetate. Additional investigation of similar procedures for the selective removal of other amino acids from protein hydrolyzates should yield profitable results.

In methodology, too, each of the assay methods is very similar, and patterned after earlier methods for vitamin assay. The selected medium, deficient in a single amino acid, is prepared at twice its final concentration. Standard solutions of the amino acid and of the samples are added to a series of test tubes in amounts calculated to produce several gradations in growth of the test organism between no growth and maximum possible growth. Contents of all tubes are diluted with water to the same volume, and an equal volume of medium is added. Tubes are then capped properly, sterilized by autoclaving, cooled, and inoculated with a suspension of the test organism obtained from an actively growing culture. The assay tubes are then incubated at a constant temperature near the optimum temperature of the organism used. After a sufficiently long incubation period, growth present in each tube is determined by one of the methods previously

** Devised for determination of glutamic acid with *L. arabinosus*. The casein hydrolyzate used has been freed from glutamic acid by converting the latter to pyrrolidone carboxylic acid and extracting with ethyl acetate.

†† Recommended for determination of valine and leucine in both protein hydrolyzates and fresh foodstuffs. When thus used, the appropriate amino acid is omitted from the medium. Test organism: *L. arabinosus*. Asparagine added in place of aspartic acid.

a Most media contain more NaCl than is indicated by these additions due to use of HCl to dissolve or preserve certain constituents of the medium, and subsequent neutralization.

b Expressed as micrograms of material 40,000 times as active by weight as an arbitrary standard, liver fraction B., 0.1γ of material of this potency is about 20 times the amount required to produce maximal growth of *L. casei* on a folic acid-free medium. Very crude concentrates of this material are suitable for use.

c No convention has been followed by different investigators concerning use of the *l-* or *dl-* forms of the amino acids. All comparisons in this table are on the basis of the weight of the naturally occurring *l-* isomer added. If *dl-* amino acids were used, the amount added was twice that indicated. This procedure seems justified, since in most cases so far investigated, the unnatural isomers of the amino acids have not been available for growth to any marked extent.

described. The growth response to increasing concentrations of the amino acid is then plotted against the concentrations of the amino acid to produce a standard curve. The amount of amino acid present in the various samples is then determined by interpolating the growth response in the sample tubes onto this standard curve. Tests for the reliability of results obtained in this fashion were discussed above.

a. *Determination of Arginine.* McMahan and Snell (1944) used the determination of arginine as a test case to see if values obtained by a microbiological procedure would agree with reliable chemical procedures for this amino acid. Growth was measured turbidimetrically on diluted cultures after three days' incubation. *L. casei* was the test organism, and

MICROGRAMS OF l(+)-ARGININE
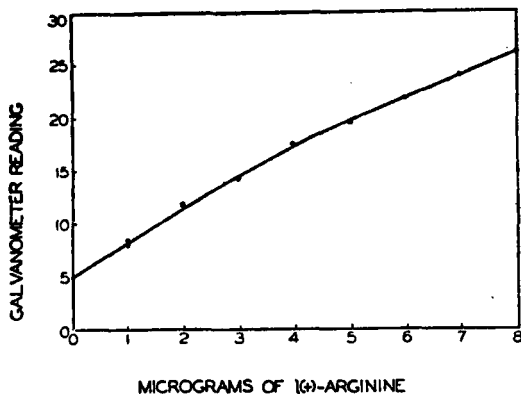
FIG. 1

the medium was that described in Table IX, with arginine omitted. Response to added l(+) arginine under these conditions is shown in Fig. 1. Assay values obtained are shown in Table X. They are in excellent agreement with those obtained by direct isolation of arginine as its monoflavianate. Quantitative recoveries of added arginine were obtained, and other criteria for reliability discussed above were fulfilled. The method thus appears to give reliable values for hydrolyzates of a variety of proteins.

In extending this or similar methods to a wider range of samples, especially non-protein samples, detailed knowledge of the specificity of the test is desirable. Kuiken, et al. (1943) showed that ornithine or citrulline would duplicate the effect of arginine on growth of *L. arabinosus.* With *L. casei,* ornithine cannot be used in place of arginine; citrulline has not been tested. d(-)Arginine is inactive (Stokes and Gunness, 1944). Values for arginine obtained on enzymatic digests of proteins are low,

TABLE X

Comparison of Microbiological and Chemical Values
for Arginine in Protein Hydrolyzates

Protein	Arginine	
	Microbiological Method*	Chemical Method
	per cent	*per cent*
Casein	3.7	3.72 (Vickery, 1940)
Ovalbumin	5.6	5.66 (Vickery, 1940)
Silk fibroin	0.97	0.95 (Bergmann, Niemann, 1938)
Horse hemoglobin	3.5	3.59 (Vickery, 1940)
Lactoglobulin	2.9	2.89 (Chibnall, 1942)
Gelatin	9.3	9.34 (Kossel, Gross, 1924)
		9.1 (Block, Bolling, 1940)

* McMahan and Snell (1944). Assay organism: *L. casei*. Assay range: 0–40γ $l(+)$ arginine per 10 cc. medium. When smaller volumes are used, correspondingly less standard and sample are required.

indicating that arginine is not always fully available when combined in peptide linkage.

b. *Determination of Valine, Leucine, and Isoleucine.* The determination of these three amino acids has been described by Kuiken, *et al.* (1943) with *L. arabinosus* and the medium described in Table IX. A number of other workers have also determined one or more of these amino acids. Results of McMahan and Snell (1944) with valine assay have been cited above to illustrate application of various criteria for accuracy to an assay. The various values reported for valine in casein are listed in Table XI. Agreement is considerably poorer than could be desired. Hegsted stated that his values were preliminary and exploratory in nature. It seems probable that the other differences are due to differences in the samples and in their preparation, rather than to major differences in the methods used. Thus values of Dunn are reported on a moisture and ash-free basis. Other authors did not specify whether or not this was true. Different workers

TABLE XI

Valine Content of Casein as Determined Microbiologically

Valine Found *per cent*	Test Organism	Reference
6.25	*L. arabinosus*	Kuiken, *et al.* (1943)
6.2	*L. arabinosus*	Schweigert, *et al.* (1944)
6.86	*L. arabinosus*	McMahan, Snell (1944)
6.7*	*L. casei*	McMahan, Snell (1944)
6.8	*L. pentosus*	McMahan, Snell (unpublished)
6.7, 6.9	*S. fecalis*	Guirard, Snell (unpublished)
6.8, 7.3	*L. arabinosus*	Dunn (1944)
5.2	*L. arabinosus*	Hegsted (1944)

* Average of six determinations; range 6.46 to 6.90; cf. Table VI.

have used different temperatures, time intervals, and acid concentrations
for hydrolysis. Some (*e.g.*, Kuiken, *et al.*) have used sulfuric acid for the
hydrolysis and removed sulfate ion with barium hydroxide, a procedure
which can lead to losses of amino acids. Others use hydrochloric acid, and
simply neutralize with sodium hydroxide before assay. These factors
must be controlled, or their effects on assay values determined before

TABLE XII

*Valine, Leucine, and Isoleucine Contents of Various Materials
as Determined Microbiologically*

Substance	Valine*	Amino Acid Present Leucine†	Isoleucine‡
	per cent	*per cent*	*per cent*
Casein	6.7 (1)	9.27 (2)	6.05 (2)
	6.25 (2)	9.1 (5)	5.9, 6.3 (5)
Ovalbumin	6.8 (1)		
Hemoglobin (horse)	8.8 (1)		
Lactoglobulin	5.8 (1)		
Gelatin	2.67 (1)		
	2.46 (2)	3.30 (2)	1.71 (2)
	2.40 (3)		
Wheat	0.58 (2)	0.93 (2)	0.53 (2)
Cottonseed meal	1.70 (2)	2.28 (2)	1.55 (2)
Soybean meal	2.06 (2)	3.23 (2)	2.29 (2)
Beef round	0.96 (4)	1.36 (4)	
Beef liver	1.08 (4)	1.72 (4)	

(1) McMahan and Snell (1944); test organism, *L. casei.*
(2) Kuiken, Norman, Hale, Lyman, and Blotter (1943); test organism, *L. arabinosus.*
(3) Guirard and Snell (unpublished); test organism, *S. fecalis* R.
(4) Schweigert, McIntire, Elvehjem, and Strong (1944); test organism, *L. arabinosus.*
(5) Dunn (1944).
* Assay range: 0 to 50γ *l*-valine per 10 cc. medium.
† Assay range: 0 to 50γ *l*-leucine per 10 cc. medium.
‡ Assay range: 0 to 50γ *l*-isoleucine per 10 cc. medium.

rigorous comparison of data secured from different laboratories by slightly
different methods can be made. It has been reported that longer hydrolysis
periods are necessary to secure maximum values for valine than for most
other amino acids tested (McMahan and Snell, 1944).

A summary of values for the valine, leucine, and isoleucine content of
various representative materials as determined by these methods is given
in Table XII. Of considerable interest is the valine and isoleucine content
of gelatin. Both amino acids had been reported absent from gelatin
(Dakin, 1920), but are listed as present in more recent compilations (cf.
Block and Bolling, 1940). The figures obtained microbiologically are
somewhat higher than those given by Block and Bolling.

With leucine, valine, and isoleucine, only those amino acids having the
l-configuration are effective in promoting growth. At the concentrations

used, simultaneous presence of the *d*-isomer (as in the *dl*-mixtures) neither stimulates nor suppresses response to the *l*-isomer (cf. Kuiken, *et al.*, 1944, and others). This is fortunate, since synthetic amino acids are used interchangeably with isolated amino acids in preparation of the media. Fox, *et al.* (1944) have shown that *d*-leucine will inhibit growth of *L. arabinosus* if added to the medium at very high levels; *l*-leucine was without effect at the same level. The concentration used was 50 to 100 times higher than that ordinarily used in assay media, and 1000 times higher than that necessary for leucine assay.

When hydrolysis is incomplete, values obtained for valine, leucine, and isoleucine are low. Similarly, leucine present in the peptides leucylglycine and glycylleucine could be determined quantitatively only following hydrolysis (Kuiken, *et al.*, 1944). Negligible quantities of these amino acids were destroyed by humin formation during acid hydrolysis, even when carbohydrate was added before hydrolysis.

c. Determination of Tryptophan. Greene and Black (1943, 1944) have described a microbiological method for tryptophan which depends upon the response of *L. arabinosus* to tryptophan in the medium described in Table IX. Since tryptophan is destroyed by acid hydrolysis, pancreatic digestion or hydrolysis with barium hydroxide is employed to release the tryptophan. The latter procedure effects complete racemization. Since *d*-tryptophan is inactive in supporting growth, results obtained following hydrolysis with barium hydroxide are lower than those obtained by enzymatic digestion, and must be multiplied by two if *l*-tryptophan is used as standard. When this is done, values obtained following enzymatic digestion are sometimes low, indicating that tryptophan is not always rendered completely available by this procedure. This is consistent with the findings reported above for other amino acids following incomplete hydrolysis.[1]

As originally reported by Snell (1943), these investigators found that indole and anthranilic acid can replace tryptophan for growth of *L. arabinosus*. Since both substances occur naturally, they must be removed before assay. This is accomplished by extracting them from the hydrolyzed sample with ether at pH 4.0 preliminary to assay.[2] Other compounds which occur naturally and are related to tryptophan in structure were ineffective in promoting growth. These included skatole, indolebutyric acid, indolepropionic acid, indoleacetic acid, kynurenic acid, and tryptamine.

[1] Woolley and Sebrell (*J. Biol. Chem.* **157**, 141 (1945)) have been unable to obtain quantitative recoveries of added tryptophan when alkaline hydrolysis is used, and have developed an enzymatic procedure for hydrolysis to eliminate the necessity for using alkali.

[2] An alternate procedure would be to use proper strains of *S. fecalis, Leuc. mesenteroides*, or *L. pentosus* for the assay organism, since these organisms cannot utilize indole or anthranilic acid in place of tryptophan (Snell, 1943).

The method gives consistent values on repeated assay, concordant values at increasing levels, and permits quantitative recovery of added tryptophan. It checks rather closely values obtained in recent investigations by other methods, but often gives values which differ from those reported in the older literature (cf. Table XIII). Sullivan and Hess (1944) have recently indicated some of the factors which contribute to variability of figures for tryptophan obtained by colorimetric procedures.

TABLE XIII

Tryptophan Content of Various Substances

| | | Tryptophan | |
Substance Assayed	Microbiological Method* per cent	Other Methods per cent	
Casein	1.19	1.21, 1.22	(Toennies, 1942; Beach, et al., 1941)
Ovalbumin	1.47	1.26, 1.6	(Brand, Kassell, 1939; Block, Bolling, 1940)
Lactalbumin	2.06	2.14	(Plimmer, Lowndes, 1937)
Gliadin	0.59	1.10	(Looney, 1926)
Zein	0.10		
White flour	1.23	0.84	(Block, Bolling, 1943)
Dried milk	1.31	1.32	(Beach, et al., 1943)

* From Greene and Black (1944). All analytical figures are based on 16% nitrogen. Assay organism: *L. arabinosus*. Assay range: 0–10 γ *l*-tryptophan per 10 cc. medium.

A similar procedure has been described by Dunn and coworkers (1945) for determination of free tryptophan in blood.

d. *Determination of Glutamic Acid.* Because glutamic acid is a prominent constituent of many proteins, appears to be an important intermediate in metabolism, and is a useful raw material in the food industries, and because the chemical methods available for its determination are tedious, several workers have attempted the microbiological assay of this compound. The method of Dunn and coworkers (1944a) has appeared in full; those of Lewis and Olcott (1944, 1945), Lyman, et al. (1944, 1945) and Hac, et al. (1945) are available only in abstract or manuscript form at the time of writing.

Determination of glutamic acid presents some points of special interest. With assays described above, growth (or acid production) appears to increase in direct proportion to the amount of amino acid added to the medium. This result would be expected if the amino acids are utilized directly for synthesis of bacterial protoplasm, and this protoplasm has a constant composition. With glutamic acid, however, standard curves obtained are sigmoidal in nature, *i.e.*, at lower concentrations of glutamic acid there is little growth above the blanks, but as the concentration is raised a rather sharp break in the curve occurs, then growth increases very

rapidly with increasing concentration until a maximum is reached. Despite this behavior, standard and hydrolyzed samples behave alike when conditions are rigorously controlled, and both Dunn, et al. and Lewis and Olcott have used such standard curves and obtain figures for the glutamic acid content of proteins which appear to be precise, and are in good agreement with values obtained by chemical and isolation procedures.

This behavior suggested to Lyman, et al. (1944) the hypothesis that at least part of the glutamic acid must be converted to some other substance before it could be utilized, and that this conversion was readily effected by the organism only when the glutamic acid concentration was high, or when vigorous growth had been initiated. Glutamine, which is well-known as a growth stimulant for many organisms (cf. McIlwain, et al., 1939; Lankford and Snell, 1943), including some lactic acid bacteria (Feeney and Strong, 1942; Pollack and Lindner, 1943; Niven, 1944), was the most obvious possibility. They found that addition of small amounts of glutamine to the assay medium permitted growth initiation, and that the growth curve then obtained with glutamic acid rose regularly with increasing concentration.

In an independent investigation of the same problem, Hac, et al. (1945) found that (a) when glutamine was used in place of glutamic acid, curves were not sigmoidal, but resembled those obtained with other amino acids; (b) the concentration of glutamic acid at which the "break" occurred was decreased by increasing the inoculum, increasing the time of incubation, or lowering the pH of the medium; (c) omission of ammonium salts from the medium increased the concentration of glutamic acid necessary for growth initiation. When conditions were chosen to permit growth initiation with the smallest possible concentration of glutamic acid (i.e., large inoculum, low pH, long incubation time, high concentration of ammonium salts), the curve obtained with glutamic acid tended to approach that obtained with glutamine. Coupled with results of Lyman, et al., these results indicate that glutamic acid must be converted to glutamine before being utilized by L. arabinosus,[1] and that presence of ammonium salts aids in the conversion.

Lyman, et al., therefore, add glutamine routinely to their assay medium (that of Kuiken, et al., Table IX, with glutamic acid omitted) in an amount sufficient to permit growth initiation, but insufficient to furnish more than a small fraction of the total requirement of the test organism for it.[2]

[1] Exactly similar behavior is noted when L. casei, S. fecalis, or Leuc. mesenteroides is used as the assay organism; the phenomenon thus appears to be of general occurrence.

[2] Most of the glutamine added is converted to inactive pyrrolidone carboxylic acid by autoclaving; only enough is left to permit growth initiation. Autoclaving time and temperature must therefore be rigorously controlled. Guirard and Snell (unpublished)

Comparative figures for the glutamic acid content of various substances as determined by these and other methods are given in Table XIV. In spite of differences in assay techniques, there is very substantial agreement between the various workers, which would undoubtedly be even better if standard samples were used and prepared for assay by a standard procedure. In some cases, agreement with figures obtained by Chibnall and coworkers by isolation procedures is excellent; in some cases, the microbiological methods yield slightly lower results than the chemical methods. The reason for this variation is not yet apparent.

Kuiken, et al. (1943a) reported that dl-glutamic acid was only 50 per cent as active as l(+) glutamic acid; other workers (Lewis and Olcott, 1945; Hac, et al., 1945) find dl-glutamic acid to be about 55 per cent as active as l(+) glutamic acid. Dunn, et al. (1944a) observed that dl-glutamic acid, although much less active than l(+) glutamic acid in the concentration range used for assay, permitted higher acid production when present in excess, and suggested that d(−) glutamic acid plays some role in the normal metabolism of these organisms. α-Ketoglutaric acid is 1–6 per cent as active as l(+) glutamic acid; α-hydroxyglutaric acid and pyrrolidone carboxylic acid are inactive. Peptides of glutamic acid, including glutathione, were less active than an equivalent amount of glutamic acid, but gave full activity after hydrolysis. Nevertheless, enzymatic protein digests or incompletely hydrolyzed samples may give falsely high values for their glutamic acid content, probably due to presence of specific peptides which replace glutamine for the organism (cf. footnote 2, p. 109). Glutamine is more active than glutamic acid, especially at low levels on assay media which are not supplemented with it (Lewis and Olcott, 1945). Lyman, et al. (1945) found very little destruction of glutamic acid by humin formation, even when carbohydrate was added before acid hydrolysis.

e. Determination of Lysine. Dunn, et al. (1944b) have recommended *Leuc. mesenteroides* P-60 for the determination of lysine in protein hydrolyzates. The medium used for glutamic acid assay (Table X) was modified[1] to permit increased acid production by the test organism. Response to added lysine was determined by titration of acid produced during 3 days' growth at 37° C. The assay range lies between 0 and 200γ of lysine per 10 cc. of medium. In 15 assays of casein, an average value of 7.72 per cent

have successfully substituted small amounts of a tryptic digest of casein for the glutamine. Such a preparation is stable to autoclaving. It is known that such enzymatic digests contain a substance (or substances) which replaces glutamine and asparagine for lactic acid bacteria (Pollack and Lindner, 1943; Wright and Skeggs, 1944).

[1] Glucose and sodium acetate concentrations were doubled; dl-alanine was increased to 20 mg. per 10 cc. and minor variations made in concentration of other amino acids. Glutamic acid was added and lysine omitted from the medium; ammonium chloride was also added.

TABLE XIV

The Glutamic Acid Content of Various Substances: Comparative Values

Preparation	Glutamic Acid				
	Microbiological Assay*				
	Dunn, et al. (1944a)	Lewis, Olcott (1945)	Lyman. et al. (1945)	Hac, et al. (1945)	Chemical Procedures
	per cent	per cent	per cent	per cent	
Casein	21.2	19.7	21.5	21.1	—
Casein, corrected for ash	22.5	—	22.4	—	22.0 (Bailey, Chibnall, et al., 1943) 22.0 (Olcott, 1944)
Silk fibroin	2.03 ±0.05	—	—	—	—
Egg albumin	—	11.6−13.7†	14.3	14.3−15.1†	16.0 (Chibnall, et al., 1943)
Gliadin	—	44.2	—	44.9	45.7 (Olcott, 1944) 46.9 (Bailey, Chibnall, et al., 1943)
Lactoglobulin	—	18.7	—	19.0§	21.5 (Chibnall, et al., 1943) 21.5 (Olcott, 1944)
Gelatin	—	10.2, 10.8	—	10.2	11.7 (Olcott, 1944)
Brewers' yeast	—	—	5.56	—	—
Torula yeast	8.0	—	—	—	—

* All investigators have used *L. arabinosus* as the test organism; media are formulated in Table IX. Assay range: In media unsupplemented with glutamine, range varies with size of inoculum and other factors, but is usually between 0 and $250\gamma l(+)$ glutamic acid per 10 cc. medium. With glutamine added to initiate growth the range is 0–150γ.

† Interfering substances were noted in egg albumin, not present in most other samples, which contributed to variability in these assays.

§Values of 19.0 and 19.1% were recently reported for the glutamic acid content of β-lactoglobulin by the isotope dilution procedure (Foster, G. L., *J. Biol. Chem.*, **159**, 431. 1945).

of lysine was found (8.3 per cent corrected for moisture and ash). The probable accuracy of this figure for lysine in casein has been discussed by Dunn, *et al.* It is considerably higher than values ordinarily obtained by isolation of lysine picrate following removal of arginine and histidine as their silver salts (*e.g.*, 6.25 per cent, Vickery and White, 1933) but is in fair agreement with values obtained by the nitrogen distribution procedure and some other methods (cf. Dunn, *et al.*). In a series of 15 consecutive assays, the maximum deviation from the average value was about 17 per cent; the average deviation was about 4 per cent of the average value. Recoveries of added lysine were quantitative in most instances, and assay values were satisfactorily constant at increasing levels of the sample. No data concerning possible activity of related compounds (*e.g.*, hydroxy-lysine, $d(-)$ lysine, peptides of lysine, etc.) are yet available. The authors found 0.56 per cent lysine in silk fibroin. Data for additional samples would be helpful in judging the general applicability of this procedure.

f. Other Amino Acids. From investigation of Table IV it is apparent that the lactic acid bacteria will be applied to determination of many amino acids for which detailed methods have not yet been suggested. A number of miscellaneous figures obtained in this fashion have already appeared. Little can be said as to the reliability of these figures until further data concerning specificity of the response, etc., are available. Some of these figures are given in Table XV.

2. Methods Using Neurospora

A method for the determination of $l(+)$ leucine, based upon the require-ment for this amino acid of the mutant strain of *N. crassa* (Regnery, 1944) has been described by Ryan and Brand (1944). Aliquots up to 5cc. of a standard solution of $l(+)$ leucine or unknown solution are meas-ured into 125 cc. Erlenmeyer flasks, then all volume are adjusted to 5 cc. with distilled water; 45 cc. of the basal medium (mineral salts, sucrose, ammonium tartrate, and biotin) are added; flasks are plugged with cotton, autoclaved, cooled, and inoculated with conidia from a stock culture. The mold is allowed to grow until no further increase in mycelial weight occurs ($8\frac{1}{2}$ days at 30° C.$\pm$0.2°), then the mycelia are filtered out through individual tared crucibles, washed, dried, and weighed. From repeated results with pure leucine, it was found that within the assay range (0–1.0 mg. of $l(+)$ leucine), mycelial weights were directly proportional to the concentration of leucine, the relationship being expressed by the straight-line equation:

$$\text{mg. leucine} = \frac{\text{mg. mycelium}}{42.66} - 0.002$$

TABLE XV

Microbiological Determination of Amino Acids: Miscellaneous Values

Amino Acid	Protein	Amino Acid	
		Microbiological Assay	Other Methods
		per cent	per cent
Aspartic acid	Casein	7.03 (Hac, Snell, 1945)*	6.65 (Bailey, Chibnall, *et al.*, 1943)
	Lactoglobulin	11.5 (Hac, Snell, 1945)§	9.88 (Chibnall, *et al.*, 1943)
	Egg albumin	9.0 (Hac, Snell, 1945)	8.13 (Bailey, Chibnall, *et al.*, 1943)
	Gliadin	2.8 (Hac, Snell, 1945)	1.40 (Bailey, Chibnall, *et al.*, 1943)
Tyrosine	Casein	5.2, 4.8 (Dunn, 1943)†	5.2 (Block, Bolling, 1940)
Methionine	Casein	3.1, 3.2 (Dunn, 1943)†	3.25 (Baernstein, 1936)
Phenylalanine	Casein	3.7 (Hegsted, 1944)†	3.88 (Foreman, 1919)
			5.8 (Block, Bolling, 1940)

* Assay organism: *Leuc. mesenteroides.*
† Assay organism: *L. arabinosus.*
§ Values of 11.3 and 11.2 per cent were recently reported by Foster (*J. Biol. Chem.*, **159**, 431, 1945) for the aspartic acid content of β-lactoglobulin, as determined by isotope dilution.

or approximately:

$$\text{mg. leucine} = 0.02335 \times \text{mg. mycelium}$$

This numerical relationship was constant, and once such a standard curve is established for the particular conditions being used, it need be checked only occasionally. Results obtained with the mold proved accurate by every test, and checked those obtained on the same protein samples by the isotope dilution and solubility product methods. Some of the results are given in Table XVI. The values also are in fair agreement with those

TABLE XVI

Leucine Assay with Neurospora Crassa (Mutant Culture)

Product	Leucine Content		
	Neurospora* per cent		Other Methods per cent
Gelatin	3.6, 3.6		3.5 (solubility product)[†]
			3.3 (*L. arabinosus*)[‡]
Egg albumin	9.6, 9.9		9.1 (solubility product)[†]
Casein	9.8 [‖]		9.27 (*L. arabinosus*)[‡]
Horse hemoglobin	15.7		15.1 (isotope dilution)[¶]
Wheat			0.82 (*L. arabinosus*)[‡]
Wheat flour	0.8		

* Ryan and Brand (1944).
† Cf. Moore and Stein (1943).
‡ Kuiken, Norman, Lyman, Hale, and Blotter (1943).
‖ Ryan and Brand obtained 8.5 per cent after 26 hours hydrolysis; Kuiken, *et al.* hydrolyzed for 24 hours. The result emphasizes need for a standard procedure for preparing samples before comparing results from different laboratories in detail.
¶ Cf. Rittenberg and Foster (1940).

obtained for leucine by Kuiken, *et al.* (Table XII), using *L. arabinosus.*

Occasionally, an individual culture flask will grow profusely even in the absence of leucine. This complete adaptation to growth in the absence of leucine is not yet understood. Partial adaptation to growth without leucine also may occur. Such partial adaptations occur only occasionally and yield values which differ from the mean of other values by amounts greater than twice the standard deviation. They are therefore discarded (Ryan and Brand, 1944).

The specificity of response of the culture to leucine has been described by Regnery (1944). α-Ketoisocaproic acid is as active in supporting and initiating growth of the test organism as *l*(+) leucine itself. *d*(−) Leucine supports growth, but does not permit its initiation. *dl*-Leucine is therefore much more than 50 per cent as active as *l*(+) leucine. α-Hydroxyisocaproic acid (leucic acid), isovaleric acid, isovaleraldehyde, and isoamyl alcohol increase weight of mycelium obtained when present together with sub-optimal amounts of *l*(+) leucine. If the method were applied to samples

which contained any of these products, it would yield falsely high values unless they were removed. Maximal figures for leucine were obtained with casein and egg albumin with 15 hours hydrolysis time (6 N HCl, reflux); values before or after this time were slightly lower.

The successful application of "leucineless" *N. crassa* to leucine assay raised the hope that other mutant strains of this organism, which require other amino acids, could be used for their determination. In an extension of their study, Ryan and Brand (1944a) have investigated a number of other cultures. Although such cultures could be used for the bioassay of amino acids in pure solution, they frequently gave completely misleading values when applied to protein hydrolyzates. Further knowledge of the metabolic interrelationships and requirements of such organisms may, however, permit future development of such methods.

3. *Other Methods*

The use of mutant cultures of *E. coli* (Roepke, *et al.*, 1944) for determination of amino acids has been suggested (Lampen, *et al.*, 1944) but details of such methods have not yet appeared. Further investigation should provide additional groups of organisms useful in this connection.

D. Conclusions

It is hoped by this review to have accomplished four purposes: (1) to show that microörganisms are available which can be used for determination of a large number of amino acids; and (2) that when properly worked out, such microbiological methods are capable of yielding results, the accuracy of which compares favorably with that of the best chemical methods; (3) to emphasize the need for careful study of each method to avoid errors caused by non-specific inhibitions or stimulation of the test organism, and to give certain criteria by means of which such interference can be detected; and (4) to summarize and compare certain data so far accumulated in this field. The advantages inherent in these methods have not been emphasized, but should be apparent. The laborious separations often necessary in protein analysis are eliminated. All the methods are very similar, and require only equipment readily available to every laboratory. They are unusually sensitive: compared with previous procedures for most amino acids, each of the microbiological assays is a micromethod. Analyses for the amino acid composition of rare proteins should now become practicable. With similar vitamin assays, it has been shown that the scale of assay can be cut 50-fold without sacrificing accuracy (Pennington, *et al.*, 1941; Lowry and Bessey, 1944); there is no apparent reason why similar adaptations in the amino acid assays cannot be made if the size of samples available for assay requires it. It is clear that continued careful study of such methods should yield results of great value to all fields of biochemistry.

REFERENCES

Baernstein, H. D. (1936). *J. Biol. Chem.* **115**, 25.

Bailey, K., Chibnall, A. C., Rees, M. W., and Williams, E. F. (1943). *Biochem. J.* **37**, 360.

Baumgarten, W., Garey, J. C., Olsen, M. J., Stone, L., and Boruff, C. S. (1944). *J. Am. Chem. Soc.* **66**, 1607.

Beach, E. F., Bernstein, S. S., Hoffman, O. D., Teague, D. M., and Macy, I. G. (1941). *J. Biol. Chem.* **139**, 57.

Beach, E. F., Munks, B., and Robinson, A. (1943). *J. Biol. Chem.* **148**, 431.

Beadle, G. W., and Tatum, E. L. (1941). *Proc. Natl. Acad. Sci.* **27**, 499.

Bergmann, M., and Niemann, C. (1938). *J. Biol. Chem.* **122**, 577.

Block, R. J., and Bolling, D. (1940). The Determination of the Amino Acids. Rev. Ed. Burgess Publishing Co. Minneapolis.

Block, R. J., and Bolling, D. (1943). *Arch. Biochem.* **3**, 217.

Brand, E., and Kassell, B. (1939). *J. Biol. Chem.* **131**, 489.

Burkholder, P. R., and McVeigh, I. (1940). *Am. J. Botany* **27**, 634.

Burrows, W. (1939). *J. Infectious Diseases* **64**, 145.

Chibnall, A. C. (1942). *Proc. Roy. Soc.* (London), B, **131**, 136.

Chibnall, A. C., Rees, M. W., and Williams, E. F. (1943). *Biochem. J.* **37**, 372.

Dakin, H. D. (1920). *J. Biol. Chem.* **44**, 499.

Dunn, M. S. (1944). Abstracts, 107th Meeting Am. Chem. Soc., Cleveland, 9A.

Dunn, M. S., Camien, M. N., Rockland, L. B., Shankman, S., and Goldberg, S. C. (1944a). *J. Biol. Chem.* **155**, 591.

Dunn, M. S., Camien, M. N., Shankman, S., Frankl, W., and Rockland, L. B. (1944b). *J. Biol. Chem.* **156**, 715.

Dunn, M. S., Shankman, S., Camien, M. N., Frankl, W., and Rockland, L. B. (1944c). *J. Biol. Chem.* **156**, 703.

Dunn, M. S., Schott, H. F., Frankl, W., and Rockland, L. B. (1945). *J. Biol. Chem.* **157**, 387.

Emmett, A. D., Bird, O. D., Brown, R. A., Peacock, G., and Vandenbelt, J. M. (1941). *Ind. Eng. Chem., Anal. Ed.* **13**, 219.

Feeney, R. E., and Strong, F. M. (1942). *J. Am. Chem. Soc.* **64**, 881.

Foreman, F. W. (1919). *Biochem. J.* **13**, 378.

Fox, S. W., Fling, M., and Bollenback, G. N. (1944). *J. Biol. Chem.* **155**, 465.

Gladstone, G. P. (1937). *Brit. J. Exptl. Path.* **18**, 322.

Graff, S., Rittenberg, D., and Foster, G. L. (1940). *J. Biol. Chem.* **133**, 745.

Greene, R. D., and Black, A. (1943). *Proc. Soc. Exptl. Biol. Med.* **54**, 322.

Greene, R. D., and Black, A. (1944). *J. Biol. Chem.* **155**, 1.

Hac, L., and Snell, E. E. (1945). *J. Biol. Chem.* **159**, 291.

Hac, L., Snell, E. E., and Williams, R. J. (1945). *J. Biol. Chem.* **159**, 273.

Hamner, K. C., Stewart, W. S., and Matrone, G. (1943). *Food Research* **8**, 444.

Hegsted, D. M. (1944). *J. Biol. Chem.* **152**, 193.

Hegsted, D. M., and Wardwell, E. D. (1944). *J. Biol. Chem.* **153**, 167.

Hutchings, B. L., Bohonos, N., and Peterson, W. H. (1941). *J. Biol. Chem.* **141**, 521.

Hutchings, B. L., and Peterson, W. H. (1943). *Proc. Soc. Exptl. Biol. Med.* **52**, 36.

Isbell, H. (1942). *J. Biol. Chem.* **144**, 567.

Knight, B. C. J. G. (1936). Bacterial Nutrition. Med. Res. Council, Spec. Rep. Ser. No. 210, London (H. M. Stationery Office).

Kossel, A., and Gross, R. E. (1924). *Z. physiol. Chem.* **135**, 167.

Kuiken, K. A., Norman, W. H., Lyman, C. M., and Hale, F. (1943a). *Science* **98**, 266.

Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L. (1943b). *J. Biol. Chem.* **151**, 615.

Lampen, J. O., Jones, M. J., and Roepke, R. R. (1944). Abstracts, 108th Meeting Am. Chem. Soc., New York, 26B.

Landy, M., and Dicken, D. M. (1942). *J. Lab. Clin. Med.* **27**, 1086.

Lankford, C. E., and Snell, E. E. (1943). *J. Bact.* **45**, 410.

Lewis, J. C. (1942). *J. Biol. Chem.* **146**, 441.

Lewis, J. C., and Olcott, H. S. (1944). Abstracts, 108th Meeting Am. Chem. Soc., New York, 27B.

Lewis, J. C., and Olcott, H. S. (1945). *J. Biol. Chem.* **157**, 265.

Looney, J. M. (1926). *J. Biol. Chem.* **69**, 519.

Lowry, O. H., and Bessey, O. A. (1944). *J. Biol. Chem.* **155**, 71.

Luckey, T. D., Briggs, G. M., and Elvehjem, C. A. (1944). *J. Biol. Chem.* **152**, 157.

Lyman, C. M., Kuiken, K., Blotter, L., and Hale, F. (1944). Abstracts, 108th Meeting Am. Chem. Soc., New York, 28B.

Lyman, C. M., Kuiken, K., Blotter, L., and Hale, F. (1945). *J. Biol. Chem.* **157**, 395.

McIlwain, H., Fildes, P., Gladstone, G. P., and Knight, B. C. J. G. (1939). *Biochem. J.* **33**, 223.

McMahan, J. R., and Snell, E. E. (1944). *J. Biol. Chem.* **152**, 83.

Meiklejohn, A. P. (1937). *Biochem. J.* **31**, 1441.

Mitchell, H. K., and Snell, E. E. (1941). *Univ. Texas Pub.* **4137**, 36.

Mitchell, H. K., Snell, E. E., and Williams, R. J. (1941). *J. Am. Chem. Soc.* **63**, 2284.

Mitchell, H. K., Snell, E. E., and Williams, R. J. (1944). *J. Am. Chem. Soc.* **66**, 267.

Möller, E. F. (1939). *Z. physiol. Chem.* **260**, 246.

Moore, S., and Stein, W. H. (1943). *J. Biol. Chem.* **150**, 113.

Niven, C. F., Jr. (1944). *J. Bact.* **47**, 343.

Niven, C. F., and Smiley, K. L. (1943). *J. Biol. Chem.* **150**, 1.

Olcott, H. S. (1944). *J. Biol. Chem.* **153**, 71.

Orla-Jensen, S. (1919). The Lactic Acid Bacteria. Copenhagen.

Pederson, C. S. (1936). *J. Bact.* **31**, 217.

Pelczar, M. J., and Porter, J. R. (1943). *Arch. Biochem.* **2**, 323.

Pennington, D., Snell, E. E., Mitchell, H. K., McMahan, J. R., and Williams, R. J. (1941). *Univ. Texas Pub.* **4137**, 14.

Peterson, W. H. (1941). *Biol. Symposia* **5**, 31.

Plimmer, R. H. A., and Lowndes, J. (1937). *Biochem. J.* **31**, 1751.

Pollack, M. A., and Lindner, M. (1943). *J. Biol. Chem.* **147**, 183.

Porter, J. R., and Meyers, F. P. (1944). *J. Bact.* **47**, 435.

Regnery, D. C. (1944). *J. Biol. Chem.* **154**, 151.

Rittenberg, D., and Foster, G. L. (1940). *J. Biol. Chem.* **133**, 737.

Roepke, R. R., Libby, R. L., and Small, M. H. (1944). *J. Bact.* **48**, 401.

Ryan, F. J., Beadle, G. W., and Tatum, E. L. (1943). *Am. J. Botany* **30**, 784.

Ryan, F. J., and Brand, E. (1944a). Abstracts, 108th Meeting, Am. Chem. Soc., New York, 5B.

Ryan, F. J., and Brand, E. (1944). *J. Biol. Chem.* **154**, 161.

Sarett, H. P., and Cheldelin, V. H. (1944). *J. Biol. Chem.* **155**, 153.

Schopfer, W. H. (1935). *Arch. Mikrobiol.* **6**, 139, 196.

Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Strong, F. M. (1944). *J. Biol. Chem.* **155**, 183.

Shankman, S. (1943). *J. Biol. Chem.* **150**, 305.

Shankman, S., Dunn, M. S., and Rubin, L. B. (1943). *J. Biol. Chem.* **150**, 477.

Sherman, J. M. (1937). *Bact. Revs.* **1**, 1.

Snell, E. E. (1943). *Arch. Biochem.* **2**, 389.

Snell, E. E. (1944). *J. Biol. Chem.* **154**, 313.

Snell, E. E., and Guirard, B. M. (1943). *Proc. Natl. Acad. Sci.* **29**, 66.

Snell, E. E., and Mitchell, H. K. (1941). *Proc. Natl. Acad. Sci.* **27**, 1.

Snell, E. E., and Peterson, W. H. (1939). *J. Biol. Chem.* **128**, xciv.

Snell, E. E., and Peterson, W. H. (1940). *J. Bact.* **39**, 273.

Snell, E. E., and Rannefeld, A. N. (1945). *J. Biol. Chem.* **157**, 475.

Snell, E. E., and Strong, F. M. (1939a). *Ind. Eng. Chem., Anal. Ed.* **11**, 346.

Snell, E. E. and Strong, F. M. (1939b). *Enzymologia* **6**, 186.

Snell, E. E., Strong, F. M., and Peterson, W. H. (1937). *Biochem. J.* **31**, 1789.

Snell, E. E., Strong, F. M., and Peterson, W. H. (1938). *J. Am. Chem. Soc.* **60**, 2825.

Snell, E. E., Tatum, E. L., and Peterson, W. H. (1937). *J. Bact.* **33**, 207.

Snell, E. E., and Wright, L. D. (1941). *J. Biol. Chem.* **139**, 675.

Stokes, J. L. (1944). *J. Bact.* **48**, 201.

Stokes, J. L., and Gunness, M. (1944). *J. Biol. Chem.* **154**, 715.

Sullivan, M. X., and Hess, W. C. (1944). *J. Biol. Chem.* **155**, 441.

Tatum, E. L., and Beadle, G. W. (1942). *Growth* **6**, 27.

Thompson, M. L., Cunningham, E., and Snell, E. E. (1944). *J. Nutrition* **28**, 123.

Thompson, R. C., Isbell, E. R., and Mitchell, H. K. (1943). *J. Biol. Chem.* **148**, 281.

Toennies, G. (1942). *J. Biol. Chem.* **145**, 667.

Vickery, H. B. (1940). *J. Biol. Chem.* **132**, 325.

Vickery, H. B., and White, A. (1933). *J. Biol. Chem.* **103**, 413.

Wildiers, E. (1901). *La Cellule* **18**, 313.

Williams, R. J. (1919). *J. Biol. Chem.* **38**, 465.

Williams, R. J. (1941). *Science* **93**, 412.

Wood, H. G., Geiger, C., and Werkman, C. H. (1940). *Iowa State Coll. J. Science* **14**, 367.

Woolley, D. W. (1941). *J. Biol. Chem.* **140**, 311.

Wright, L. D., and Skeggs, H. R. (1944). *J. Bact.* **48**, 117.

The Amino Acid Composition of Food Proteins

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CONTENTS

I. INTRODUCTION

The elucidation of the specific nutritive roles played by certain amino acids for growth, reproduction, lactation, maintenance, etc., by Osborne and Mendel, Rose, Almquist, and others indicated the need for information concerning the amino acid composition of food proteins as they are commonly consumed by man and animals, rather than knowledge of the amino acid composition of certain purified proteins, which often account for only a small percentage of the total proteins of the foodstuff.

It is generally recognized that one of the chief sources of error in protein analysis is the loss by destrucrion of amino acids during hydrolysis. The evidence for this loss and methods for its avoidance are discussed in detail by Block and Bolling (1945), and by Bolling and Block (1943), and will be mentioned only briefly here. It is obvious that the goal of protein analysts is the use of methods which do not require preliminary hydrolysis. Certain beginnings along this line have been made; thus, Holiday (1936) and later investigators have been able to estimate the quantities of tyrosine and tryptophan in the intact protein by spectrographic methods. Bates (1937) used a modification of the Voisenet-Rhode *p*-dimethylaminobenzaldehyde

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method for determining tryptophan in intact proteins provided the latter were soluble in 0.1 N NaOH. Mirsky and Anson (1934–35) and others (cf. 17) have utilized the reducing action of the sulfhydryl group of cysteine to estimate the quantities of this amino acid and of cystine in proteins without hydrolysis. A more promising field is the observation of Horowitz (1944) that the neurospora mutant "leucineless" developed by Beadle and Tatum (1941) is capable of determining leucine in unhydrolyzed casein. Extension of this line of investigation is awaited with interest.

The amount of destruction during the conventional acid hydrolysis of a protein is increased by the presence of non-protein substances, especially carbohydrates and lipids. The latter are relatively easy to remove by extraction with the appropriate organic solvents. The former present a more difficult problem and will be discussed below.

II. SEPARATION OF CARBOHYDRATES FROM PROTEINS IN FOODSTUFFS

1. *Extraction of Protein with Dilute HCl*

Miller (1935) in an excellently conceived study found that 88 per cent of the proteins in dry grass could be solubilized by boiling the grass with 4 per cent HCl for 48 hours under reflux. After removal of the excess HCl, the solubilized proteins were ready for determination of the basic amino acids by the Kossel procedure (cf. Block and Bolling, 1945).

2. *Extraction of Protein with Formic Acid*

Wilkins (1937) found that extraction of air-dried grass in a Soxhlet apparatus with 90 per cent formic acid brought most of the sample and all of the nitrogen into solution. Mazur and Clarke (1938) used repeated digestion with 90 per cent HCOOH on the steam bath to effect solution of the proteins of marine algae, while Albanese (1944) removed a portion of the carbohydrates soluble in the formic acid by precipitation with two volumes of ethanol. In each case the solubilized proteins were prepared for further hydrolysis after removal of the HCOOH. This procedure cannot be used as a preliminary method when acid labile tryptophan is to be determined.

3. *Extraction of Starch with HCl*

Csonka (1940) extracted the starch and part of the protein from ground defatted corn with cold 21 per cent HCl. The starch was then precipitated from the acid solution by addition of an equal volume of ethanol. Any protein extracted by the cold 21 per cent HCl was then added back to the proteinaceous residue after removal of the solvent.

4. *Digestion of Starch with Amylolytic Enzymes*

A procedure which has given good results in our hands is the digestion of the gelatinized starch in finely ground grains (wheat, corn, rice, oats) by salivary ptyalin. The defatted grain is ground in a Raymond hammer mill and the resulting powder is pasted with 8 to 9 volumes of hot water until no lumps remain. Acetic acid is then added to pH 4.5 and the suspension is heated in boiling water with stirring for one hour after the internal temperature has reached 90° C. The suspension is then diluted with 3 volumes of water and the pH is adjusted to 7.0 with NaOH. After cooling to 37° C., an excess of fresh, centrifuged saliva is added. The digestion is allowed to proceed for 7 days at 37° C. Toluene and chloroform are the preservatives. At the end of this period the insoluble protein is removed by filtration, thoroughly washed with water, and dried. Diatomaceous earth aids in the filtration and does not interfere with the subsequent amino acid determinations.

Purification of the proteins of the cereal grains by this procedure raises the protein content from initial levels of 8–15 per cent to 65–75 per cent on the moisture and ash-free basis. Approximately 90 per cent of the original nitrogen remains in the insoluble residue.

Analysis of the dried filtrate for leucine, isoleucine, valine, threonine, and glutamic acid (amino acids which are harmed but little by acid hydrolysis in the presence of large quantities of carbohydrates) indicate that the distribution of amino acids in the solubilized portion of the cereal grain nitrogen is similar if not identical with that in the insoluble residue.

III. METHODS OF HYDROLYSIS OF PROTEINS

1. *Enzymes*

Various proteolytic enzymes have been used to hydrolyze proteins in order to avoid destruction and/or racemization of the amino acids. There are, however, several disadvantages to their use among which are: (*a*) The hydrolysis seldom goes to completion. (*b*) Many days are often required to get satisfactory digestion. (*c*) Enzymes are proteins and often undergo partial autolysis with the result that a portion of the amino acid to be estimated may have arisen from the enzyme preparation (cf. Block and Bolling, 1945).

2. *Alkalies*

Hydrolysis with strong alkalies (NaOH, Ba(OH)$_2$, KOH, etc.) results in complete racemization of the amino acids and in the partial or complete destruction of arginine, cystine, threonine, and certain other amino acids. Alkaline hydrolysis is, however, necessary in the determination of tryptophan and is conveniently employed in the estimation of tyrosine and phenylalanine.

3. *Acids*

HCl, H₂SO₄, HI, and a mixture of HCl and HCOOH are commonly used to hydrolyze the protein as the prerequisite for determination of the acid-stable amino acids. In general, the concentration of hydrolyzing acid used is inversely proportional to the time and temperature of the reaction (for details cf. Block and Bolling, 1945).

In order to prevent or decrease the formation of the brown-black protein-carbohydrate decomposition products called humin, Hlaziwetz and Habermann (1873) introduced tin or $SnCl_2$ into the hydrolysis mixture to produce a reducing atmosphere while Kossel and Kutscher (1900) used a mixture of HI and HPO_2 for this purpose. The use of Kossel's HI-hypophosphorous acid mixture for the hydrolysis of proteins in the presence of large quantities of carbohydrates has been again suggested by Holland, *et al.* (1944). Holland, *et al.* (1944) extract the carbohydrate reduction products with chloroform before complete hydrolysis of the protein.

IV. Approximate Amino Acid Composition of Food Proteins

The data given in the following tables are considered the best at present available. The reader should bear in mind that these figures probably will be subject to considerable revision in the future not only because of improvements in methods of analysis but also in the case of the plant proteins because of amino acid variations in differing species of the same plant. Thus, Hamilton and Nakamura (1940) have shown differences in the cystine content of various soybeans while Block and Bolling (unpublished data) have found variations in the amino acid composition of yeasts.

The amino acids are listed first according to their essentiality and secondly according to their chemical grouping or mode of determination. Thus the basic amino acids: arginine, histidine, and lysine, lead the list, followed by the aromatic amino acids: tyrosine, tryptophan, and phenylalanine. The two sulfur amino acids: cystine and methionine, come next, followed by threonine, the only essential hydroxyamino acid. Leucine, isoleucine, and valine, come next, then glycine, the last nutritionally important amino acid. Alanine, serine, and the dicarboxylic amino acids follow with the two imino acids, proline and hydroxyproline, completing the list.

All the amino acid data in the tables are given in g. of amino acid per 16.0 g. of nitrogen; therefore, it is only necessary to know the nitrogen content of the protein food in order to recalculate the data in the tables to give the approximate amino acid composition of the preparation. Thus, if the preparation contains 12.2 per cent of nitrogen, then the amino acid values would be multiplied by the factor

$$\frac{12.2}{16.0} \text{ or } 0.76$$

to give the percentage composition.

The inherent error in calculating protein from nitrogen, especially in certain plants such as yeasts, soybeans, etc., has long been known. If the plant proteins are isolated in the purified form and then analyzed for their constituent amino acids, and if the results so obtained are used to calculate the amino acid composition of the tissue from the nitrogen content of the latter, the error may be considerable. But if the tissue is itself analyzed for the amino acids, this error is minimized. Results so obtained show the approximate amino acid composition of the proteinaceous substances irrespective of the presence of nonprotein nitrogen. The majority of the analyses of proteins and protein-containing foods summarized in this chapter are to be used to indicate the amino acid composition of foods and not for considerations of protein structure. Therefore, the relative amino acid composition of the various food proteins is of paramount importance and not the absolute amounts of each essential amino acid in per cent of total nitrogen.

TABLE I
Approximate Amino Acid Content of Animal Proteins
(Calculated to 16.0 g. of Nitrogen)*

Amino Acid in g.	Collagen Gelatin	Elastin	Egg Albumin Crystalline	Vitellin
Arginine	8.8_{K17}	0.9_{K94}	5.8_{K102}	8.4_{K26}
Histidine	1.0_{K17}	0.0_{K94}	2.4_{K92}	1.9_{K84}
Lysine	4.5_{K17}	$?_{K94}$	5.1_{K30}	5.8_{K26}
Tyrosine	0.3_{M48}	1.5_{M94}	4.4_{M25}	5.3_{M26}
Tryptophan	0.1_{H61}	0.0_{M94}	1.3_{T25}	1.3_{T26}
Phenylalanine	2.1_{A17}	3.1_{A58}	5.6_{A3}	
Cystine	$0.1\text{–}0.2_{W36}$	0.2_{W94}	2.3_{W98}	1.8_{BS62}
Methionine	1.0_{BS4}	0.4_{BS94}	5.0_{BS62}	3.1_{BS62}
Threonine	1.5_{B17}	2.5_{B18}		4.9_{B80}
Leucine	3.7_{FB17}		9.4_{X96}	
Isoleucine	1.7_{L68}		5.6_{FB17}	
Valine	2.1_{FB17}		6.8_{L73}	
Glycine	23.6_{BM13}	27.5_{BM94}	3.3_{BM96}	0.8_{Z89}
Alanine	9.2_{X44}	0.0_{BM94}	7.4_{X103}	
Serine	3.3_{N78}		7.6_{N21}	9.4_{N80}
Glutamic Acid	10.3_{X81}	2.5_{I94}	16.3_{I30}	12.7_{I84}
Aspartic Acid	5.9_{L18}	0.0_{I94}	8.2_{I30}	
Hydroxyproline	13_{MC72}	1.9_{X94}		
Proline	15.3_{BM95}	14.2_{BM94}	4.3_{X25}	

* Nitrogen percentages of purified proteins, corrected for moisture and ash, are as follows: Gelatin 18.3; elastin 17.1; crystalline egg albumin 15.8; vitellin 16.3; casein 15.6 (Osborne), 15.75 (Vickery 1944), 15.5 (Vickery 1940); gliadin 17.66; zein 16.1; edestin 18.7; cottonseed globulin 18.6; arachin 18.0.

TABLE I (continued)

Amino Acid in g.	Egg White	Egg Yolk	Whole Egg	Casein
Arginine	5.8K[27]	8.2K[27]	7.0K[17]	4.2K[99]
Histidine	2.2K[27]	1.4K[27]	2.4K[17]	2.5K[31]
Lysine	6.5K[27]	5.5K[27]	7.2K[18]	7.9K[100]
Tyrosine	5.4M[71]	5.8M[27]	4.3M[90]	6.9M[66]
Tryptophan	1.7T[27]	1.7T[27]	1.5M[17]	1.4M[66]
Phenylalanine	5.5A[103]	5.7A[17]	5.9A[17]	5.2A[58]
Cystine	2.6w[17]	2.3w[71]	2.4w[17]	0.3w[36]
Methionine	4.4x[10]		4.9S[18]	3.5BS[4]
Threonine	4.3B[17]		4.9B[17]	4.1B[69]
Leucine			9.2L[18]	9.9L[68]
Isoleucine			8.0L[18]	6.5L[68]
Valine			7.3L[18]	6.7L[68]
Glycine			2.2z[90]	0.6z[91]
Alanine				2.8x[35]
Serine				7.5N[80]
Glutamic Acid				24.2T[41]
Aspartic Acid				6.3I[7]
Hydroxyproline				0.0MC[68]
Proline				8V[32]

Amino Acid in g.	Lactalbumin	Whole Milk	Human Milk	Beef Muscle
Arginine	3.9K[9]	4.3K[17]	6.8K[18]	7.7K[18]
Histidine	2.1K[18]	2.6K[18]	2.8K[18]	2.9K[18]
Lysine	9.6K[18]	7.5K[17]	7.2K[17]	8.1K[11]
Tyrosine	4.4M[18]	5.5M[17]	5.1M[18]	3.4M[18]
Tryptophan	2.5M[18]	1.6M[17]	1.5–3.1M[18]	1.3M[18]
Phenylalanine	5.4A[18]	5.3A[17]	5.9A[18]	4.9A[18]
Cystine	4.1W[56]	1.0w[18]	2.3–3.9w[18]	1.3w[18]
Methionine	3.1BS[9]	3.3S[18]	2.5S[18]	3.3S[18]
Threonine	5.4B[79]	4.6B[17]	4.5B[18]	4.6B[11]
Leucine	10.4L[18]	11.3L[18]	10.1L[18]	7.7L[18]
Isoleucine	6.4L[18]	6.2L[18]	7.5L[18]	6.3L[18]
Valine	6.4L[18]	6.6L[18]	8.8L[18]	5.8L[93]
Glycine	0.0z[89]			5.0z[17]
Alanine				4.x[86]
Serine	4.9N[79]			5.4N[11]
Glutamic Acid	13.4I[55]			15.4I[86]
Aspartic Acid	9.7I[55]			6.0I[57]
Hydroxyproline				
Proline				6.0I[86]

TABLE I (continued)

Amino Acid in g.	Chicken Muscle	Fish Muscle	Shell Fish Muscle	Heart
Arginine	7.1_{K11}	7.4_{K18}	7.6_{K92}	7.4_{K11}
Histidine	2.3_{K11}	2.4_{K17}	1.9_{K85}	2.7_{K17}
Lysine	8.4_{K11}	9.0_{K11}	8.3_{K11}	7.4_{K17}
Tyrosine	4.3_{M11}	4.4_{M11}	4.7_{M11}	4.4_{M11}
Tryptophan	1.2_{M11}	1.3_{T92}	1.2_{M11}	1.4_{M11}
Phenylalanine	4.6_{A11}	4.5_{A11}	4.8_{A11}	5.1_{M11}
Cystine	1.3_{cu11}	1.2_{S92}	1.3_{S92}	1.2_{S11}
Methionine	3.2_{cu11}	3.5_{S11}	3.4_{BS4}	3.2_{S11}
Threonine	4.7_{B11}	4.5_{B11}	4.0_{B11}	4.7_{B11}
Leucine		7.1_{L18}		8.4_{L93}
Isoleucine		6.0_{L18}		
Valine		5.8_{L18}		6.3_{L92}
Glycine				
Alanine				
Serine	4.7_{N11}	4.9_{N11}	4.0_{B11}	5.9_{B11}
Glutamic Acid	16.5_{I83}			13.3_{I29}
Aspartic Acid				6.9_{I29}

Amino Acid in g.	Liver	Kidney
Arginine	6.6_{K11}	6.3_{K14}
Histidine	3.1_{K14}	2.7_{K14}
Lysine	6.7_{K14}	5.5_{K14}
Tyrosine	4.6_{M11}	4.8_{M65}
Tryptophan	1.8_{M11}	1.7_{T65}
Phenylalanine	6.1_{A11}	5.5_{A17}
Cystine	1.3_{cu11}	1.5_{w17}
Methionine	3.2_{cu11}	2.7_{cu11}
Threonine	4.8_{B11}	4.6_{B11}
Leucine	8.4_{L93}	7.9_{L93}
Isoleucine		
Valine	6.2_{L93}	5.3_{L93}
Glycine	8.5_{z17}	
Alanine		
Serine	7.3_{N11}	6.1_{B11}
Glutamic Acid	12.2_{I54}	
Aspartic Acid	6.9_{I29}	
Hydroxyproline		0.0_{x64}

The small numbers in the tables are the references, while the letters indicate the general analytical procedures used. These abbreviations are given on the next page.

A detailed discussion of the reliability or lack of reliability of these methods is given in Block and Bolling (1945).[1] However, the following comments may be helpful.

A: Kapeller-Adler for *phenylalanine*. Approximate only. Often high.

B: Block-Nicolet for *threonine* can be made to yield accurate results if carefully carried out. Shinn-Nicolet very accurate.

BM: Bergmann's solubility methods are highly accurate.

BS: Baernstein for *cystine* and *methionine*. Accurate.

C: Cohen-Krebs for *glutamic acid*. Accurate.

Cu: Hopkins' cuprous oxide for *cystine* and *methionine*. Satisfactory if properly carried out.

F: Fleming-Vassel for *cystine*. Good unless cystine destroyed during hydrolysis.

FB: Fromageot-Block for *leucine, isoleucine*, and *valine*. Useful only in the absence of any other method. Now replaced by microbiological methods.

H: Hopkins, Voisenet, Rhode aldehyde methods for *tryptophan*. Often too high.

I: Isolation methods usually give minimal results.

J: Jansen's arginase methods for *arginine*. Accurate.

K: Kossel-Kutscher isolation method for *arginine, histidine*, and *lysine*. Has had many modifications. Accurate minimal values only.

L: Microbiological methods. Appear accurate, but are too new for a final evaluation. Probably better for some amino acids than others. They are discussed in detail by Snell elsewhere in this volume.

M: Modifications of Millon's test for *tyrosine* and *tryptophan*. Very accurate.

Mc: McFarlane's hydrogen peroxide oxidation for *proline* and *hydroxyproline*. Approximate only.

N: Nicolet's method is very accurate for *serine*.

P: Modifications of Pauly's diazo reaction. Can be made quite accurate for *histidine* and *tyrosine*.

S: Sullivan-McCarthy procedure for *methionine*. Accurate. Sullivan *cystine* method. Accurate.

T: Folin's colorimetric method for *tryptophan*. Only useful with purified proteins; otherwise will give completely erroneous results.

V: Van Slyke amino nitrogen. Approximate for lysine.

W: Winterstein-Folin phosphotungstic acid method for *cystine* and *cysteine*. Can be made very accurate. Many substances give high results.

X: Miscellaneous methods.

Z: Zimmermann's phthaldialdehyde method for *glycine*. Approximate only.

V. DISCUSSION

The data presented in the tables are self-explanatory; however, several striking points may be brought out:

Gelatin (Collagen) and Elastin are deficient or devoid of many essential amino acids. Their presence in foods and feeds may impair nutritive value.

Egg proteins are well balanced, rich in sulfur, and of the highest biological value.

[1] See also the review by Martin and Synge in the present volume.

Casein (Cheese) is very rich in lysine, tryptophan, and the "leucines" but somewhat low in the sulfur amino acids.

Lactalbumin is unusually rich in tryptophan and lysine, but somewhat low in histidine.

Muscle proteins from different animal sources are almost identical. They all are rich in lysine.

Kidney proteins are lower in lysine but higher in tryptophan than muscle proteins.

Cereal grains. Rice and oat proteins are superior to wheat and corn. The proteins of whole grains are superior to those of the endosperm due to the relatively high amino acid value of the germ proteins. Lysine deficiency is the salient lack in the cereal grains.

Leafy vegetables supply well-balanced proteins, but the quantities of these proteins consumed by humans are very low. The amino acid values obtained from various leafy vegetables such as spinach, beet tops, chard, alfalfa leaf, clover, etc. were so similar in composition that all values have been averaged and included under the above heading.

Cottonseed proteins are somewhat low in lysine and methionine while peanut proteins are low in lysine, tryptophan, methionine, and threonine.

Linseed and sesame seed proteins are unusually rich in tryptophan when compared to most plant proteins.

Sunflower seed proteins appear to be superior in lysine, methionine, and threonine to peanut and cottonseed proteins.

TABLE II

APPROXIMATE AMINO ACID CONTENT OF PLANT PROTEINS

(*Calculated to 16.0 g. of Nitrogen*)

Amino Acid in g.	Flour	Wheat	Corn	Corn Gluten
Arginine	3.9_{K17}	3.8_{K17}	4.8_{K17}	3.1_{K17}
Histidine	2.2_{K17}	2.1_{K17}	2.5_{K17}	1.6_{K17}
Lysine	1.9_{K17}	2.7_{K17}	2.0_{K17}	0.8_{K17}
Tyrosine	3.8_{M17}	4.4_{M17}	5.6_{M17}	6.7_{M17}
Tryptophan	1.3_{L46}	1.2_{M17}	0.8_{L46}	0.7_{M17}
Phenylalanine	5.5_{A17}	5.7_{A17}	5.0_{A17}	6.4_{A17}
Cystine	1.9_{W17}	1.8_{F18}	1.5_{W17}	1.1_{F18}
Methionine	3_{X17}			4_{S18}
Threonine	2.7_{B17}	3.3_{B17}	3.7_{B17}	4.1_{B17}
Leucine		5.8_{L68}	22_{FB17}	24_{FB17}
Isoleucine		3.3_{L68}	4_{FB17}	5_{FB17}
Valine		3.6_{L68}	5_{FB17}	5_{FB17}
Glycine	7.2_{Z17}			4.3_{Z17}
Glutamic Acid				24.5_{C18}

TABLE II (continued)

Amino Acid in g.	Rice	Oats	Corn Germ	Wheat Germ
Arginine	7.2_{K18}	6.0_{K18}	8.1_{K17}	6.0_{K17}
Histidine	1.5_{K18}	2.2_{K18}	2.9_{K17}	2.5_{K17}
Lysine	3.2_{K18}	3.3_{K18}	5.8_{K17}	5.5_{K17}
Tyrosine	5.6_{M18}	4.6_{M18}	6.7_{M17}	3.8_{M17}
Tryptophan	1.3_{M18}	1.2_{M18}	1.3_{M17}	1.0_{M17}
Phenylalanine	6.7_{A18}	6.6_{A18}	5.5_{A17}	4.2_{A17}
Cystine	1.4_{F18}	1.8_{F18}	1.8_{W17}	0.8_{W17}
Methionine	3.4_{S18}	2.4_{S18}	1.6_{S17}	
Threonine	4.1_{B18}	3.5_{B18}	4.7_{B17}	3.8_{B17}
Leucine	9.0_{L18}	8.3_{L18}	13_{FB17}	
Isoleucine	5.3_{L18}	5.6_{L18}	4_{FB17}	
Valine	6.3_{L18}	6.3_{L18}	6_{FB17}	
Glycine	10.3_{Z18}			
Glutamic Acid	24.1_{L18}			

Amino Acid in g.	Leafy Vegetables	Cottonseed Meal	Peanut Meal	Linseed Meal
Arginine	7.0_{K99}	7.4_{K17}	9.9_{K17}	6.2_{K17}
Histidine	2.1_{K17}	2.6_{K17}	2.1_{K17}	1.5_{K17}
Lysine	5.7_{K99}	2.7_{K17}	3.0_{K17}	$2 5_{K17}$
Tyrosine	5.4_{M66}	3.2_{M17}	4.4_{M17}	5.1_{M17}
Tryptophan	1.9_{M66}	1.3_{M17}	1.0_{M17}	1.9_{M17}
Phenylalanine	4.5_{A17}	6.8_{M17}	5.4_{M17}	5.6_{A17}
Cystine	2.0_{BS67}	2.0_{W17}	1.6_{W17}	1.9_{W17}
Methionine	2.3_{BS67}	1.6_{S17}	0.9_{S17}	2.3_{S17}
Threonine	4.1_{B69}	3.0_{B17}	1.5_{B17}	5.1_{B17}
Leucine		5.0_{L68}	7_{FB17}	7_{FB17}
Isoleucine		3.4_{L68}	3_{FB17}	4_{FB17}
Valine		3.7_{L68}	8_{FB17}	7_{FB17}
Glycine		5.3_{Z18}	5.6_{Z17}	
Glutamic Acid	13.1_{I75}			
Aspartic Acid	5.3_{I75}			
Proline	2.5_{I75}			

Amino Acid in g.	Sesame Seed	Sunflower Seed	Brewer's Yeast	Food Yeast
Arginine	9.2_{K18}	8.2_{K18}	5.3_{K18}	4.0_{K18}
Histidine	1.5_{K18}	1.7_{K18}	3.0_{K18}	2.3_{K18}
Lysine	2.8_{K18}	3.8_{K18}	7.4_{K18}	8.0_{K18}
Tyrosine	4.3_{M18}	2.6_{M18}	3.5_{M18}	3.4_{M18}
Tryptophan	1.9_{M18}	1.3_{M18}	1.5_{M18}	1.2_{M18}
Phenylalanine	8.3_{A18}	5.4_{A18}	4.6_{A18}	2.9_{A18}
Cystine	1.3_{F18}	1.3_{F18}	0.9_{F18}	1.1_{F18}
Methionine	3.1_{S18}	3.4_{S18}	2.8_{S18}	2.8_{S18}
Threonine	3.6_{B18}	4.0_{B18}	5.3_{B18}	5.1_{B18}
Leucine	7.5_{L18}	6.2_{L18}	7.1_{L18}	6.8_{L18}
Isoleucine	4.8_{L18}	5.2_{L18}	6.0_{L18}	5.8_{L18}
Valine	5.1_{L18}	5.2_{L18}	4.7_{L18}	5.4_{L18}
Glycine	9.3_{Z18}			

TABLE II (continued)

Amino Acid in g.	Soybean Meal	Gliadin	Zein	Edestin
Arginine	5.8_{K17}	2.7_{J45}	1.6_{K102}	14.3_{K30}
Histidine	2.3_{K17}	1.9_{P48}	0.8_{K101}	2.1_{K30}
Lysine	5.4_{K17}	1.1_{17}	0.0_{K101}	2.2_{K99}
Tyrosine	4.1_{M17}	2.8_{M37}	5.9_{M38}	3.9_{M37}
Tryptophan	1.2_{L46}	0.8_{T37}	0.2_{T38}	1.3_{T37}
Phenylalanine	5.7_{A17}		6.6_{I72}	4.2_{L73}
Cystine	0.9_{F18}	2.3_{S6}	1.0_{W39}	1.2_{W39}
Methionine	2.0_{S2}	2.7_{BS4}	2.5_{BS5}	2.0_{BS6}
Threonine	4.0_{B17}	2.7_{B105}	2.5_{B20}	
Leucine	6.6_{L68}		25_{FB17}	6.6_{FB42}
Isoleucine	4.7_{L68}		5_{FB17}	
Valine	4.2_{L68}		3_{FB17}	5.1_{FB42}
Glycine	$(17.4)_{I57}$		0_{X82}	1.6_{Z89}
Alanine	$(\ 8.8)_{I57}$		9.9_{X103}	4.8_{X35}
Glutamic Acid	$(21.0)_{X81}$	42.2_{I7}	35.6_{I22}	17.8_{I30}
Aspartic Acid		1.3_{I7}	3.4_{X63}	10.3_{I30}
Hydroxyproline			0_{X34}	
Proline		12_{I88}	9_{V34}	5.4_{X43}

Amino Acid in g.	Cottonseed Globulin	Arachin
Arginine	12.2_{K40}	12.2_{K102}
Histidine	3.0_{K40}	1.9_{K53}
Lysine	5.2_{K40}	1.5_{K53}
Tyrosine	3.4_{M40}	4.1_{M17}
Tryptophan	1.3_{M40}	0.6_{X24}
Phenylalanine	7.8_{A40}	4.4_{A24}
Cystine	1.1_{BS40}	1.3_{BS23}
Methionine	2.3_{BS40}	0.6_{BS23}
Threonine	2.7_{B40}	2.3_{B23}
Leucine	7.5_{FB40}	
Isoleucine	2.3_{FB40}	
Valine	6.7_{FB40}	
Glycine		1.8_{Z89}
Alanine		4_{X53}
Serine	2.7_{N40}	4.6_{N23}
Glutamic Acid	21.2_{X81}	22.1_{X81}
Aspartic Acid		4.9_{I57}

Yeast proteins vary somewhat in composition with the type analyzed, but all are quite similar in composition to cow's milk casein.

Soybean proteins are well balanced in all respects except for a slight deficiency in methionine.

Gliadin, Zein, Edestin, Cottonseed Globulin, and Arachin are included to permit the evaluation of experimental diets built upon one or more of these purified proteins.

TABLE III

APPROXIMATE AMINO ACID CONTENT OF SOME PREPARED BREAKFAST CEREALS

(Calculated to 16.0 g. of Nitrogen)

Amino Acid in g.	Whole Wheat	Puffed Wheat	Toasted Corn	Puffed Rice	Puffed Oats
Arginine	5.0_{K17}	2.2_{K17}			5.0_{K18}
Histidine	1.7_{K17}	2.2_{K17}			2.1_{K18}
Lysine	3.0_{K17}	0.9_{K17}	$<1_{K18}$	1.4_{K18}	2.2_{K18}
Tyrosine	3.0_{M17}	2.1_{M17}	5.6_{M18}	6.4_{M18}	4.0_{M18}
Tryptophan	1.0_{M17}	0.5_{M17}	0.5_{M18}	1.3_{M18}	1.1_{M18}
Phenylalanine	5.6_{A17}	4.6_{A17}	6.2_{A18}	6.3_{A18}	6.0_{A18}
Cystine	1.1_{F18}	0.7_{F17}	1.5_{F18}	1.2_{F18}	1.4_{F18}
Methionine	2.5_{S18}		3.4_{S18}	3.1_{S18}	2.5_{S18}
Threonine	3.3_{B18}	3.8_{B17}	2.9_{B18}	3.9_{B18}	3.5_{B18}
Leucine	9.1_{L18}		21.0_{L18}	9.0_{L18}	8.8_{L18}
Isoleucine	4.5_{L18}		4.5_{L18}	5.1_{L18}	5.6_{L18}
Valine	5.0_{L18}		5.2_{L18}	6.4_{L18}	6.2_{L18}

Prepared Breakfast Cereals. The results in Table III when compared to those in Table II suggest that the heat and mechanical treatments used in the preparation of many breakfast foods may result in some destruction of lysine and tryptophan in the case of wheat and corn, of lysine in the case of rice, and of cystine in the case of oats. These changes do not, however, account for the large loss in biological value observed on puffing or toasting.

VI. NUTRITIONAL EVALUATION OF CHEMICAL ANALYSES

A brief discussion of the correlation between amino acid composition of a protein food and its nutritive or biological value as determined by animal tests may be appropriate. Although the writer is not aware of any publication in which the results of animal experiments are directly correlated with protein composition since the classical studies of Osborne and Mendel on gliadin, zein, casein, etc., considerable correlative information is on hand.

Thus the nutritive poverty of gelatin, observed early in the nineteenth century by Magendie and by Prout, can be directly related to its multifold deficiencies in essential amino acids. The minor deficiency in the sulfur amino acids, cystine plus methionine, in casein, which was first pointed out by Osborne and Mendel, is also evident from the analytical values. The high biological value of egg proteins found by Murlin and his collaborators is also indicated in the well balanced distribution of the essential amino acids. In fact, at a "Symposium on Nutritional Qualities of Proteins" at the Thirtieth Annual Meeting of the American Association of Cereal Chemists, Minneapolis, May 25, 1944, papers by H. J. Almquist, P. R. Cannon, and R. H. Barnes all called attention to the close correlation between amino acid composition and biological value of food proteins providing, of course, that the proteins were digestible.

The data in Table III, when compared with some of the figures in Table II, indicate that during the processing of certain cereal grains, which involves high temperatures and pressures, there is a loss in lysine and tryptophan in the case of puffed wheat and toasted corn, of lysine in the case of puffed rice, and of cystine in the case of puffed oats. These losses in essential amino acids can be directly correlated with the poorer growth-promoting ability of some of the heated and puffed products (cf. Murlin, Nasset, and Marsh, 1938). The slight loss of cystine in the case of puffed oats would not, however, account for the marked loss in nutritional value which takes place in oats on puffing (cf. Stewart, Hensley, and Peters, 1943). This discrepancy, which may be tentatively explained on the basis of the rate or completeness of digestion, awaits further experimental elucidation.

Thus if the degree of digestibility and the essential amino acid composition of protein foods are known, even approximately, it should be possible to replace an expensive or scarce food by an available one of equal nutritive value. Furthermore, the total protein of the diet can often be drastically reduced without loss of nutritive value by ascertaining that the amino acids are supplied in a *balanced mixture* where each is utilized to maximum efficiency rather than furnishing a considerable excess of total protein and trusting to chance and practical experience that the minimum amino acid requirements are being met.

VII. Amino Acid Requirements of Man

It is generally recognized that as long as a man has enough to eat, there is not likely to be much wrong with the average total protein intake. In fact, it appears that whatever the diet is, especially if there is plenty of bread and flour, the average individual will ingest 70 to 80 g. of protein. So, there is nothing wrong with the total protein in the average diet; the question then is with the quality of protein, *i.e.*, its essential amino acid composition. Block and Bolling (1943, 1944) have discussed the essential amino acid requirements of man, and have concluded that the average quantity of protein is usually ample, and even when the cereal grains, especially wheat and rye, furnish the greater part of the total protein intake, only one amino acid, namely, lysine, is apt to be lacking. The addition of small amounts of lysine to whole wheat or to white flour will more than double their nutritive and biological value, converting them from inferior protein foods to foods having a nutritive value almost as good as many of the more expensive animal protein foods.

REFERENCES

1. Albanese, A. A. (1944). *Ind. Eng. Chem Anal. Ed.* **16**, 609.
2. Almquist, H. J., Mecchi, E., Kratzer, F. H., and Grau, C. R. (1942). *J. Nutrition* **24**, 385.
3. Arnow, L. E., Burns, J., and Bernhart, F. W. (1939). *Proc. Soc. Exptl. Biol. Med.* **41**, 499.
4. Baernstein, H. D. (1932). *J. Biol. Chem.* **97**, 669.
5. Baernstein, H. D. (1936). *J. Biol. Chem.* **115**, 25.
6. Bailey, K. (1937). *Biochem. J.* **31**, 1396.
7. Bailey, K., Chibnall, A. C., Rees, M. W., and Williams, E. F. (1943). *Biochem. J.* **37**, 360.
8. Bates, R. W. (1937). *Proc. Am. Soc. Biol. Chem.; J. Biol. Chem.* **119**, VII.
9. Beach, E. F., Bernstein, S. S., Hoffman, O. D., Teague, D. M., and Macy, I. C. (1941). *J. Biol. Chem.* **139**, 57.
10. Beach, E. F., and Teague, D. M. (1942). *J. Biol. Chem.* **142**, 277.
11. Beach, E. F., Munks, B., and Robinson, A. (1943). *J. Biol. Chem.* **148**, 431.
12. Beadle, G. W., and Tatum, E. L. (1941). *Proc. Natl. Acad. Sci.* **27**, 499.
13. Bergmann, M., and Stein, W. H. (1939). *J. Biol. Chem.* **128**, 217.
14. Block, R. J., Jervis, G. A., Bolling, D., and Webb, M. (1940). *J. Biol. Chem.* **134**, 567.
15. Block, R. J. (1943). *Yale J. Biol. Med.* **15**, 723.
16. Block, R. J., and Bolling, D. (1944). *J. Am. Diet. Assoc.* **20**, 69.
17. Block, R. J., and Bolling, D. (1945). The Amino Acid Composition of Proteins and Foods. C. C. Thomas, Springfield, Ill.
18. Block, R. J., and Bolling, D. Unpublished data.
19. Bolling, D. and Block, R. J. (1943). *Arch. Biochem.* **2**, 93.
20. Borchers, R., Totter, J. R., and Berg, C. P. (1942). *J. Biol. Chem.* **142**, 697.
21. Boyd, M. J., and Logan, M. A. (1942). *J. Biol. Chem.* **146**, 279.
22. Brazier, M. A. B. (1930). *Biochem. J.* **24**, 1188.
23. Brown, W. L. (1942). *J. Biol. Chem.* **142**, 299.
24. Brown, W. L. (1944). *J. Biol. Chem.* **155**, 277.
25. Calvery, H. O. (1931–1932). *J. Biol. Chem.* **94**, 613.
26. Calvery, H. O., and White, A. (1931–1932). *J. Biol. Chem.* **94**, 635.
27. Calvery, H. O. (1932). *J. Biol. Chem.* **95**, 297.
28. Cannan, R. K., Palmer, A. H., and Kibrick, A. C. (1942). *J. Biol. Chem.* **142**, 803.
29. Chibnall, A. C., Rees, M. W., Williams, E. F., and Boyland, E. (1940). *Biochem. J.* **34**, 285.
30. Chibnall, A. C. (1942). The Bakerian Lecture. *Proc. Roy. Soc. (London)* B, **131**, 136.
31. Csonka, F. A. (1939). *J. Agr. Research*, **59**, 765.
32. Dakin, H. D. (1918). *Biochem. J.* **12**, 290.
33. Dakin, H. D. (1920). *J. Biol. Chem.* **44**, 499.
34. Dakin, H. D. (1923). *Z. physiol. Chem.* **130**, 159.
35. Desneulle, P. (1938). *Enzymologia* **5**, 37.
36. Folin, O., and Looney, J. M. (1922). *J. Biol. Chem.* **51**, 421.
37. Folin, O., and Marenzi, A. D. (1929). *J. Biol. Chem.* **83**, 89.
38. Folin, O., and Ciocalteu, V. (1927). *J. Biol. Chem.* **73**, 627.
39. Folin, O., and Marenzi, A. D. (1929). *J. Biol. Chem.* **83**, 103.
40. Fontaine, T. D., Olcott, H. S., and Lowy, A. (1942). *Ind. Eng. Chem.* **34**, 116.

41. Foreman, F. W. (1914). *Biochem. J.* **8**, 463.
42. Fromageot, C., and Mourgue, M. (1941). *Enzymologia* **9**, 329.
43. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35**, 1369.
44. Gordon, A. H., Martin, A. J. P., and Synge, R. L. M. (1943). *Biochem. J.* **37**, 92.
45. Graff, S., Maculla, E., and Graff, A. M. (1937). *J. Biol. Chem.* **121**, 71.
46. Greene, R. D., and Black, A. (1944). *J. Biol. Chem.* **155**, 1.
47. Hamilton, T. S., and Nakamura, F. I. (1940). *J. Agr. Research* **61**, 207.
48. Hanke, M. T. (1925). *J. Biol. Chem.* **66**, 489.
49. Hlaziwetz, H., and Habermann, J. (1873). *Ann.* **169**, 150.
50. Holland, B. R., Lyman, C. M., and Hale, F. (1944). Div. Biological Chemistry, New York Meeting, Am. Chem. Soc., Sept. 1944.
51. Horowitz, N. H., and Beadle, G. W. (1943). *J. Biol. Chem.* **150**, 325.
52. Horowitz, N. H. (1944). *J. Biol. Chem.* **154**, 141.
53. Johns, C. O., and Jones, D. B. (1918). *J. Biol. Chem.* **36**, 491.
54. Johnson, J. M. (1940). *J. Biol. Chem.* **132**, 781.
55. Jones, D. B., and Johns, C. O. (1921). *J. Biol. Chem.* **48**, 347.
56. Jones, D. B., Gersdorff, C. E. F., and Moeller, D. (1924–1925). *J. Biol. Chem.* **62**, 183.
57. Jones, D. B., and Moeller, O. (1928). *J. Biol. Chem.* **79**, 429.
58. Kapeller-Adler, R. (1932). *Biochem. Z.* **252**, 185.
59. Kossel, A., and Kutscher, F. (1900). *Z. physiol. Chem.* **31**, 165.
60. Kossel, A., and Gross, R. E. (1924). *Z. physiol. Chem.* **135**, 167.
61. Kraus, I. (1925). *J. Biol. Chem.* **63**, 157.
62. Kuhn, R., and Desnuelle, P. (1938). *Z. physiol. Chem.* **251**, 19.
63. Laine, T. (1939). *Suomen Kemistilehti* **12 B**, 23.
64. Lang, K. (1933). *Z. physiol. Chem.* **219**, 148.
65. Lee, W. C., and Lewis, H. B. (1934). *J. Biol. Chem.* **107**, 649.
66. Lugg, J. W. H. (1938). *Biochem. J.* **32**, 2123.
67. Lugg, J. W. H. (1939). *Biochem. J.* **33**, 110.
68. Lyman, C. M., Kuiken, K. A., Norman, W. H., Hale, F., and Blotter, L. (1943). *Science* **98**, 266; *J. Biol. Chem.* **151**, 615.
69. Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35**, 294.
70. Mazur, A., and Clarke, H. T. (1938). *J. Biol. Chem.* **123**, 729.
71. McFarlane, W. D., Fulmer, H. L., and Jukes, T. H. (1930). *Biochem. J.* **24**, 1611.
72. McFarlane, W. D., and Guest, G. H. (1939). *Can. J. Research* **B, 17**, 139.
73. McMahan, J. R., and Snell, E. E. (1944). *J. Biol. Chem.* **152**, 83.
74. Miller, E. J. (1935). *Biochem. J.* **29**, 2344.
75. Miller, E. J. (1936). *Biochem. J.* **30**, 273.
76. Mirsky, A. E., and Anson, M. L. (1934–1935). *J. Gen. Physiol.* **18**, 307–323.
77. Murlin, J. R., Nasset, E. S., and Marsh, M. E. (1938). *J. Nutrition* **16**, 249.
78. Nicolet, B. H., and Shinn, L. A. (1941). *J. Biol. Chem.* **139**, 687.
79. Nicolet, B. H., and Shinn, L. A. (1942). *J. Biol. Chem.* **142**, 139.
80. Nicolet, B. H., Shinn, L. A., and Saidel, L. J. (1942). *Am. Chem. Soc. Abstr.*, Sept., 22 B.
81. Olcott, H. S. (1944). *J. Biol. Chem.* **153**, 71.
82. Osborne, T. B., and Clapp, S. H. (1907–1908). *Am. J. Physiol.* **20**, 477.
83. Osborne, T. B., and Heyl, F. W. (1909). *Am. J. Physiol.* **23**, 81.
84. Osborne, T. B., and Jones, D. B. (1909). *Am. J. Physiol.* **24**, 153.
85. Osborne, T. B., and Jones, D. B. (1909). *Am. J. Physiol.* **24**, 161.
86. Osborne, T. B., and Jones, D. B. (1909). *Am. J. Physiol.* **24**, 437.

87. Osborne, T. B., and Jones, D. B. (1910). *Am. J. Physiol.* **26**, 212.
88. Osborne, T. B. and Guest, H. H. (1911). *J. Biol. Chem.* **9**, 425.
89. Patton, A. R. (1935). *J. Biol. Chem.* **108**, 267.
90. Patton, A. R., and Palmer, L. S. (1936). *J. Nutrition* **11**, 129.
91. Plimmer, R. H. A., and Lawton, J. H. T. (1939). *Biochem. J.* **33**, 530.
92. Pottinger, S. R., and Baldwin, W. H. (1939). *Proc. Sixth Pacific Sci. Congr.*, 453.
93. Schweigert, B. S., McIntire, J. M., Elvehjem, C. A., and Strong, F. M. (1944). *J. Biol. Chem.* **155**, 183.
94. Stein, W. H., and Miller, E. G., Jr. (1938). *J. Biol. Chem.* **125**, 599.
95. Stein, W. H., and Bergmann, M. (1940) *J. Biol. Chem.* **134**, 627.
96. Stein, W. H. (1943). *Cereal Chem. Trans.* **57**, 11.
97. Stewart, R. A., Hensley, G. W., and Peters, F. N. (1943). *J. Nutrition* **26**, 519.
98. Tompsett, S. L. (1931). *Biochem. J.* **25**, 2014.
99. Tristram, G. R. (1939). *Biochem. J.* **33**, 1271.
100. Van Slyke, D. D. (1913–1914). *J. Biol. Chem.* **16**, 531.
101. Vickery, H. B. (1938). *Compt. rend. trav. lab. Carlsberg* **22**, 519.
102. Vickery, H. B. (1940). *J. Biol. Chem.* **132**, 325.
103. Virtanen, A. I., Laine, T., and Toivonen, T. (1940). *Z. physiol. Chem.* **266**, 193.
104. Wilkins, H. L. (1937). *Science* **85**, 526.
105. Winnick, T. (1942). *J. Biol. Chem.* **142**, 461.

The Relationship of Protein Metabolism to Antibody Production and Resistance to Infection

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CONTENTS

I. INTRODUCTION

The particular feature which differentiates acquired from natural resistance is the antibody mechanism. For the more complete understanding of acquired resistance, therefore, more information is necessary concerning antibodies. Much progress has been made in recent years, especially with regtard to their chemical nature. In fact, according to Heidelberger "The protein nature of antibodies is now generally accepted since analytical, ultracentrifugal, and electrophoretic measurements all show highly purified antibodies to be typical proteins" (1). Assuming that this assertion is correct and that antibodies are modified plasma globulins whose only differences from normal globulins are "mainly those of configuration or arrangement of the component amino acids" (2), it follows that the process of antibody production must be essentially similar to or even identical with that for the production of normal plasma globulin. In other words, the problem of antibody production must be the problem of plasma globulin synthesis. The present review will concern itself largely, therefore, with certain chemical and biological aspects of the problem of synthesis and function of normal and antibody plasma globulin.

II. Serum Protein Synthesis a Problem of Nutrition

Inasmuch as plasma globulin is a tissue-protein its synthesis must be accomplished by the mechanisms of protein metabolism; in particular this synthesis must depend upon the building up of complex polypeptide chains from dietary amino acids. Although it has been thought by some that diet, while determining albumin synthesis, has but little influence upon that of globulin, there is now good reason to believe that both processes require an abundant and varied assortment of dietary amino acids. For example, in a review by Madden and Whipple (3) it was stated that "the same 100 g. of beef serum which produced 38 g. of total plasma protein produced approximately 21 g. of albumin and 17 g. of globulin. The addition of 100 g. bran flakes to a kidney basal diet results in the formation of about 12 g. albumin and 11 g. globulin. Since it has been shown that 100 g. casein will yield only 5 g. albumin and 7 g. globulin, and 100 g. gelatin 5 g. or less of each, it becomes apparent that, as measured by plasmapheresis, diet regulates globulin production equally as well as albumin." These facts indicate that globulin synthesis, also, is basically a nutritional problem.

Nevertheless, most of the studies on the problem of plasma protein production have been concerned for the most part with the synthesis of plasma albumin. Predominant emphasis upon this protein may be attributed to the fact that in the course of induced protein deficiency disease the reduction in concentration of plasma albumin precedes that of plasma globulin. Inasmuch, however, as both plasma albumin and plasma globulin have certain chemical similarities it is not unlikely that there may be a similarity also in their mode of synthesis. The findings of Whipple and his associates with regard to the influence of dietary protein upon plasma protein production have agreed in the main with those of Weech (4, 5) and of Melnick, Cowgill, and Burack (6). More recently it has been demonstrated in dogs (7, 8) that satisfactory rates of plasma protein production may even be accomplished by the ingestion in adequate quantities of the ten amino acids essential for growth of the rat. It is evident, therefore, that amino acids can also enter directly into the synthesis of plasma protein, including albumin and globulin.

The direct incorporation of ingested amino acids into plasma protein has been shown, moreover, by the feeding of isotopic amino acids. Schoenheimer, et al. (9, 10, 11) demonstrated by this technique that plasma proteins participate in general metabolic reactions and that "the rate of this process in the plasma proteins when compared with that in the kidney, liver, and intestinal tract of the same animals is approximately the same, and all fractions of the plasma protein, fibrinogen, euglobulin, pseudo-

globulin, and albumin participate to about an equal extent." They found, also, that when isotopic amino acids were fed to actively immune rats and rabbits "antibody, like the other serum and body proteins, participates in metabolic reactions involving the uptake of dietary nitrogen," "amino acid replacement and nitrogen transfer among individual amino acids occur in antibody and normal serum proteins in the same manner as has been indicated in organ proteins," and "the half life of an antibody molecule is about two weeks, approximately the same as that of the average serum protein." That these interchanges are a part of the normal process of globulin metabolism is indicated by the fact that no such interchanges were observed with passively injected antibody, because they found that in a rabbit already actively immune to another antigen "the absence of uptake of dietary nitrogen by the passive antibody is in pronounced contrast to the appearance of marked nitrogen in the active antibody."

III. Chemical Composition of Normal Serum Globulin

Further elucidation of the question of globulin synthesis requires information concerning the chemical nature of globulin and, particularly, of its amino acid composition. Because of imperfect analytical methods complete amino acid analyses are not yet available, but there is at least some evidence that serum globulin contains several of the amino acids essential both for adequate growth of the rat and maintenance of nitrogen equilibrium in man. Thus in one incomplete analysis of serum globulin (12), 14 amino acids were isolated in the following percentage amounts: glycine, 3.5; alanine, 2.2; leucine and isoleucine, 18.7; histidine, 0.9; arginine, 5.2; cystine, 1.0; proline, 2.8; phenylalanine, 3.8; tyrosine, 6.7; tryptophan, 2.3; glutamic acid, 8.2; aspartic acid, 2.5; and lysine, 6.2. Of particular interest, moreover, is the fact that this analysis revealed the presence of five of the eight amino acids demonstrated by Rose, et al. (13) to be essential in man for the maintenance of nitrogen equilibrium. A more recent analysis of purified human γ-globulin by Brand, Kassell, and Saidel (14) has demonstrated the presence of 12 amino acids, including five of those essential for man (lysine, methionine, tryptophan, threonine, and leucine). Thus from only two analyses of serum globulin, both incomplete, it has been shown that seven of the eight amino acids essential for nitrogen equilibrium in man were present.

From these analytical results it becomes evident that serum globulin is an exceedingly complex protein which contains several and possibly all of those amino acids which the human body itself cannot synthesize but must obtain preformed in the food. How does antibody-globulin compare with normal serum globulin when analyzed by essentially similar methods?

IV. Chemical Composition of Antibody Serum Globulin

Breinl and Haurowitz reported in 1930 "that the precipitate containing about 10 per cent of haemoglobin, formed by haemoglobin and antihaemoglobin serum, contained the same percentages of tyrosine, histidine, and cystine, as serum globulin, within the limits of error of their methods" (15). Smith, Brown, and Gross (16) found the nitrogen contents practically identical in normal horse serum pseudoglobulin, purified type 1 pneumococcus antibody, and purified diphtheria antiserum. Hewitt (17) found a remarkably close agreement with respect to the percentage amounts of amide nitrogen and of cystine, tyrosine, and tryptophan in horse serum globulin fractions and toxin-antitoxin floccules. According to him "the floccules, . . . probably represent an approximation to chemically pure diphtheria antitoxin and these preparations were subjected to analysis. Even in the case of these floccules, the analytical results fall within the range of values obtained for serum globulin." Calvery (18) compared normal horse serum globulin and purified type 1 pneumococcus antibody with the following results: the values for amide nitrogen and for arginine, histidine, cystine, and lysine showed almost identical values. As he said, "in a general way the values agree with those reported in the literature for serum globulins but specific comparisons are almost impossible since no single globulin fraction has been analyzed for the constituents reported here." These results, nonetheless, suggest a close chemical similarity if not identity between normal serum globulin and antibody globulin, although they are based, admittedly, on inadequate analytical methods.

V. Electrophoretic Analysis

Although much information has been obtained from sulfate fractionation of normal and antibody serum proteins, the methods are still too crude for many purposes. Indeed some workers doubt that the different fractions obtained actually afford a true picture of the state of the plasma proteins in the body fluids. Further advances have had to await the development of better and gentler analytical methods. One such was found in the procedure of electrophoretic analysis perfected by Tiselius (19). By this method he demonstrated that "serum is not a more or less continuous mixture, but contains well-defined protein fractions, albumin and three globulins; a, β, and γ." Of particular interest, also, was his demonstration that "a highly potent anti-egg albumin serum from rabbit showed that the antibody fraction migrated with the γ-globulin fraction only." Since this work of Tiselius, rapid progress has been made in the application of the electrophoretic technique to important immunological problems (20, 21, 22, 23) and as a result it is now obvious that one can no longer think exclusively of serum "globulin"; rather one must consider at least three types of

globulin of differing mobilities in the electrophoretic apparatus in order to appreciate more completely the details of globulin metabolism. This is all the more true with respect to the problem of antibody globulin inasmuch as most antibodies thus far studied by the technique of electrophoresis have been found either in the γ-fraction or in one related closely to it. In considering the problem of antibody production, therefore, one must think in terms of the synthesis and nature of γ-globulin rather than of total globulin alone.

VI. SIGNIFICANCE OF γ-GLOBULIN IN RELATION TO ANTIBODIES

Several examples of the significance of γ-globulin in infection, as demonstrated by electrophoretic analysis, may be cited. For instance, the serum of the new-born calf is deficient in γ-globulin, presumably because of the impermeability of the cow's placenta to this protein (24). Coincidentally the calf also may be extremely susceptible to colon bacillus infection, at least until antibodies present in the colostrum have been ingested and absorbed (25). But within 18–24 hours after the ingestion of colostrum, γ-globulin can be demonstrated in the serum (24). Another example of the role of γ-globulin is evidenced by the increase in the γ-fraction in the course of hyperimmunization. If, however, the specific antibody is absorbed by antigen according to the method of "optimal proportions," much of the γ-globulin is also removed. Tiselius and Kabat (26) demonstrated, similarly, that "rabbit and monkey antibody (pneumococcal) and rabbit anti-egg-albumin antibody were quantitatively contained in the γ-fraction, that removal of the antibody with antigen produced a decrease in the γ-globulin and that this decrease corresponded quantitatively to the amount of antibody removed."

Although it is not yet certain that all immune bodies are in the γ-fraction or in a fraction closely related to it, most of those which have thus far been examined by electrophoretic analysis in the rabbit, horse, rat, monkey, and man have been in the fractions of slow mobility. Furthermore, there is no evidence to suggest any important chemical dissimilarity of specific antibody globulin in different species, inasmuch as immune serums from the horse, cow, or rabbit can be used interchangeably with human serum for specific antibacterial therapy in man. In man, moreover, the concentrations of total serum globulin and of the γ-fractions tend as a rule to increase concomitantly in infections characterized by hyperproteinemia, as, for example, in tuberculosis, sarcoidosis, leprosy, lymphogranuloma, leishmaniasis, rheumatoid arthritis, lupus erythematosus, etc. It is not known, however, to what extent the γ-globulin increase is due to antibody alone.

Particularly significant is the recent demonstration of the presence in globulin fractions from the blood sera of normal persons of a large variety

of antibodies. Thus Enders and his collaborators (27) have found, in globulin fractions containing over .90 per cent of γ-globulin, at least sixteen different types of antibodies, including ones specific for diphtheria toxin, typhoid bacilli, pertussis, herpes simplex, scarlatina, vaccinia, measles, mumps, influenza, poliomyelitis, and lymphocytic choriomeningitis. Moreover, these fractions have been used successfully for the treatment of measles (28, 29); indeed, according to Cohn (30) "a large scale public health experiment has now supplemented earlier clinical studies in demonstrating the value of the γ-globulins of human plasma in the modification or prevention of measles." In fact, from the standpoint of its relation to public health he believes that "γ-globulin may come to be recognized as the most important fraction of the plasma."

Of the total globulin concentration in human serum about one-third may be γ-globulin (23). It is obvious, therefore, that changes in the α- and β-components may affect the development of either hyper or hypoglobulinemia, regardless of any changes in the γ-fraction *per se*. This fact illustrates the fallacy of drawing conclusions from the albumin-globulin ratio as to the concentration of γ-globulin in the serum or of its relationship to antibody production. Thus it has been shown that in nephrotic sera (31) the γ-globulin fraction may be almost negligible in amount and yet, because of the high amount of the β-component, the quantity of total globulin may be within the normal range. There is need, therefore, of a simpler method for determining the content of γ-globulin in order to get a better understanding of the antibody-producing potentialities of the individual.

VII. THE SITE OF ORIGIN OF ANTIBODY GLOBULIN

Consideration of the nature of γ-globulin requires consideration also of its site of origin. Although the problem of the locus of antibody formation is as old as immunology itself, the view has gained ground steadily since the early work of Metchnikoff that antibodies arise from cells rather than from humoral constituents of the blood. Evidence has gradually accumulated, moreover, indicating that antibodies are the products of phagocytic cells, coming, for the most part, from the macrophages of the spleen, liver, lymph nodes, and bone marrow. This evidence has been secured in various ways, including ablation of organs, injury to the macrophages, blockade procedures, and tissue culture.

With the recent demonstrations that antibody is specifically modified serum globulin it has become probable also that the macrophage is the principal fabricator of serum globulin and, presumably, also of γ-globulin. Sabin (32) has suggested that both normal serum globulin and antibody globulin arise by a process of shedding of surface films of cytoplasmic

material from monocytes or macrophages. She came to these conclusions
as a result of experiments in which she injected a dye-protein into rabbits
and observed the cytoplasmic changes coincident with the appearance of
circulating antibodies. According to Sabin, "the appearance of antibodies
in the serum correlates with the time when the dye-protein is no longer
visible within the cells and with the phenomenon of a partial shedding of
their surface films." She concluded, furthermore, "that the cells of the
reticulo-endothelial system normally produce globulin and that antibody
globulin represents the synthesis of a new kind of protein under the influ-
ence of an antigen."

More recently, however, it has been suggested that antibodies originate,
not from macrophages alone, but also from lymphocytes. This concept, if
correct, would profoundly alter current views regarding antibody produc-
tion. In support of it Rich (33) a few years ago pointed out that the parent-
eral injection of a foreign protein leads to a marked lymphoid prolifera-
tion in the spleen and regional lymph nodes, a proliferation which increases
with the increase in antibody titer. He remarked, moreover, that "in the
present imperfect state of our knowledge regarding the site of origin of
antibody, the fact that the lymphocyte is concerned in some way with the
earliest reaction of the body to foreign proteins warrants its consideration
in relation to the problem of the cells concerned in antibody forma-
tion." This point of view, based largely on morphological observations,
is in good agreement with that of Ehrich and his associates (34, 35, 36)
who, as a result of antibody titrations of lymphocytic extracts from immun-
ized animals, have suggested that "the lymphocytes either produce anti-
bodies or take them up from the lymph plasma." Because, however, of
their inability to demonstrate either adsorption or absorption of antibodies
by lymphocytes, these latter workers concluded that although "lympho-
cytes are instrumental in the formation of antibodies" the possibility
remains that "the lymphocyte goes into action only after the raw mate-
rial, i.e., bacteria or other formed antigens, has been properly prepared by
the action of micro- or macrophages."

A somewhat different point of view has been suggested recently by
another group of investigators (37) who, while not stating that lymphocytes
actually form antibody, attribute a function of the lymphocyte to be that
of "a storehouse of antibody protein." Their conclusions are based on
experiments in which they found, in extracts of lymphocytes from normal
rabbits, the presence of a protein which they regard as "probably identical
with the γ-globulin of normal rabbit serum." Moreover, in the examination
of extracts of lymph nodes from mice which had been injected with sheep's
erythrocytes they found that "a significant portion of antibody protein is
present in lymphocyte elements"; they concluded, therefore, that "anti-

body is concentrated chiefly within lymphocytes." They look upon the lymphocyte as a carrier of antibody protein and believe that this antibody may be released at an increased rate "in circumstances of augmented adrenal cortical secretion" (38).

These newer concepts are, indeed, surprising, in view of the long-time persistence of a solid immunity after antigenic stimulation and the necessity of relating the antibody mechanism so directly to a type of tissue as labile structurally and functionally as is lymphoid tissue. But if antibodies arise from lymphocytes or are absorbed by lymphocytes, that fact would add further emphasis to the adverse effect of undernutrition upon the mechanisms of acquired resistance, in view of the well-known tendency of malnutrition to engender atrophy of lymphoid tissues. For example, Jackson (39) has stated that "the characteristic involution of the lymphoid tissue during malnutrition usually results in atrophy of the lymph nodes," and "in emaciated human adults (the lymphatic glands) are in many cases extremely atrophic." On the contrary, however, he calls attention to the fact that "upon adequate refeeding after inanition the lymphatic glands in general recuperate promptly, showing rapid increase in weight, associated with active mitosis and recovery of normal structure in the lymphoid tissue."

VIII. The Biological Evaluation of Proteins

For the production of a complex tissue protein such as serum globulin it is obvious that proteins must be consumed which can supply the globulin-producing tissues with most and possibly all of the essential amino acids and in adequate amounts. In attempting to ascertain the amino acid composition of a protein, however, chemical evidence does not necessarily demonstrate its biological potentialities because, in some instances, changes in amino acids in a protein molecule may not be revealed by chemical analysis; in order to determine nutritive value, therefore, the protein must be tested in a living animal. A protein's nutritive quality, assuming that it is well-digested and absorbed, depends presumably in large measure on its qualitative and quantitative content of essential amino acids. Animal proteins, therefore, are, on the whole, superior to most vegetable proteins because of their generally higher content of each of the essential amino acids. This does not mean, however, that both types of protein may not be highly important for the synthesis of serum globulin, depending, of course, upon the relative contents of essential amino acids available.

For the evaluation of protein quality two principal methods have been relied upon, viz., determination of (1) growth rates in young rats, or (2), of nitrogen balance. Because both of these methods are time-consuming, and for other reasons, we have developed an additional method (40) which

depends upon the principle of feeding known amounts of protein over a period of seven and fourteen days to adult protein-deficient male white rats and ascertaining the effects upon weight recovery and regeneration of total serum protein. This method, in our opinion, has proved to be useful as a sort of "screen test" for demonstrating the nutritive potentialities of proteins and protein derivatives of varied origins.

The principle utilized is similar to that used frequently by biologists, *viz.*, the production of a biological deficit in order to measure the replacement value of a material to be tested. Thus for the assay of various kinds of hormonal preparations it is common practice first to produce hypothyroidism, hypogonadism, etc., and then administer the hormone preparation to be tested; similarly for the study of blood regeneration it is necessary first to produce anemia. The method, in principle, is a modification for the rat of the method used in the dog by Weech and his associates; it has in common with their method the advantages of speed and relative simplicity; in addition it has the advantage of suitability for the testing of small quantities of protein materials in large numbers of animals. It differs from Weech's method in at least two important particulars, *viz.*, it determines regeneration of *total* serum protein rather than albumin alone and it simultaneously measures hemoglobin regeneration and weight recovery. In fact, for many purposes the degree of weight recovery may furnish all the evidence desired. The method, furthermore, can demonstrate variations in protein quality in from one to two weeks, rather than, as in the usual growth rate methods, in from four to eight weeks. This presupposes, of course, a supply of protein-deficient test animals; but once this has been established it is usually as easy to feed a colony of rats on a protein-deficient ration as on any other special type of ration. Moreover, the use of a hungry test-animal whose protein stores have been severely depleted favors both consumption of the test-food and repletion of the protein stores in accordance with tissue needs. However, if during the repletion feeding period there is a deficiency in the test-protein of certain essential amino acids, as, for example, lysine or tryptophan, the tissue needs cannot be adequately satisfied and the protein deficiency will be manifested in a poor weight recovery and a low total serum protein regeneration or hemoglobin output. Finally, this method has now been compared with the rat-growth method for the nutritional evaluation of three types of food-proteins of widely differing nutritive values and has yielded exceedingly good agreement (unpublished experiments, Quartermaster Corps, U. S. A.).

The detailed procedures have been published elsewhere (40), but since then a few modifications have been made, principally in the use of a basal ration practically devoid of utilizable protein. To this basal ration the protein to be tested is added at a 9 per cent protein concentration. All

rations are made isocaloric and differ from one another only with respect
to the kind of protein they contain. They are fed in 15 g. amounts per day
for periods of 7 and 14 days. A rat consuming all the ration gets approxi-
mately 48 cal. per day, and in 7 days consumes approximately 9 g. of test
protein. A rat supplied with a high-quality protein, such as meat or lactal-
bumin, will usually consume all of his daily ration and in 7 days will regain
from 35 to 40 g. of weight; in 14 days he will frequently have recovered all
of his lost weight. Moreover, even if his caloric intake is restricted by
allowing him to eat only about four-fifths of the daily ration (12 g. per day),
still he will regain from 30 to 35 g. of weight in 7 days, thus demonstrating
that under these conditions calorie restriction does not cause any marked
diversion of test protein for caloric needs. In contrast, when a protein-
deficient animal is supplied with a poor-quality protein, such as that in
white flour, but eaten in amounts comparable to those for rations containing
high-quality protein, he will regain only from 10 to 14 g. of weight in 7 days.
If, however, the white flour is supplemented with a small amount of syn-
thetic lysine, a 50 per cent improvement in weight recovery results (unpub-
lished experiments). This would suggest, therefore, that the poor nutri-
tional quality of the protein in white flour is to be attributed rather to
differing availabilities of essential amino acids necessary for weight recov-
ery, than to poor palatability or digestibility.

By this method we have been able to feed proteins of unknown nutritive
value as the principal source of nitrogen and thus get some measure of
their nutritive potentialities. For example, we have fed purified bovine
albumin, γ-globulin and several purified fractions of human plasma to
protein-deficient rats with the following results (40); of the various fractions
tested the best weight recovery and the most effective serum protein regen-
eration occurred in animals fed fibrinogen and certain globulin fractions;
the poorest responses occurred in rats fed purified albumin and several
albumin fractions. Presumably, therefore, the larger globulin molecules
of these plasmas contain a greater content or assortment of essential amino
acids utilizable for weight recovery and for total serum protein fabrication.
With respect to γ-globulin these results supplement the chemical evidence
in indicating the presence in it of most and possibly all of the essential
amino acids.

In summary, these facts derived from amino acid analysis, electro-
phoresis, and biological techniques all indicate that both normal serum
globulin and antibody globulin are essentially similar types of protein.
The conclusion seems warranted, also, that both types of globulin are com-
posed of molecules which contain many if not all of the amino acids essen-
tial for mammals, including man, the rat, and the dog. Nutritional con-
ditions necessary for the fabrication of one would seem, therefore, to be

necessary also for the fabrication of the other. Moreover, conditions interfering with the production of one should influence adversely the production of the other.

IX. The Effects of Dietary Protein Deficiency upon the Fabrication of Serum Globulin

A prolonged reduction in the intake of dietary protein must lead in time to a reduction also in the amount of reserve protein in various tissues. The term "reserve protein" is used, not to describe a particular type of protein chemically distinct from other types of body protein and having as a specific function the storage of nitrogenous materials somewhat as fats or carbohydrates are stored, but rather in the sense intended by Whipple and his associates, that is, to describe the presence within various body cells, (liver, muscle, etc.) of a reservoir of protein which is in "dynamic equilibrium" with the plasma protein. According to their viewpoint (41), "As this reserve protein must be largely or wholly within the cell boundaries it is probably specific *cell protein*, yet in some way different from integral cell proteins because it can be withdrawn or supplemented on occasion." Because of its lability, they believe that a "constant ebb and flow exists between plasma and cell proteins" thus establishing a *"dynamic equilibrium"*.

This concept is in accord with that of Schoenheimer and his associates (42) and of Borsook, *et al.* (43). In fact, Schoenheimer has said, in reference to it, "This concept of a dynamic state of blood and organs . . . is supported by all our results."

In this general sense, therefore, one may think of the protein reserves as being influenced quantitatively either in the direction of an increase or decrease in the total amounts of potentially available nitrogenous materials. In the latter sense, as the tissue protein reserves become more and more depleted, hypoproteinemia and anemia develop because of the dynamic relationship between plasma and tissue proteins whereby the loss of one or the insufficient production of the other leads eventually to a lowered protein concentration in both. When the pool of tissue protein reserves reaches a critically low level, further processes of protein metabolism become definitely impaired. In the blood there develops a hypoalbuminemia, followed by a hypoglobulinemia. Impairment of production of either type of plasma protein, however, interferes seriously with its functional effectiveness, as illustrated by the development of nutritional edema as the result of a severe hypoalbuminemia and by a decreased resistance to bacterial infection because of hypoglobulinemia. Because more interest has been shown in the synthesis and functions of the plasma albumins,

however, the effects of undernutrition upon the function of the plasma globulins have been too frequently minimized.

X. The Effects of Protein Deficiency and Protein Repletion upon the Ability of Experimental Animals to Fabricate Antibody

If antibodies are specifically modified plasma globulins, a prolonged inadequacy of protein intake severe enough to cause a marked depletion of the protein reserves should also lessen the capacity of the antibody-producing tissues to synthesize specific antibody. This we have found to be the case in rabbits and white rats (44, 45), and for such types of antibodies as agglutinins, precipitins, and hemolysins. In our earlier experiments, protein deficiency was brought about in young and adult male rabbits by feeding a ration low in protein, the latter being obtained from raw carrots. On such a ration adult rabbits steadily lose weight and become hypoproteinemic; with young rabbits the weight remains almost stationary over a period of two months and the animals also become hypoproteinemic. The ability of such animals to form agglutinins was then determined by the subcutaneous injection of a formaldehyde-killed suspension of typhoid bacilli and measurement of agglutinin titers in comparison with those from sera of well-fed rabbits of corresponding age, similarly injected. As stated in our first publication (44) "our results with young rabbits indicate that protein deficiency during the period of active growth was associated with a lowered activity of the antibody-producing mechanism. Furthermore, adult rabbits made hypoproteinemic by protein deficiency and plasmapheresis, produced antibodies less abundantly than did well-fed animals. In both age groups, however, the results show definitely the superior ability of well-fed rabbits to fabricate agglutinins." In other words, in the absence during the process of immunization of an adequate supply of essential amino acids obtainable either from the tissue-protein stores or from the daily ration, specific antibody cannot be normally fabricated. This fact suggests the probability, moreover, that whenever an animal with a pronounced protein deficiency becomes infected with pathogenic bacteria, its ability to acquire resistance quickly or to mobilize a specific immune mechanism effectively may also be impaired.

In our experiments with rabbits we had as a primary purpose the determination of the effects of severe starvation upon the antibody mechanism, and although the rations fed induced severe protein deficiency, no attempt was made to supply all the vitamins which might be desirable for optimal nutrition. In later experiments in which white rats were used to ascertain the effects of protein deficiency upon their ability to fabricate hemolysins, more rigorous measures were taken to ensure adequacy of vitamin intake (45). The animals were fed isocaloric rations either high or low in protein

concentration but with all other constituents identical, including adequate amounts of all known vitamins considered necessary for the rat. When the animals had become markedly hypoproteinemic they were injected intravenously with a suspension of washed sheep's erythrocytes and their hemolysin output measured in terms of hemolytic units. The results indicated that "the animals with abundant protein reserves and an adequate protein intake were able, on the average, to fabricate approximately ten times as much antibody as were those with depleted reserves."

If our conclusion is correct that a lessened antibody output is due to severe protein deficiency and a depletion of the protein reserves, repletion of these reserves should restore the capacity of the antibody-producing tissues to form antibody. This also we have found to be the case. For example, when rats which are markedly hypoproteinemic are fed high-quality proteins, such as dehydrated beef or lactalbumin, for a period of seven days, and are then subjected to antigenic stimulation, they quickly regain their capacity to elaborate specific antibody and, in fact, almost equal the antibody output of the control animals.

It is of interest, moreover, that proteins of vegetable origin, as, for example, soyflour or wheat flour, are also able to bring about a considerable degree of restoration of the antibody-producing capacity; we have not yet made quantitative studies of the relative potentialities of proteins of varying degrees of amino acid completeness to restore the antibody-producing capacity. Proteins which are particularly incomplete, however, such as gelatin, are unable either to engender protein repletion or reëstablishment of the normal antibody-producing capacity. We assume, therefore, that in an otherwise healthy animal, repletion of the protein pools by ingestion of proteins furnishing a proper assortment of all the amino acids which go into the antibody molecule will enable the animal to fabricate specific antibodies almost as well as a normal animal.

It should be emphasized that protein repletion may affect both the protein reserves and the antibody-producing tissues whose structural and functional integrity may have been impaired by severe and prolonged starvation. Thus the phagocytic cells of the liver, spleen, lymph nodes and lymphoid tissues, and bone marrow require a constant supply of amino acids and other nutrient materials. In severe inanition, however, these cellular reserves also undergo atrophy, as illustrated by the marked hypocellularity of the bone marrow in severe starvation or debilitating disease. Under such adverse circumstances the leukocytic tissues tend to revert to myeloblastic levels; in times of stress, therefore, such a bone marrow is less prepared to meet an infectious emergency by responding with an outpouring of mature phagocytic cells, no matter how much specific antibody might be available for the particular microörganisms causing the infection. A

similar effect may occur also with respect to the production of mononuclear phagocytes. Protein repletion, therefore, helps not only to restore the protein reserves to be drawn upon for the production of specific antibodies, but it may also make available materials which can be utilized for the restoration of depleted phagocytic tissues in the bone marrow and reticuloendothelial system (46).

XI. RELATIONSHIP OF PROTEIN DEFICIENCY
TO REDUCED RESISTANCE TO BACTERIAL INFECTION

If the above evidence is valid there should be indications from clinical and experimental sources of the influence of protein deficiency upon resistance to infection. Clinical evidence for this, however, is largely circumstantial, although highly probable in view of the long-known sequential relationship between hunger, famine, and infection (47). As Jackson has said (39), "Whether it be a direct effect of inanition, or indirectly caused by toxins in circulation, a lowered resistance to infection is a well-known result of various types of inanition, both total and partial. Thus the immediate cause of death following inanition is frequently an infectious complication, such as bronchopneumonia in the human species." There are obviously many other factors which affect resistance to infection, including multiplicity of contacts with infectious agents, massiveness of infecting dose, invasiveness or virulence of the microörganisms, exposure, fatigue, etc.; nevertheless, there is accumulating evidence that protein deficiency may influence both the acquisition and retention of resistance to bacterial agents of many kinds. This phase of the subject will not be emphasized in detail inasmuch as the author has recorded elsewhere some of the evidence pointing to this relationship (48, 49, 50). It should be kept in mind, however, that antibacterial resistance may not only be acquired; it may also be lost as a consequence of unfavorable circumstances of life. The important question is: In what ways may protein metabolism influence both the acquisition and retention of the capacity of the antibody mechanism and of the phagocytic tissues to cope with infectious agents?

In considering this latter question it is assumed that resistance to infection depends upon a combination of the forces of natural and acquired immunity. Although acquired resistance enhances resistance to infectious agents against which natural resistance may be inadequate, this does not mean that the value of the latter is to be underestimated nor that it cannot be increased by optimal nutrition. The increasing emphasis upon protein metabolism in relation to infection and resistance, therefore, must be evaluated with respect to both types of immune processes.

It is particularly in conditions of chronic infection, where the balance between infection and resistance may be readily upset by interference

with various resistive mechanisms, that one should find evidence of an adverse influence of undernutrition. Inasmuch as one of the commonest and most widespread chronic infectious diseases is tuberculosis, it is with respect to this disease that the most attention has been paid because of the long-held belief that its course may be adversely affected by poor nutrition. For example, Long has said (51), "malnutrition is popularly supposed to predispose to tuberculosis, and feeding in the effort to restore weight and strength has been one of the mainstays of treatment since early times. Inasmuch as the loss of adipose tissue is conspicuous in advancing tuberculosis, excessive administration of fat-building foods was common in the past. At present more attention is concentrated on proteins and vitamins."

Since World War I, several writers have pointed out the possible relationship of protein shortage to mortality from tuberculosis. Cobbett, for example, has suggested that food shortage in World War I was probably the most important factor leading to a decreased resistance to tuberculosis (52). He pointed out that the effects of malnutrition are most adverse in individuals already infected with tubercle bacilli because in "those already consumptive (their) lives hang in a delicate balance that is easily upset." He emphasized the fact in particular that in England and Wales during World War I deaths from tuberculosis in lunatic asylums more than doubled although the number of inmates declined. Other diseases, also, especially dysentery, increased enormously in these asylums during the war.

In discussing further the possible effects of protein inadequacy upon resistance to tuberculosis, Long has cited the following suggestive facts: (1) In World War I there was a sharp rise in the death rate from tuberculosis throughout Europe coincident with the fact that, of all the dietary elements, the reduction in protein and fat was most significant. The inference is that in many instances a low protein, low fat diet would not prevent breakdown in patients with slight lesions who would have been safely protected by normal diets. (2) In Denmark, during this time, where the death rate from tuberculosis rose early in the war, there was a sharp drop in 1918, due, presumably, to the fact that the strict blockade of 1917 had compelled the Danes to consume their own abundant supplies of meat, milk, and butter. He refers to Faber's conclusion that, because the tuberculosis death rate went down as the malnutrition disappeared, the increase in mortality of the war years was in close relation to the shortage of meat and fish and that the original rise in the tuberculosis death rate was the result of protein deficiency. (3) Even in a brief period of famine a rising death rate from tuberculosis may appear. Thus Long refers to observations of Høygaard of a sharp rise in the tuberculosis rate and an increase

in the severity of the disease in an Eskimo tribe temporarily deprived of meat and fish during a brief famine.

These observations all point to the need for further study of all nutritional aspects of tuberculosis; furthermore, they are in accord with the idea that impaired protein metabolism, as a part of the process of malnutrition, may interfere seriously with the resistive mechanisms of both natural and acquired resistance which must be operative in tuberculosis.

Less attention has been paid to the possible effects of undernutrition and malnutrition upon other chronic infectious processes. It should be mentioned, however, that Coburn and Moore have recently called attention to a possible role of protein metabolism in rheumatic disease (53). They observed a relationship between protein inadequacy and the development of rheumatic disease in underprivileged children and concluded that there was "a significant association between the level of protein in the diet and rheumatic susceptibility" and that, moreover, "the association between greater susceptibility and a deficient intake of protein is statistically significant."

Experimental evidence pointing to the adverse influence of protein inadequacy upon resistance to infection is still meager, although there are some observations which suggest such a correlation. For example, Kohman (54), in her early experiments dealing with the relationship of nutritional edema to protein deficiency said, "Post mortem examinations were made of all the animals that died as a result of being fed the low-protein-carrot diet . . . a very common finding was pneumonic lungs." Whipple and his associates, since their earliest experiments with hypoproteinemic dogs, have repeatedly called attention to the fact that "dogs after long periods of plasma depletion show a lowered resistance to infection" (55). These dogs are also unusually susceptible to intoxications of various sorts. Meyer (56) has reported that when rats were injected with diphtherial toxin the fatality rate was approximately four times as high in animals fed either a stock diet or a high carbohydrate or fat diet as it was with animals on a high protein intake (casein). Watson (57), and Robertson and Doyle (58) have reported that young mice and rats are definitely more resistant to infection or intoxication with mouse typhoid or enteritis microörganisms when fed rations containing high levels of dried separated milk or casein. Watson concluded that, "It is probable that the factor responsible for this increase in resistance is the dried separated milk," while Robertson and Doyle concluded that "rats fed a diet high in casein have a considerably higher resistance to enteritidis infection (intraperitoneal) than controls fed either wheat gluten or soybean flour." Sako (59) has recently observed, in mice infected with multiple lethal doses of virulent pneumococci, that resistance to infection depended considerably upon diet

and that "animals which had been maintained on a very low protein intake showed a greatly decreased post-inoculation time, as compared with control animals. On the other hand, those which had been subjected to an excessively high protein intake showed a distinct lengthening of the survival time after inoculation. Wissler (unpublished experiments) has also demonstrated in hypoproteinemic rabbits and rats an impaired capacity to acquire resistance to infection with virulent pneumococci. Finally, we have pointed out (45) that in the white rats unable to fabricate antibodies adequately because of severe protein depletion there was also "a definitely increased tendency to develop spontaneous infection. For example, in the first experiment, three of the twelve hypoproteinemic rats died during the following three weeks; at the end of this period, moreover, four others exhibited varying degrees of chronic abscessive pneumonia. In the second experiment nine of the twelve hypoproteinemic rats showed, when sacrificed, a chronic abscessive pneumonia. In contrast, none of the control rats in either group (on a 22 per cent casein intake) showed any gross evidence of pulmonary infection."

These experimental facts afford encouragement to surgeons concerned with problems of postoperative infection. Hitherto not enough consideration has been given to the fact that patients are frequently operated upon after prolonged periods of weight loss and associated loss of body nitrogen. There is evidence, indeed (60) that postoperative fatality rates may, at times, be dependent in some measure upon the degree of preoperative weight loss. Moreover, after some types of operation there is a period of several days during which but little if any food can be consumed, and obviously during this time, considerable amounts of nitrogen are lost (61, 62, 63). If, furthermore, wound healing is delayed or if the wound becomes infected, a favorable outcome is definitely less likely. It is at this critical time, therefore, both before and immediately after operation, that particular attention should be focused on the patient's protein needs. This does not mean that caloric, mineral, and vitamin requirements should be neglected; too often, however, only these latter are attended to, nitrogenous requirements are ignored and the patient steadily loses nitrogen. There can be but little doubt that some of the spectacular surgical advances in recent years, notably those relating to surgery of the lungs, esophagus, pancreas, and stomach, may be attributed to a considerable degree to the generous use of blood and plasma transfusions and to intravenous and enteral alimentation by means of protein hydrolyzates.

Utilization of these hydrolyzates, particularly for intravenous feeding, is of great interest at the present time; their development, however, has created several additional problems. The most widely used preparations now are casein hydrolyzates and it has been shown that, by parenteral

administration of some of them in adequate amounts, a patient can be kept in nitrogen equilibrium. Attempts are also being made to prepare protein hydrolyzates frcm other proteins, as, for example, lactalbumin, fibrin, fish muscle, and mixtures of animal and vegetable protein. They are prepared either by enzymatic or acid hydrolysis. No matter how they are prepared, however, or from what original material, if they are to be nutritionally satisfactory they must be (a) non-antigenic and (b) complete with respect to the assortment and amounts in them of at least those eight amino acids essential for the maintenance of nitrogen balance in man. At the present stage of development of these hydrolyzates, therefore, it is imperative that each manufacturer demonstrate both their safety for intravenous use, and their amino acid completeness.

We have tested several of these hydrolyzates by our assay method and have found considerable variability in their nutritive potentialities. In general, those derived frcm casein by enzymatic digestion have been superior although scme of the others have also been excellent with respect to their ability to cause weight-recovery and regeneration of serum protein in protein-deficient rats. If acid hydrolysis is utilized, however, the hydrolyzate must be supplemented with adequate amounts of tryptophan. At any rate, there is now abundant evidence that these protein hydrolyzates, if properly prepared, can function effectively as a parenteral source of utilizable amino acids.

In conclusion it should be emphasized that, although the facts presented deal mainly with the problem of protein metabolism in its relation to antibody production, it is taken for granted that globulin synthesis must also require the intermediation of synthesizing enzymes, minerals and other focd constituents. Just as polypeptides cannot be built up without the necessary amino acid "building blocks," so they cannot be synthesized without the necessary enzymes, including vitamins. It is a suggestive fact, moreover, that many protein-rich foods are themselves rich in vitamins, such as, thiamine, riboflavin, and vitamin A. Particular emphasis upon the role of amino acids, therefore, should not cause one to underrate the importance of the dynamic mechanisms within the tissues which accomplish protein synthesis, including that of normal globulin and antibody-globulin.

REFERENCES

1. Heidelberger, M. (1943). Addendum to the Chemistry of the Amino Acids and Proteins, p. 1259. Edited by Carl L. A. Schmidt. Charles C. Thomas, Springfield, Ill.
2. Heidelberger, M. (1938). The Chemistry of the Amino Acids and Proteins, p. 963. Edited by Carl L. A. Schmidt. Charles C. Thomas, Springfield, Ill.
3. Madden, S. C., and Whipple, G. H. (1940). *Physiol. Revs.* **20**, 194.

4. Weech, A. A. (1938-39). *Harvey Lectures*, p. 57.
5. Weech, A. A. (1942). *Bull. Johns Hopkins Hosp.* **70**, 157.
6. Melnick, D., Cowgill, G. R., and Burack, E. (1936). *J. Exptl. Med.* **64**, 877, 897.
7. Madden, S. C., Carter, J. R., Kattus, A. A., Jr., Miller, L. L., and Whipple, G. H. (1943). *J. Exptl. Med.* **77**, 277.
8. Madden, S. C., Woods, R. R., Shull, F. W., and Whipple, G. H. (1944). *J. Exptl. Med.* **79**, 607.
9. Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M. (1942). *J. Biol. Chem.* **144**, 541.
10. Schoenheimer, R., Ratner, S., Rittenberg, D., and Heidelberger, M. (1942). *J. Biol. Chem.* **144**, 545.
11. Heidelberger, M., Treffers, H. P., Schoenheimer, R., Ratner, S., and Rittenberg, D. (1942). *J. Biol. Chem.* **144**, 555.
12. Calvery, H. O. (1938). Chemistry of the Amino Acids and Proteins, p. 217. Edited by Carl L. A. Schmidt. Charles C. Thomas, Springfield, Ill.
13. Rose, W. C. (1944). Personal communication.
14. Brand, E., Kassell, B., and Saidel, L. J. (1944). *J. Clin. Investigation* **23**, 437.
15. Marrack, J. R. (1934). The Chemistry of Antigens and Antibodies. Special Report Series No. 194. Medical Research Council, London, p. 50.
16. Smith, M. L., Brown, A. M., and Gross, C. (1932). *Biochem. J.* **26**, 1473.
17. Hewitt, L. F. (1934). *Biochem. J.* **28**, 2080.
18. Calvery, H. O. (1935-36). *J. Biol Chem.* **112**, 167.
19. Tiselius, A. (1937). *Biochem. J.* **31**, 1464.
20. Boyd, W. C. (1943). Fundamentals of Immunology. Interscience Publishers, Inc., New York, N. Y.
21. Treffers, H. P. (1944). *Advances in Protein Chemistry* **1**, 70.
22. Abramson, H. A., Moyer, L. S., and Gorin, M. A. (1942). Electrophoresis of Proteins and the Chemistry of Cell Surfaces. Reinhold Publishing Corp., New York, N. Y.
23. Kabat, E. A. (1943). *J. Immunol.* **47**, 513.
24. Jamieson, E., Alvarez-Tostado, C., and Sortor, H. H. (1942). *Proc. Soc. Exptl. Biol. Med.* **51**, 163.
25. Smith, T., and Little, R. B. (1922). *J. Exptl. Med.* **36**, 181.
26. Tiselius, A., and Kabat, E. A. (1939). *J. Exptl. Med.* **69**, 119.
27. Enders, J. F. (1944). *J. Clin. Investigation* **23**, 510.
28. Stokes, J., Jr., Maris, E. P., and Gellis, S. S. (1944). *J. Clin. Investigation* **23**, 531.
29. Ordman, C. W., Jennings, C. G., and Janeway, C. A. (1944). *J. Clin. Investigation* **23**, 54.
30. Cohn, E. J. (1945). *Science* **101**, 51.
31. Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A. (1939). *J. Exptl. Med.* **70**, 399.
32. Sabin, F. R. (1939). *J. Exptl. Med.* **70**, 67.
33. Rich, A. R. (1944). The Pathogenesis of Tuberculosis, pp. 414 and 593. Charles C. Thomas, Springfield, Ill.
34. Ehrich, W. E., and Harris, T. N. (1942). *J. Exptl. Med.* **76**, 335.
35. Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E. (1945). *J. Exptl. Med.* **81**, 73.
36. Ehrich, W. E. and Harris, T. N. (1945). The Site of Antibody Formation. *Science* **101**, 28.

37. Dougherty, T. F., Chase, J. H. and White, A. (1944). The Demonstration of Antibodies in Lymphocytes. *Proc. Soc. Exptl. Biol. Med.* **57**, 295.
38. Dougherty, T. F., White, A. and Chase, J. H. (1944). Relationship of the Effects of Adrenal Cortical Secretion on Lymphoid Tissue and on Antibody Titer. *Proc. Soc. Exptl. Biol. Med.* **56**, 28.
39. Jackson, C. M. (1925). The Effects of Inanition and Malnutrition upon Growth and Structure. P. Blakiston's Son and Co., Philadelphia, Pa.
40. Cannon, P. R., Humphreys, E. M., Wissler, R. W., and Frazier, L. E. (1944). *J. Clin. Investigation* **23**, 601.
41. Madden, S. C., Turner, A. P., Rowe, A. P., and Whipple, G. H. (1941). *J. Exptl. Med.* **73**, 571.
42. Schoenheimer, R. (1942). The Dynamic State of Body Constituents. Harvard University Press, Cambridge, Mass.
43. Borsook, H., and Dubnoff, J. W. (1943). *Ann. Rev. Biochem.* **12**, 183.
44. Cannon, P. R., Chase, W. E., and Wissler, R. W. (1943) *J. Immunol.* **47**, 133.
45 Cannon, P. R., Wissler, R. W., Woolridge, R. L., and Benditt, E. P. (1944). *Ann. Surg.* **120**, 514.
46. Cannon, P. R. (1945). *J. Am. Med. Assoc.* **128**, 360.
47. Cannon, P. R. (1943). *Sci. Monthly* **56**, 5.
48. Cannon, P. R. (1942). *J. Immunol.* **44**, 107.
49. Cannon, P. R. (1944). *J. Am. Diet. Assoc.* **20**, 133.
50. Cannon, P. R. (1944). *J. Mich. State Med. Soc.* **43**, 323.
51. Long, E. R. (1941). *Arch. Path.* **32**, 122, 286.
52. Cobbett, L. (1930). *J. Hyg.* **30**, 79.
53. Coburn, A. F., and Moore, L. V. (1943). *Am. J. Diseases Children* **65**, 744.
54. Kohman, E. A. (1920). *Am. J. Physiol.* **51**, 378.
55. McNaught, J. B., Scott, V. C., Woods, F. M., and Whipple, G. H. (1936). *J. Exptl. Med.* **63**, 277.
56. Meyer, A. R. (1939). *Proc. Soc. Exptl. Biol. Med.*, **41**, 404.
57. Watson, M. (1937). *J. Hyg.* **37**, 420.
58. Robertson, E. C., and Doyle, M. E. (1936–37). *Proc. Soc. Exptl. Biol. Med.* **35**, 374.
59. Sako, W. S. (1942). *J. Pediat.* **20**, 475.
60. Studely, H. O. (1936). *J. Am. Med. Assoc.* **106**, 458.
61. Brunschwig, A., Clark, D. E. and Corbin, N. (1942). *Ann. Surg.* **115**, 1091.
62. Elman, R. (1942). *J. Am. Med. Assoc.* **120**, 1176.
63. Mulholland, J. H., Co Tui, Wright, A. M., and Vinci, V. J. (1943). Nitrogen Metabolism, Caloric Intake and Weight Loss in Postoperative Convalescence. *Ann. Surg.* **117**, 512.

Terminal Amino Acids in Peptides and Proteins

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I. INTRODUCTION

Three levels of study of proteins have been vigorously prosecuted. One of these is the elucidation of the chemistry of the component amino acids by the prodigious labors of Emil Fischer and the extension of such work by his students and followers. The other approaches which have been liberally employed by protein investigators are those of behavior of proteins themselves, and the determination of physical constants. The first type of work has especially attracted organic chemists who found in the amino acids units which could be handled in the style to which they were accustomed. The larger aspects have been developed mainly by physical chemists, whose tools have proved adaptable to the natural materials of huge construction which we know as proteins.

The intermediate field of study of the exact arrangements in which the amino acids are linked together to form larger molecules has, however, not been appreciably cultivated. There is reason to believe that much of the biological significance of proteins may correlate with such arrangement. The need for proper implementation of such an attack has provoked the preparation of this review.

It has already been pointed out by Synge (1943) that important obstacles in the development of knowledge of protein fine structure are those of technic, particularly in the isolation of pure products of partial hydrolysis of protein. It is quite likely that the development of isolation procedures will be aided by the development of proper methods for marking or identifying terminal amino acids. One reason for believing this is the fact that amino acids condensed with various reagents are found to possess properties which differ considerably from those of unsubstituted amino acids obtained as hydrolytic products. One might expect therefore to mark the terminal amino acid chemically, hydrolyze the large molecule, and then take advantage of solubility differences to separate the amino acid derivative. To cite one example, the β-naphthalenesulfonyl derivatives of leucine and phenylalanine are easily soluble in ether, in contrast to the unsubstituted amino acids (Fischer and Bergell, 1902). Alcohol solubility is found also for β-naphthalenesulfonyl peptides (Fischer, 1903). It may therefore prove most economical to develop the chemistry of the terminal amino acid prior to or concurrently with the better means of separation that are sorely needed.

There are also other reasons than the desire to elucidate protein structure, for seeking good methods of identification of terminal amino acids.

II. Actual and Proposed Purposes
of Determining Terminal Amino Acids

A number of reasons seem worthy of consideration.

1. For Characterization of Proteins

Adequate study of terminal amino acids might provide useful tools for characterizing some of the chemical differences· underlying biological specificities. It would in general be especially desirable to have an added index of difference or similarity between protein molecules that could be used more precisely than amino acid composition. Our present analytical methods, for example, do not permit us to decide if the casein from a cow in Iowa is the same, molecularly, as that from a cow in New Jersey. Amino acid assays reveal similarities in this and related comparisons, but such assays are in general insufficiently precise to enable the investigator to pick out differences which are definitely not ascribable to errors of assay. Many of the methods for terminal amino acids might be expected to prove sufficiently precise, however, to differentiate proteins or protein mixtures on this basis.

An early study of this sort was carried out by Dakin and coworkers (Dakin, 1912; Dakin and Dudley, 1913; Dakin and Dale, 1919) on the antigenically different egg albumins of duck and domestic fowl. Differences in terminal amino acids were found by a method to be described later (p. 162). No efforts to purify the proteins to single individuals were made (the methodology available then was markedly inadequate). The last publication would have been improved by the inclusion of details of the amino acid analysis of the proteins. Although the purity of the proteins and the significance of the identification method are open to question, differences in terminal amino acids were found.

Leaving out of consideration comparison between biologically similar proteins, it is conceivable that the terminal amino acid in any single protein may have inherent serological significance, at least as a locus of coupling, as with the synthetic haptens of Harington (1940).

2. To Determine Amino Acid Sequence

The ideal solution to this problem would involve removal of terminal amino acids from a peptide of any length, one at a time, without alteration of the remaining peptide. Such a method has been devised by Bergmann and Zervas (1936), and approximated by a simpler procedure developed by Abderhalden and Brockmann (1930). It appears quite likely that practical difficulties have prevented wider use of these methods by others, especially since they might have been employed on proteins as a partial

check of the periodicity hypothesis (Bergmann and Niemann, 1937, 1938).

Simple marking technics might however be used in the following manner.

A tetrapeptide, A–B–C–D, could be hydrolyzed to fragments B–C and C–D. If A, B, and C are then designated chemically as terminal amino acids in each of these three peptides, it becomes possible to formulate the structure of the original peptide. To cite an actual example, it was possible to reconstruct the tripeptide glutathione from the two dipeptides which were identified after hydrolysis, by marking procedures alone. The same type of determination of sequence should hold for larger peptides.

3. TO REVEAL OTHER FEATURES OF PROTEIN STRUCTURE

The terminal amino acid approach offers promise of providing a chemical means of testing some of the hypotheses of peptide arrangement in proteins.

Of various conceivable types of peptide arrangement in protein, the closed or cyclic peptide structure would be established in a negative manner, *i.e.*, by a demonstration of the absence of terminal amino acid. The closed peptide structure has been suggested for gramicidin (Hotchkiss, 1944), and the absence of amino nitrogen suggests that it may apply to zein also (Hopkins and Wormall, 1934). Hydrolysis sufficient to open one peptide linkage followed by chemical designation of a terminal amino acid which was not present in the original molecule would then constitute negative evidence of the kind alluded to above.

The absence of free α-amino nitrogen in proteins like zein can also be explained by the occurrence of proline or hydroxyproline at one terminus. In such cases, distinction might be established by a terminal amino acid method which requires a free carboxyl group. On the other hand, the amino terminus would have to be marked when the carboxyl is masked as amide.

The two features of protein structure which have been definitely established are the constituent amino acid residues and the recurrent peptide linkage. A protein which would involve no other covalent bonds would then be a giant polymer of a variously substituted glycine residue and would possess one α-amino group and one α-carboxyl group. Methods for designation of these two groups should then specify one terminal *amino* acid,[1] and one terminal amino *acid*, and no other.

There is reason to believe that this simple polypeptide picture will not suffice for many proteins, although it may be tenable for some (Chibnall, 1942). Chibnall has suggested a hypothetical imide coupling of peptide chains:

[1] *Amino* acid refers to terminal amino acid with free amino group; amino *acid* refers to terminal amino acid with carboxyl group free.

$$NH_2 \qquad R \qquad R \qquad R$$
$$CO-(CH_2)_x-CH-CO-NH-CH-CO-NH-CH-CO-NH-CH-COOH$$
$$NH$$
$$\quad R \qquad R \qquad R \qquad R$$
$$CO-CH-NH-CO-CH-NH-CO-CH-NH-CO-CH-NH-CO-CH-(CH_2)_x-CO$$
$$NH_2 \qquad NH$$
$$R \qquad R \qquad R \qquad R \qquad R \qquad R$$
$$H_2N-CH-CO-NH-CH-CO-NH-CH-CO-NH-CH-CO-NH-CH-CO-NH-CH-CO$$

This pattern combines peptide chains with imide linkages at the acid termini, so that although the whole molecule contains several free α-amino groups, it possesses but one α-carboxyl. Such a structure could accordingly be checked by an adequate terminal amino *acid* method.

In the picture below, the peptide chains have been designated "subunits" by Chibnall and he has suggested two hypothetical means of joining of the subunits. These are the ester and thiol-ester linkages, depicted as follows:

$$R$$
$$H_2N-CH-CO-NH-CH-CO-\cdots-NH-CH-CO-\cdots-COOH$$
$$(CH_2)_2 \qquad\qquad (CH_2)_2$$
$$CO-S \qquad\qquad CO-O$$
$$CH_2 \qquad\qquad CH_2$$
$$HOOC\cdots-CO-CH-\cdots-CO-CH-NH-\cdots-NH_2$$

Should either of these types of structure be widespread in nature, some proteins should then yield more than one terminal *amino* acid and more than one terminal amino *acid*. The proper terminal amino acid methods would supply contributing evidence on a protein of established purity. The above considerations apply also for "subunits" held together by hydrogen bonds (Mirsky and Pauling, 1936) or by disulfide linkages.

Although it appears to be assumed generally that proteins do not contain branched main-chains, rejection of such a structural picture does not seem warranted. The branched-chain molecule among high polymers is for example known in the case of polysaccharides (Haworth, Hirst, and Isherwood, 1937; Staudinger and Eilers, 1936; Meyer, 1942). Cohn (1939) has indicated that the peptide linkages of proteins occur only through α-amino and α-carboxyl groups, but it is strikingly true that in glutathione the

glutamic acid is not attached through the α-carboxyl group. Were the glutathione type of linkage of glutamic acid to occur as a unit in protein, a branched main-chain could then be visualized thus:

$$\text{HOOC-CHR-NH-}\cdots\text{-OC-CH-(CH}_2\text{)}_r\text{-CO-NH-CHR}_r\text{-CO-}\cdots\text{-NH-CHR}_r\text{-COOH}$$

$$\begin{array}{c} | \\ \text{NH} \\ | \\ \vdots \\ | \\ \text{CO} \\ | \\ \text{CHR}_1 \\ | \\ \text{NH}_2 \end{array}$$
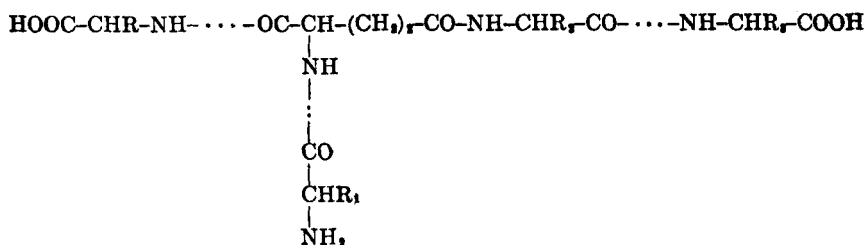
It has been pointed out (Grassmann, Dyckerhoff, and Eibeler, 1930) that the ω-carboxyl linkage of glutamic acid with the cysteine residue in glutathione has not been hydrolyzed by proteolytic enzymes to which it has been subjected. This has been taken as evidence against such bonding in protein. On the other hand, the existence of such a linkage in a widespread naturally occurring peptide is unequivocally established, and it seems reasonable to suppose that the mechanism responsible for the synthesis of glutathione could synthesize other peptides as well.

Branches or other features in protein structure are likely to be expressed by particular arrangements and numbers of terminal amino acids. In the further elucidation of protein structure, the terminal amino acid should prove of contributory value, as has the "end-group" in the clarification of polysaccharide structure, with the added advantage that greater differences in end-group may be expected since the protein units are more diverse in structure.

It is a pleasure to acknowledge the benefit of discussions of the above topics with Dr. R. E. Rundle.

Each of the methods which follows for designating terminal amino acids should be evaluated in the light of the purpose for which it is intended.

III. MEANS OF IDENTIFYING TERMINAL AMINO ACIDS

1. ENZYMIC METHODS

Proteolytic enzymes which split off terminal amino acids should be ideally adaptable for purposes of identification. This ideal may some day be attained, but application of existing knowledge suffers from one very fundamental criticism which applies to much of our knowledge of protein chemistry. This is of course the difficulty of insufficient and inadequately established purity of the enzymes (proteins) which are employed. The

specificity of the enzymes, knowledge of which is a prerequisite to their use in this connection, has also not been generally established. This may represent merely another manifestation of insufficient purity. It may, on the other hand, correspond to groupings of structural specificities which our conventional chemical thinking does not permit us easily to recognize. The specificity of the impure preparations ordinarily employed has rested on shifting ground, e.g., Linderström-Lang (1929b). Considerable uncertainty existed regarding the specificity of carboxypeptidase preparations (Abderhalden and Abderhalden, 1938). With the description of a crystalline carboxypeptidase (Anson, 1937), it became possible to investigate the specificity more carefully. Hofmann and Bergmann (1940) found that some of the original doubts of enzymic inhomogeneity were unjustified. On the other hand, thrice recrystallized carboxypeptidase was found to attack carbobenzoxyglycyl-l-glutamic acid slowly only, and to attack ε-hippuryl-l-lysine. In this latter case, a free amino group was required for hydrolysis, whereas absence of a free amino group was previously considered to have been a requirement.

A serious objection to the enzymic method is the possibility of resynthesis (Behrens and Bergmann, 1939). One might thus find sequences which did not occur in the original protein (Cohn and Edsall, 1943).

In spite of these drawbacks, it has been possible to obtain reliable information on terminal amino acids in glutathione and in protamines (Grassmann, Dyckerhoff, and Eibeler, 1930; Waldschmidt-Leitz, 1935). The glycine terminus of glutathione was demonstrated by carboxypeptidase action. Arginine was shown to be terminal in both clupein and salmine, by the action of protaminase (Waldschmidt-Leitz, Ziegler, Schäffner, and Weil, 1931).

The general method of enzymological investigation was reviewed by Linderström-Lang in 1933. This author stated as follows: "Hat man daher ein reines Enzym mit wohldefiniertem Spezifitätsbereiche in Händen, so besitzt man gleichzeitig ein ideales Werkzeug zur Aufklärung von Struktur und Bindungsart einer zur Untersuchung vorliegenden Substanz. Wie bekannt, ist man noch weit von der Verwirklichung dieses Ideales entfernt."

The work of Bergmann and collaborators (Fruton, 1941) has served to increase the knowledge of the specificity of the proteinases since 1933, but much remains to be done both on this score and on purification. Such enzymes as carboxypeptidase, aminopeptidase, and protaminase should be especially pertinent to structural studies. It is to be hoped that the availability of crystalline carboxypeptidase may lead the way to a rigid establishment of homogeneity of this enzyme, and then to an experimental evaluation of its substrate specificity which will make it more generally useful.

In the further development of this mode of study, the finding of peptidases which are specific for individual amino acids (Maschmann, 1941) could be especially helpful. By the use of leucylpeptidase, for instance, it has been possible to differentiate leucylglycine and alanylglycine.

2. PHYSICO-CHEMICAL METHODS

Three main types of physical chemical constants have been employed: dissociation constants, optical rotatory power, and reaction equilibrium constants.

a. *Titration Constants*

The first suggestion of the correct structure of glutathione arose from a study of the titration constants of the various dissociable groups. The position of the groups was selected on the basis of the influence of neighboring structures (Harris, 1923–24; Simms, 1927–28; Pirie and Pinhey, 1929). These considerations led to two suggested structures of which the favored one was subsequently proved by chemical means to be correct. For this technic, glutathione probably presented an unique opportunity. One might expect considerably more difficulty in allocation when drawing conclusions from the titration of a peptide composed only of monoamino monocarboxylic acids.

b. *Racemization*

The second method depends upon the relative resistance to racemization of the terminal amino acid. Dakin and coworkers (1912, 1913, 1919) employed a racemization of proteins with cold alkali. The amino acids within the main-chain were believed to have racemized readily, in contrast to the terminal amino acids. After hydrolysis, the separated amino acids were examined for optical rotatory power, and those which remained active were considered to be terminal amino acids. This type of study might profitably be extended to more definitely pure proteins, and to peptides of natural origin.

Cahill and Burton (1940) suggested the use of racemization by ketene or acetic anhydride to allocate the terminal amino acid in peptides. They found such racemization to occur rapidly with *l*-leucine and *l*-glutamic acid when either acetylation agent was employed without neutralization of the acid solution resulting from acetylation of the sodium salt of the amino acid. If an initial alkalinity were maintained, no racemization occurred. Glycyl-*l*-leucine was racemized whereas under the same conditions *l*-leucylglycine was not. Cahill and Burton's experiments did not include dipeptides of two racemizable amino acids, however, and it would be interesting to see if the same principles be applicable to such peptides.

c. *Equilibrium Constants*

Lack of general utility constitutes a criticism of the method employing the equilibrium constant. During the study of the reaction of amino acids, peptides, and glucose, it was found that the equilibrium constant of the reaction fell into narrow ranges of values, depending upon which amino acid bore the amino group (Frankel and Katchalsky, 1937, 1938; Katchalsky, 1941). These principles held for simpler amino acids such as glycine, alanine, and leucine, but in more complex peptides containing phenylalanine or glutamic acid, predictions were not borne out.

It is conceivable that the various physical chemical methods thus developed can however be refined to cover most or·all of the needs in this field.

3. CHEMICAL MEANS

a. *At Amino Terminus*

The largest number of chemical methods have operated at the locus of the free amino group, which possesses certain advantages. The chemistry of the amines is well-known and serves as good background information. Hydrolyzed condensation products would be expected to have free carboxyl groups or derivatives thereof, and such compounds may be induced to crystallize more readily than amines which have lower melting points.

The ideal agent should possess the following characteristics:

(1) It should be readily available.

(2) It should condense in a single reaction.

(3) It should condense under mild conditions.

(4) The condensation product should be unaffected by hydrolysis of the whole molecule. It is on this criterion, especially, that most of the reagents used have fallen short of the ideal.

(5) The terminal condensation product should be readily separable.

(6) The condensation product finally obtained should be easily identified (as by melting point).

(7) It should not react adversely with the amino acid side-chains in peptides and proteins.

(8) It should not react with any group other than the amino or carboxyl which is sought. The peptide enol should be left untouched.

The relative importance of each of these characteristics of course depends upon the purpose in view.

The first reagents to be applied were those which had been used to form characteristic acyl derivatives of amino acids alone. For much of the early work naphthalenesulfonation was employed. β-Naphthalenesulfonyl

chloride was frequently used by Fischer, Abderhalden, and their coworkers (Plimmer, 1913). The Abderhalden group were especially active in applying this reagent to the determination of amino acid sequence in peptides isolated from silk fibroin and other proteins (Synge, 1943). β-Naphthalenesulfonyl chloride and other reagents applicable to the amino group are listed below:

β-Naphthalenesulfonyl Chloride. The β-naphthalenesulfonyl group is coupled to the amino group in alkaline solution by the Schotten-Baumann technic. The resultant β-naphthalenesulfonyl peptide is

$$\text{(NaOH)} \longrightarrow$$

$-SO_2Cl + H_2N \cdot CHR \cdot CO \cdot NH-----$

$-SO_2 \cdot NH \cdot CHR \cdot CO- \mid NH*-----$
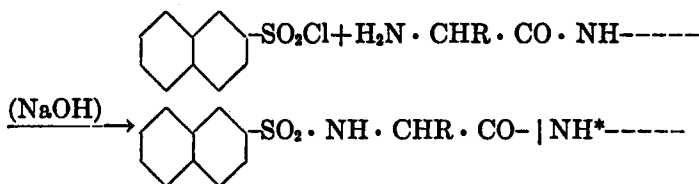
hydrolyzed more readily at the peptide linkage than at the sulfonylamide linkage. The β-naphthalenesulfonyl amino acid can generally be readily identified. The structures of glycylalanine and alanylglycyltyrosine in silk fibroin (Fischer and Bergell, 1903; Fischer and Abderhalden, 1907; Abderhalden, 1911a, 1911b) were thus demonstrated. The peptides were typically obtained after cold hydrolysis with 70 per cent sulfuric acid for several days.

The β-naphthalenesulfonyl chloride reagent must be used in excess if good yields are to be obtained. The linkage appears to be more resistant to hydrolysis than the peptide bond generally. β-Naphthalenesulfonyl glycine, however, can be hydrolyzed in three hours at 110° C. in concentrated hydrochloric acid solution (Fischer and Bergell, 1902).

Benzenesulfonyl Chloride. Benzenesulfonyl chloride behaves similarly to the naphthalene homolog.

$$C_6H_5 \cdot SO_2Cl + H_2N \cdot CHR \cdot CO \cdot NH-----$$

$$\text{(NaOH)} \longrightarrow \quad C_6H_5 \cdot SO_2 \cdot NH \cdot CHR \cdot CO- \mid NH-----$$
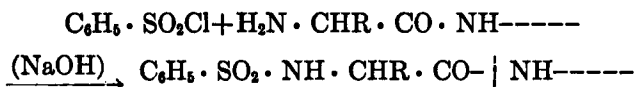
Gurin and Clarke (1934) have applied this reagent in an attempt to allocate the free amino groups of gelatin. It was possible by the hydrolysis of the benzenesulfonylgelatin to obtain ε-benzenesulfonyllysine in quantity. Hydrolysis was conducted in aqueous formic acid containing mineral acid. No derivatives of the α-amino group were isolated. This work emphasizes the necessity for accounting for ε-amino groups of the side-chains.

---

* The vertical line indicates the point of greatest hydrolyzability after the peptide is marked.

Benzoyl Chloride. Benzoyl chloride has been reacted with alanyltyrosine obtained from silk, by Abderhalden and Heyns (1931).

$$C_6H_5 \cdot COCl + H_2N \cdot CHR \cdot CO \cdot NH-----$$

$$\underline{(NaOH)}_{\longrightarrow} \ C_6H_5 \cdot CO \cdot NH \cdot CHR \cdot CO-| \ NH-----$$
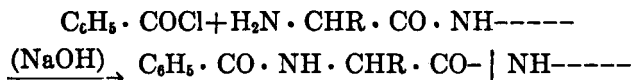
The phenolic hydroxyl was also benzoylated. After four hours at reflux temperature with 20 per cent sulfuric acid, there was isolated from the *N*-benzoylalanyl-*O*-benzoyltyrosine: *benzoylalanine*, tyrosine, and benzoic acid in 74, 82, and 91 per cent yields, respectively. Recoveries of this magnitude make it difficult to understand why the reaction has not been used more often, especially when one considers the smoothness of benzoylation, and the easy characterizability of benzamino acids. What the effect of this or any other acyl halide is in the Bamberger reaction of the imidazole moiety in proteins needs to be clarified (cf. Harington and Overhoff, 1933). Some qualitative data is however available for benzenesulfonyl chloride, in the work of Gurin and Clarke (1934).

The relative hydrolyzability of benzamide and peptide linkages has been studied (Goldschmidt and Füner, 1930).

Glycine Color Reagents. Reagents which react with glycine bound by its carboxyl group in peptide linkage have been reported. E. Abderhalden and Neumann (1936) employed *o*-phthalaldehyde. R. Abderhalden (1938) used alloxan and ninhydrin for the same purpose. This type of allocation has obviously limited value.

Nitrous Acid. The relative difficulty of obtaining crystalline hydroxy acids would probably interfere with the fullest utilization of nitrous acid:

$$HNO_2 + H_2N \cdot CHR \cdot CO \cdot NH------- \ \ \longrightarrow HO \cdot CHR \cdot CO \, | \, NH---$$
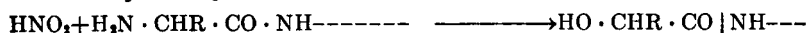
It has however been employed on glutathione (Quastel, Stewart, and Tunnicliffe, 1923). The use of nitrous acid in the van Slyke amino-nitrogen analysis of peptides is mentioned later.

Methylation Reagents. Both methyl sulfate (Zimmermann, 1935a, 1935b) and diazomethane (Abderhalden and Sickel, 1926) have been employed for the methylation of peptides. The latter authors used diazomethane for the methylation of leucylproline to *N*-dimethylleucylproline methyl ester. This was however not hydrolyzed.

Zimmermann methylated proteins

$$(CH_3)_2SO_4 + H_2N \cdot CH_2 \cdot CO \cdot NH \cdot CHR \cdot CO \cdot NH---$$

$$\longrightarrow \ H\bar{O}(CH_3)_3N^+ \cdot CH_2 \cdot CO- \, | \, NH---$$
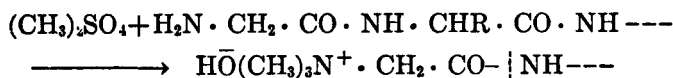
and showed that when betaine could be isolated, the terminal *amino* acid was glycine. If this device may be employed for glycine only, its utility is as limited as that of the color reactions mentioned above. The recoveries

of betaine were low and tediously acquired. In the case of glycylglycine, for instance, 0.67 g. of betaine hydrochloride was found when 3.6 g. were expected theoretically.

Glutathione properly gave no betaine. A peptide of proline and glycine (Abderhalden and Suzuki, 1923) was shown to be glycylproline. Peptone and "glutokyrin" (Siegfried and Schunke, 1916) were both shown to possess terminal glycine by this technic.

Nitrophenyl Chlorides. Attempts to use 2, 4-dinitrophenyl chloride by Abderhalden and Stix (1923) gave a mixture which could not be suitably fractionated after hydrolysis, even though the dinitrophenyl derivatives of amino acids could be readily characterized (Abderhalden and Blumberg, 1910).

$$O_2N-\langle\rangle-Cl+H_2N \cdot CHR \cdot COOH$$
$$NO_2$$

$$\longrightarrow \quad O_2N-\langle\rangle-NH \cdot CHR \cdot COOH$$
$$NO_2$$
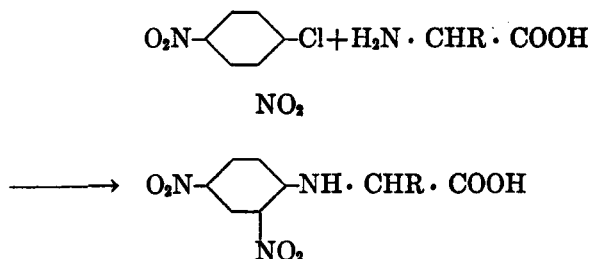
Picryl chloride has also been found to combine with amino acids (Hirayama, 1909) in low yields (Fox, 1941).

Hypobromites. Hypobromite reacts with the amino end of peptides to give different products depending upon whether an acid or alkaline medium is employed (Goldschmidt, Wiberg, Nagel, and Martin, 1927). In the former case the reactions involved are as follows:

$$R_1-CH(NH_2) \cdot CO \cdot NH \cdot CHR_2 \cdot COOH$$
$$\downarrow 2 \text{ HOBr}$$
$$R_1-CHNBr_2 \cdot CO \cdot NH \cdot CHR_2 \cdot COOH \text{ (unstable)}$$
$$\downarrow -HBr$$
$$R_1-C(=NBr) \cdot CO \cdot NH \cdot CHR_2 \cdot COOH$$
$$\downarrow +H_2O$$
$$R_1-CO \cdot CO \mid NH \cdot CHR_2 \cdot COOH$$
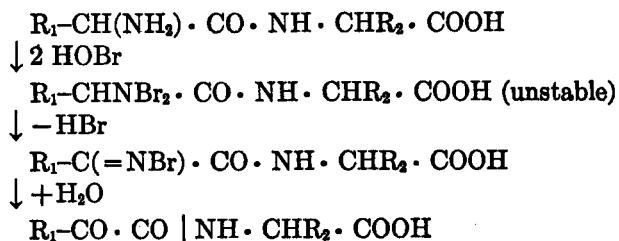
Upon hydrolysis, the keto acid is released, and it can be identified as the phenylhydrazone. From leucylglycine 93 per cent of the theoretical quantity of keto acid was isolated.

Alkaline hypobromite simultaneously hydrolyzes the peptide linkage and releases the nitrile corresponding to the terminal *amino* acid with one less carbon:

$$R_1\text{-CH(NH}_2) \cdot CO \cdot NH \cdot CHR_2 \cdot COOH$$
$$\downarrow \quad (NaOBr)$$
$$R_1\text{-CH}=NBr+HOOC \cdot NH \cdot CHR_2 \cdot COOH$$
$$\downarrow -HBr$$
$$R_1\text{-CN}$$
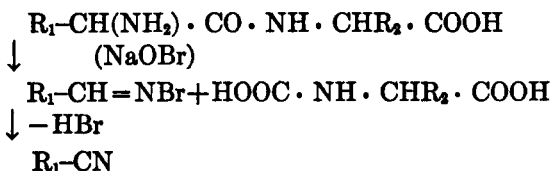
Secondary bromination products result from this reaction, however. From 200 g. of egg albumin it was possible by this reaction to obtain 2.5 g. of valeronitrile, and other products. The hypobromite reaction would thus seem not to be sufficiently delicate for purposes of allocation in large molecules.

The following peptides have, however, been successfully studied in this way (Goldschmidt and Strauss, 1929, 1930): alanylvalylglycine, alanyl-leucylglycine, alanylalanylleucine, dialanylglycine, trialanylglycine, valyl-alanylglycine, and leucylalanylvalylglycine. With tripeptides and tetra-peptides, dehydrohydantoins form from the peptide residue.

Aryl Isocyanates. Phenyl isocyanate (Bergmann, Miekeley, and Kann, 1927) has many of the desired properties of a suitable condensing agent. This reagent condenses at room temperature, or below, to yield hydantoins upon hydrolysis, and these are readily identified after generally easy separation. The reaction is schematically:

$$C_6H_5 \cdot N : CO+H_2N \cdot CHR \cdot COOH \longrightarrow C_6H_5 \cdot NH \cdot CO \cdot NH \cdot CHR \cdot COOH$$

phenylureide
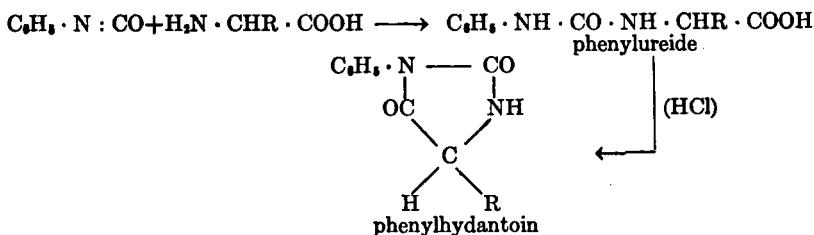
phenylhydantoin

With any marking reagent of promise, such as phenyl isocyanate, it is desirable to test the hydrolyzability of the derivative, under the conditions of hydrolysis of protein. For glycine phenylhydantoin the rate of hydrolysis in concentrated hydrochloric acid solution under reflux has been tested in this laboratory (Fox, 1944). The results are shown in the following table:

Time of Hydrolysis hours	Breakdown to One Free Amino Group per cent
2	18
4	25
6	33
8	38

It is evident that the group is opened by prolonged hydrolysis, although not nearly as rapidly as the peptide linkage.

An example of the utilization of isocyanates in determining terminal amino acids is found in the work of Jensen and Evans (1935). These authors condensed phenyl isocyanate with insulin. After acid hydrolysis of the coupled product, the phenylhydantoin of phenylalanine precipitated. This not only served to establish the terminal amino acid of a relatively pure protein, but provided the first evidence for the occurrence of phenylalanine in insulin. Naphthyl isocyanate proved to be similarly applicable.

The utilization of the phenyl isocyanate technic reached its fullest development in the hands of Abderhalden and Brockmann (1930). By hydrolysis of a phenyl isocyanate tripeptide in methanolic hydrochloric acid at 60–65° C. for one-half hour, these authors were able to isolate the phenylureido acid without marked hydrolysis of the remaining peptide linkage.

Alanylglycylleucine was thus converted to the phenylhydantoin of alanine, and glycylleucine. The residual peptide was then similarly treated to yield the glycine phenylhydantoin.

Under the hydrolytic conditions employed, leucylglycine was shown to be split to the extent of 6 per cent. This is of course enough to preclude the use of the reported method for large peptides.

Abderhalden and Brockmann were able to combine this method with another used to designate the amino *acid,* described on p. 169.

The conditions for condensation with isocyanates are favorable, and the derivatives obtained after hydrolysis are easily separated and characterized. The concomitant hydrolysis of peptide linkages more than one amino acid residue distant from the ureido linkage limits the utility of this relatively desirable technic as a stepwise procedure.

Trinitrotoluene. A relatively non-hydrolyzable derivative of the amino group was introduced by Barger and Tutin (1918). Although this agent has been successfully used to elucidate the structure of anserine, carnosine, and glutathione, other inadequacies of the procedure have perhaps prevented its being more widely used. One of these drawbacks is that some of the derivatives are not readily crystallizable, as found by Quastel and coworkers (1923) in attempting to determine the structure of glutathione. Similar experience has been obtained in this laboratory (Bollenback and Fox, 1943).

With any of the trinitrotoluenes in which two nitro groups are in ortho position, one will react with free amino groups on gentle heating, with the elimination of nitrous acid:

$$\downarrow CH_3 \cdot C_6H_2(NO_2)_3 + H_2N \cdot CHR \cdot COOH$$

$$CH_3 \cdot C_6H_2(NO_2)_2 - NH \cdot CHR \cdot COOH + HNO_2$$
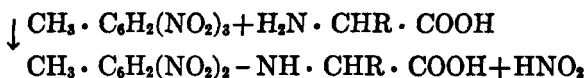
When this reaction is performed on a peptide, the dinitrotolyl peptide can be hydrolyzed without appreciable breakage of the linkage at the previously free amino group.

The possession of this critical property of non-hydrolyzability would seem to make the trinitrotoluene structure a logical chemical embarkation point in the elaboration of a simple and therefore generally useful reagent.

b. *At Carboxyl Terminus*

The following reagents have been employed for identifying the amino *acid:*

Benzylamine. Benzylamine is another reagent which has been introduced by Abderhalden (Abderhalden and Brockmann, 1930). The peptide is converted to an ester, which is heated in benzylamine suspension. There results a peptidebenzylamide:

$$H_2N \cdot CHR \cdot CO \cdot NH \cdot CHR_1 \cdot COOC_2H_5 + H_2N \cdot CH_2 \cdot C_6H_5$$

$$\rightarrow H_2N \cdot CHR \cdot CO- | NH \cdot CHR_1 \cdot CO \cdot NH \cdot CH_2 \cdot C_6H_5$$
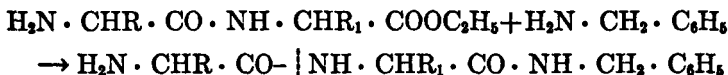
This product hydrolyzes far less readily than the true peptide linkage and crystalline compounds may be isolated.

β-Naphthol. This method was discovered by Lichtenstein, Hestrin, Dimant, and Brzoza (1938).

$$H_2N \cdot CHR \cdot CO \cdot NH \cdot CHR_1 \cdot CO \cdot NH \cdot CHR_2 \cdot COOH$$

$$\xrightarrow{\beta\text{-naphthol}} H_2N \cdot CHR_2 \cdot COOH + \quad \begin{array}{c} HN - CHR - CO \\ | \qquad\qquad | \\ OC - CHR_1 - NH \end{array}$$
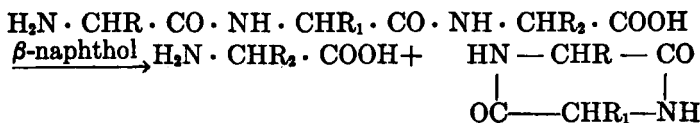
When tripeptides are heated in β-naphthol at 135–150° C., the amino acid bearing the free carboxyl is liberated, while the residue condenses to a substituted diketopiperazine. The two may be separated readily by solution of the ring compound in alcohol. With leucylglycylglycylleucine only the glycine-leucine anhydride was recovered. This method may be applicable only to tripeptides, therefore, partly because of the expected deleterious effect of such high temperatures on larger compounds.

Thiohydantoin Reagents. This method bears certain similarities to the isocyanate procedure, but works on the acid end. Schlack and Kumpf (1926) have applied it to benzoylleucylglycine and benzoyltriglycine:

Benzoylleucylglycine+NH$_4$NCS +(CH$_3$CO)$_2$O

↓

C$_6$H$_5$ · CO · NH·CH(C$_4$H$_9$)-CO-N——CH$_2$

$\qquad\qquad\qquad\qquad\qquad$ SC$\qquad$CO

$\qquad\qquad\qquad\qquad\qquad\qquad$ N

$\qquad\qquad\qquad\qquad\qquad\qquad$ H

↓ (cold NaOH solution)

C$_6$H$_5$ · CO · NH · CH(C$_4$H$_9$) · COOH+thiohydantoin
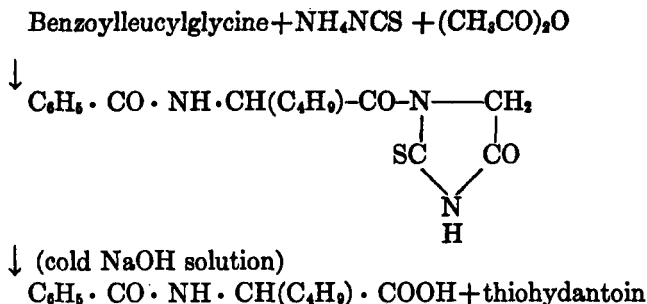
The particular thiohydantoin obtained corresponds to the original amino acid. This method has been successfully used on glutathione. One may question its utility on whole protein because of the vigorous reagents employed and the likelihood of their attack upon the large molecule. The free amino group, for instance, must be protected, as with a benzoyl group above. This degradation seems however to deserve further study.

Grignard Reaction. The Grignard reaction has proved useful in identifying amino acid residues in amounts of less than 100 mg. It seems therefore to be applicable to semimicro quantities, a property which is desirable in any reagent. Bettzieche (1926) and Bettzieche and Menger (1926) have developed this reaction, in which the terminal amino acid may be related to the ketone obtained:

↓ H$_2$N · CH$_2$ · CO · NH · CH(CH$_3$) · COOC$_2$H$_5$+2 C$_6$H$_5$ · MgBr

H$_2$N · CH$_2$· CO · NH· CH(CH$_3$) – C(OMgBr)(C$_6$H$_5$)$_2$ (+C$_2$H$_5$ · OMgBr)

↓ $\quad$ H$_2$O

H$_2$N · CH$_2$ · CO · NH · CH(CH$_3$) – C(OH)(C$_6$H$_5$)$_2$ (+MgOHBr)

↓ $\quad$ H$_2$O

H$_2$N · CH$_2$ · COOH+NH$_3$+CH$_3$ · CO · CH(C$_6$H$_5$)$_2$
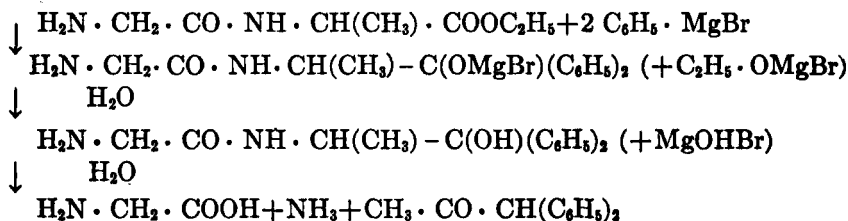
In the case of glycylalanine, illustrated by the reaction above, a 52 per cent yield of the diphenylacetone was recovered.

This method, also, has been applied to glutathione.

Bergmann-Zervas Degradation. The most elegant method for identifying terminal amino acids which has appeared is undoubtedly the Bergmann-Zervas degradation (Bergmann and Zervas, 1936) which relies heavily upon Curtius reactions. It has been used successfully in degrading a tetrapeptide, glycylalanylleucylglutamic acid. The peptide ester was benzoylated for protection, and converted through the hydrazide and azide to the benzylurethane, and then hydrogenated to give an aldehyde with one less carbon than the terminal amino *acid*. This left the benzoyl-

peptideamide as residue and the process was repeated. The process is obviously not simple and degradation has not been carried through more peptide links than the corresponding Bergmann-Zervas synthesis (Bergmann and Zervas, 1932), perhaps for similar reasons (Pacsu and Wilson, 1942).

The sequence of reactions can be represented schematically by the degradation of N-benzoyl-l-leucine methyl ester:

$$C_6H_5 \cdot CO \cdot NH \cdot CH(C_4H_9) \cdot COOCH_3$$
$$\downarrow \quad NH_2-NH_2$$
$$C_6H_5 \cdot CO \cdot NH \cdot CH(C_4H_9) \cdot CO \cdot NH \cdot NH_2$$
$$\downarrow \quad HNO_2$$
$$C_6H_5 \cdot CO \cdot NH \cdot CH(C_4H_9) \cdot CON_3$$
$$\downarrow \quad +C_6H_5 \cdot CH_2 \cdot OH$$
$$C_6H_5 \cdot CO \cdot NH \cdot CH(C_4H_9) \cdot NH \cdot COO \cdot CH_2 \cdot C_6H_5$$
$$\downarrow \quad H_2(Pd)$$
$$C_6H_5 \cdot CO \cdot NH_2+C_4H_9 \cdot CHO+NH_3+CO_2+CH_3 \cdot C_6H_5$$
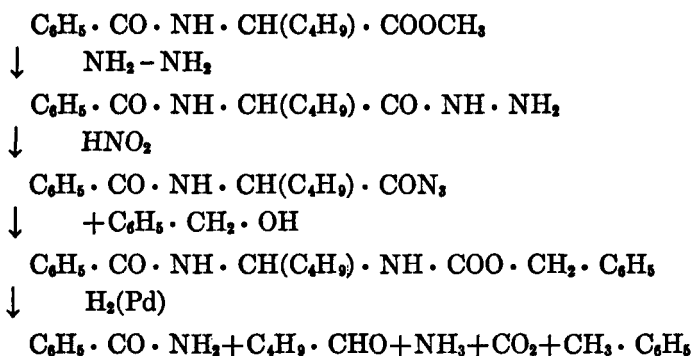
The yield of aldehyde in this instance was approximately 10 per cent of the starting compound.

4. OTHER METHODS

a. Comparison with Synthetic Peptides

In a systematic investigation of isolated peptides it is now practical, as it was not during the period in which most terminal amino acids were identified, to compare the isolated product with a synthetic material. This is possible because of the development of the carbobenzoxy synthesis (Bergmann and Zervas, 1932), which combines l-amino acids, and is not restricted to the simplest amino acids.

The principle of comparison with synthetic materials has however been applied. One example of this is the finding of glycylphenylalanine in intestinal contents by Abderhalden (1912).

b. Van Slyke Analysis

The van Slyke reaction has been used for determining the amino acid sequence in a hydroxyproline-arginine dipeptide isolated from clupein by Dirr and Felix (1932). This study required determining only the presence or absence of amino N. It should however be expedient to determine the terminal amino acid of small peptides by nitrous acid analysis. Correc-

tions for some hydrolysis would probably be necessary (Lieben and Loo, 1942).

Prior to such determination it is of course necessary to identify the amino acid components of the isolated peptide. The new amino acid bacterial assay technics (Shankman, Dunn, and Rubin, 1943), could be applied for systematic microdetermination and identification.

IV. Applications of Methods for Identifying Terminal Amino Acids

1. GLUTATHIONE

The most thorough test of the methods developed prior to 1929 proved to be the elucidation of the structure of glutathione. As in other structural studies, special methods also figured, but quite a number of the procedures alluded to in the preceding text were given the test of practical utility.

A special bit of information was the finding that glutathione could be hydrolyzed to a dipeptide containing cysteine and glycine in aqueous solution at 62° C. (Kendall, Mason, and McKenzie, 1930b). This was condensed with 2, 3, 4-trinitrotoluene, which product on hydrolysis yielded free glycine.

The terminal glutamic acid component of glutathione with the free a-amino group was first demonstrated by Quastel, Stewart, and Tunnicliffe (1923). This was done by condensing 2, 3, 4-trinitrotoluene with the glutamic acid-cysteine moiety of glutathione and hydrolyzing the product. The dinitrotolyl derivative of glutamic acid could not be obtained in crystalline form, but cystine was isolated, indicating it was the original amino *acid* of the dipeptide.

Some of the same peptide was treated with nitrous acid, and from 0.5 g. of material, 0.1 g. of a-hydroxyglutaric acid was isolated.

Glutathione is an anomalous peptide in that it has both an a-amino and a-carboxyl of the same amino acid free. This was confirmed by oxidation with hydrogen peroxide, leading to the expected succinic acid after hydrolysis.

Since Quastel and coworkers had been working with only a part of the glutathione molecule, it remained for others to confirm and extend the amino acid sequence in the natural peptide. Kendall, McKenzie, and Mason (1929) repeated the nitrous acid treatment, hydrolyzed, and obtained cystine and glycine only.

These same workers (1930a) used the Bettzieche-Grignard method to indicate a terminal glycine residue.

Nicolet (1930a, b, c) employed the Schlack and Kumpf thiohydantoin procedure to designate the terminal carboxyl as belonging to glycine.

This was also confirmed by the use of carboxypeptidase (Grassmann, Dyckerhoff, and Eibeler, 1930).

All of the evidence was substantiated by the synthesis of glutathione by Harington and Mead (1935), and comparison of properties with the natural γ-glutamylcysteylglycine.

2. ANSERINE AND CARNOSINE

The sequence of amino acids in these peptides (Fox, 1943) has be n determined by the trinitrotoluene procedure (Barger and Tutin, 1918; Keil, 1930). A crystalline derivative of β-alanine was isolated in each case. A characterized derivative of histidine was also reported, for comparative purposes. Barger and Tutin also reported the isolation of free histidine from the hydrolysis of dinitrotolylcarnosine.

3. PROTEIN HYDROLYTIC PRODUCTS

This subject has been covered in the review by Synge (1943). Elsewhere in the present review instances of the identification of amino acid sequences in proteins such as clupein, gelatin, insulin, and fibroin have been alluded to. The methods of partial hydrolysis of proteins need to be developed much further. To obtain peptides one may employ such devices as treatment with cold concentrated acids (hydrochloric and sulfuric) for periods of several days, or treatment with dilute acids at elevated temperatures for lesser times. A rather elegant study of the conditions of hydrolysis is to be found in the publication of Felix, Hirohata, and Dirr (1933). At this time, however, the potentialities for finely controlled fissions would appear to be the greatest for enzymically catalyzed hydrolysis. The conceivability of resynthesis is here again a source of considerable doubt (cf. p. 161).

The separation of peptides resulting from hydrolysis of proteins should be greatly improved by the further application of such tools as diffusion, adsorption (Felix and Lang, 1929), electrophoresis (Albanese, 1940), and liquid-liquid extraction (Martin and Synge, 1941). It is noteworthy that Felix was able to separate hydrolytic products of clupein as flavianates rather than as peptides.

V. CONCLUSION

The value of any individual method for identification of a terminal amino acid depends upon the purpose to which it is to be put and the type of material to be studied. These latter can probably best be divided into the small peptides, the large peptides, and the proteins.

Most of the methods reviewed above should be adequate for identifying dipeptides. It is of interest that in the establishment of amino acid sequence

in the natural dipeptides anserine and carnosine, and in the natural tri-peptide glutathione the trinitrotoluene condensation proved of key value for all three peptides. The possible utility of trinitrotoluene in sequence studies in protein hydrolytic products appears to have been neglected.

Need for data of the effect of the various chemical reagents on functional groups other than a-carboxyl and a-amino exists. A maximum of difficulty might be expected from ε-amino groups reacting simultaneously in the same way as the a-amino groups. Such an effect was found in benzene-sulfonation of gelatin (Gurin and Clarke, 1934). Clutton, Harington, and Mead have demonstrated that selective suppression of the reactivity of the ε-amino groups can, however, be brought about by pH control. Such control was accomplished in the reaction of gelatin with O-β-glucosido-N-carbobenzyloxytyrosyl azide.

It is reasonable to suppose that judicious choice of conditions will provide the necessary control with marking reactants which condense with the a-amino group.

In the amino *acid* group of reagents the Bergmann-Zervas degradation has been shown experimentally to offer no unusual difficulty even though an ω-carboxyl of glutamic acid existed free in the tetrapeptide which was degraded. Inspection of the reactions of the Schlack-Kumpf procedure reveals that the combined a-amino group is essential for the thiohydantoin formation. It has thus been demonstrated that difficulties arising from reactions with non-a-amino groups or non-a-carboxyl groups are not serious.

The study of terminal amino acids alone, in protein, would appear to have a working basis in procedures already developed. For the problem of amino acid sequence, the protein chemist has a number of large natural peptides on which described methods and others to be developed can be tested and strengthened. These peptides would include gramicidin (Hotch-kiss, 1934), secretin (Hammersten, Jorpes, and Agren, 1933), protease inhibitors (Northrop, 1934-35), and allergenic compounds (Abramson, Moore, and Gettner, 1942).

A complete structure has been suggested for the protamine clupein (Felix and Mager, 1937). This work employed more definitely pure mate-rial than that which had been used by Waldschmidt-Leitz (1935). The structure offered relied upon the Bergmann-Niemann hypothesis, which the careful analytical work of Chibnall (1942) has shown to be yet unworthy of acceptance as a theory. The presence of amino acid-diarginyl sequences in clupein was demonstrated almost four decades ago (Kossel and Pringle, 1906). Felix, Hirohata, and Dirr (1936) were able to isolate a small amount of triarginylarginine from a carefully controlled clupein hydrolyzate. Clupein is thus an excellent material for further study, although the high

proportion of arginine (88–89 per cent) makes this an atypical substance for the general study of amino acid sequence in proteins.

To unravel the amino acid sequence of the peptides and the proteins, the terminal amino acid would seem indeed to be a logical point at which to begin.

REFERENCES

Abderhalden, E. (1911a). *Z. physiol. Chem.* **72**, 1.

Abderhalden, E. (1911b). *Z. physiol. Chem.* **72**, 13.

Abderhalden, E. (1912). *Z. physiol. Chem.* **81**, 315.

Abderhalden, E., and Abderhalden, R. (1938). *Fermentforschung* **16**, 48.

Abderhalden, E., and Blumberg, P. (1910). *Z. physiol. Chem.* **65**, 318.

Abderhalden, E., and Brockmann, H. (1930). *Biochem. Z.* **225**, 386.

Abderhalden, E., and Heyns (1931). *Z. physiol. Chem.* **202**, 37.

Abderhalden, E., and Neumann, A. (1936). *Z. physiol. Chem.* **238**, 177.

Abderhalden, E., and Sickel, H. (1926). *Z. physiol. Chem.* **159**, 163.

Abderhalden, E., and Stix, W. (1923). *Z. physiol. Chem.* **129**, 143.

Abderhalden, E., and Suzuki, H. (1923). *Z. physiol. Chem.* **127**, 281.

Abderhalden, R. (1938). *Z. physiol. Chem.* **252**, 81.

Abramson, H. A., Moore, D. H., and Gettner, H. H. (1942). *J. Phys. Chem.* **46**, 192.

Albanese, A. A. (1940). *J. Biol. Chem.* **134**, 467.

Anson, M. L. (1937). *J. Gen. Physiol.* **20**, 663.

Barger, G., and Tutin, F. (1918). *Biochem. J.* **12**, 402.

Behrens, O. K., and Bergmann, M. (1939). *J. Biol. Chem.* **129**, 587.

Bergmann, M., and Niemann, C. (1937). *J. Biol. Chem.* **118**, 301.

Bergmann, M., and Niemann, C. (1938). *J. Biol. Chem.* **122**, 577.

Bergmann, M., Miekeley, A., and Kann, E. (1927). *Ann.* **458**, 56.

Bergmann, M., and Zervas, L. (1932). *Ber.* **65**, 1192.

Bergmann, M., and Zervas, L. (1936). *J. Biol. Chem.* **113**, 341.

Bettzieche, F. (1926). *Z. physiol. Chem.* **161**, 178.

Bettzieche, F., and Menger, R. (1926). *Z. physiol. Chem.* **161**, 37.

Bollenback, G. N., and Fox, S. W. (1943). Unpublished experiments.

Cahill, W. M., and Burton, L. F. (1940). *J. Biol. Chem.* **132**, 161.

Chibnall, A. C. (1942). *Proc. Roy. Soc. (London)* B, **131**, 136.

Clutton, R. F., Harington, C. R., and Mead, T. H. (1937). *Biochem. J.* **31**, 764.

Cohn, E. J. (1939). *Chem. Revs.* **24**, 203.

Cohn, E. J., and Edsall, J. T. (1943). Proteins, Amino Acids, and Peptides, New York.

Dakin, H. D. (1912). *J. Biol. Chem.* **13**, 357.

Dakin, H. D., and Dale, H. H. (1919). *Biochem. J.* **13**, 248.

Dakin, H. D. and Dudley, H. W. (1913). *J. Biol. Chem.* **15**, 263.

Dirr, K., and Felix, K. (1932). *Z. physiol. Chem.* **209**, 5.

Felix, K., Hirohata, R., and Dirr, K. (1933). *Z. physiol. Chem.* **218**, 269.

Felix, K., and Lang, A. (1929). *Z. physiol. Chem.* **182**, 125.

Felix, K., and Mager, A. (1937). *Z. physiol. Chem.* **249**, 111.

Fischer, E. (1903). *Ber.* **36**, 2094.

Fischer, E., and Abderhalden, E. (1907). *Ber.* **40**, 3544.

Fischer, E., and Bergell, P. (1902). *Ber.* **35**, 3779.

Fischer, E., and Bergell, P. (1903). *Ber.* **36**, 2592.

Fox, S. W. (1941). Unpublished experiments.

Fox, S. W. (1943). *Chem. Revs.* **32**, 58.

Fox, S. W. (1944). Unpublished experiments.

Frankel, M., and Katchalsky, A. (1937). *Biochem. J.* **31**, 1595.

Frankel, M., and Katchalsky, A. (1938). *Biochem. J.* **32**, 1904.

Fruton, J. S. (1941). *Cold Spring Harbor Symposia Quant. Biol.* **9**, 211.

Goldschmidt, S., and Füner, W. (1930). *Ann.* **483**, 190.

Goldschmidt, S., and Strauss, K. (1929). *Ann.* **471**, 1.

Goldschmidt, S., and Strauss, K. (1930). *Ber.* **63**, 1218.

Goldschmidt, S., Wiberg, E., Nagel, F., and Martin, K. (1927). *Ann.* **456**, 1.

Grassmann, W., Dyckerhoff, H., and Eibeler, H. (1930). *Z. physiol. Chem.* **189**, 112.

Gurin, S., and Clarke, H. T. (1934). *J. Biol. Chem.* **107**, 395.

Hammersten, E., Jorpes, E., and Ågren, G. (1933). *Biochem. Z.* **264**, 272.

Harington, C. R. (1940). *J. Chem. Soc.* **1940**, 119.

Harington, C. R., and Mead, T. H. (1935). *Biochem. J.* **29**, 1602.

Harington, C. R., and Overhoff, J. (1933). *Biochem. J.* **27**, 338.

Harris, L. J. (1923–24). *Proc. Roy. Soc. (London),* B, **95**, 440.

Haworth, W. N., Hirst, E. L., and Isherwood, F. A. (1937). *J. Chem. Soc.* **1937**, 577.

Hirayama, K. (1909). *Z. physiol. Chem.* **59**, 290.

Hofmann, K., and Bergmann, M. (1940). *J. Biol. Chem.* **134**, 225.

Hopkins, S. J., and Wormall, J. (1934). *Biochem. J.* **28**, 235.

Hotchkiss, R. D. (1944). *Advances in Enzymol.* **4**, 166.

Jensen, H., and Evans, E. A., Jr. (1935). *J. Biol. Chem.* **108**, 1.

Katchalsky, A. (1941). *Biochem. J.* **35**, 1024.

Keil, W. (1930). *Z. physiol. Chem.* **187**, 1.

Kendall, E. C., McKenzie, B. F., and Mason, H. L. (1929). *J. Biol. Chem.* **84**, 657.

Kendall, E. C., Mason, H. L., and McKenzie, B. F. (1930a). *J. Biol. Chem.* **87**, 55.

Kendall, E. C., Mason, H. L., and McKenzie, B. F. (1930b). *J. Biol. Chem.* **88**, 409.

Kossel, A., and Pringle, H. (1906). *Z. physiol. Chem.* **49**, 301.

Lichtenstein, N., Hestrin, S., Dimant, E., and Brzoza, H. (1938). *J. Am. Chem. Soc.* **60**, 560.

Lieben, F., and Loo, Y. C. (1942). *J. Biol. Chem.* **145**, 223.

Linderström-Lang, K. (1929a). *Z. physiol. Chem.* **182**, 151.

Linderström-Lang, K. (1929b). *Z. physiol. Chem.* **188**, 48.

Linderström-Lang, K. (1933). *Ergeb. Physiol.* **35**, 415.

Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35**, 91.

Maschmann, E. (1941). *Naturwissenschaften* **29**, 370.

Meyer, K. H. (1942). High Polymers, IV, 87. New York.

Mirsky, A. E., and Pauling, L. (1936). *Proc. Natl. Acad. Sci.* **22**, 439.

Nicolet, B. H. (1930a). *J. Biol. Chem.* **88**, 389.

Nicolet, B. H. (1930b). *J. Biol. Chem.* **88**, 395.

Nicolet, B. H. (1930c). *J. Biol. Chem.* **88**, 403.

Northrop, J. H. (1934–35). *Harvey Lectures* **1934–35**, 229.

Pacsu, E., and Wilson, E. J., Jr. (1942). *J. Org. Chem.* **7**, 117.

Pirie, N. W., and Pinhey, K. G. (1929). *J. Biol. Chem.* **84**, 321.

Plimmer, R. H. A. (1913). Chemical Constitution of the Proteins. II, 80. New York.

Quastel, J. H., Stewart, C. P., and Tunnicliffe, H. E. (1923). *Biochem. J.* **17**, 586.

Schlack, P., and Kumpf, W. (1926). *Z. physiol. Chem.* **154**, 125.

Shankman, S., Dunn, M. S., and Rubin, L. B. (1943). *J. Biol. Chem.* **141**, 163.

Siegfried, M., and Schunke, W. (1916). *Z. physiol. Chem.* **97**, 233.

Simms, H. S. (1927–28). *J. Gen. Physiol.* 11, 629.

Staudinger, H., and Eilers, H. (1936). *Ber.* 69, 819.

Synge, R. L. M. (1943). *Chem. Revs.* 32, 135.

Waldschmidt-Leitz, E. (1935). *Monatsh.* 66, 357.

Waldschmidt-Leitz, E., Ziegler, F., Schäffner, A., and Weil, L. (1931). *Z. physiol. Chem.* 197, 219.

Zimmermann, W. (1935a). *Z. physiol. Chem.* 231, 19.

Zimmermann, W. (1935b). *Z. physiol. Chem.* 231, 25.

The Copper Proteins

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CONTENTS

I. INTRODUCTION

Although the element copper has been known to man since earliest times, its presence in plant and animal tissues was not demonstrated until a little over a century ago. Bucholz (1816) and Meissner (1817) appear to have been the first to report the presence of small amounts of copper in

plants, and a few years later Boutigny (1833) reported its presence in animal tissues. These early studies stimulated a new interest in the element copper, and many workers since that time have concerned themselves with investigating many different plant and animal tissues for the presence and biological function of the element.

No attempt will be made in this review to trace in detail the historical development of research in this field during the past century, for this has been done elsewhere (Lehmann, 1895; Dhéré, 1915; Redfield, 1934; Elvehjem, 1935; Rawlinson, 1943). It should be pointed out, however, that for a long time most workers considered that the copper present in biological material was merely accidental and had no definite function. This undoubtedly was due largely to the fact that the early methods of analysis in many cases were faulty and not sufficiently sensitive. The amounts of copper involved were often very small. As a result much conflicting and erroneous copper data came into the literature. It was not possible to establish a definite correlation between copper content and biological function until these limitations in the methods of analysis had been overcome. Thus, although Lehmann (1895) reviewed and summarized the known facts regarding the distribution of copper, it was not until 1920 that the universal distribution of copper in plant life was established without question. This came about largely as the result of the work of Maquenne and Demoussy (1920), Guerithault (1920) and Fleurent and Levi (1920). They found from 3 to 40 parts per million of copper in all plant materials which they analyzed and suggested that copper must be an essential element in plant metabolism. Most of the copper was found in the more active parts of the plants, such as young shoots and leaves, and it was therefore concluded that copper in some way had a role in the life processes of plants. Guerithault (1920) and Bertrand (1920) suggested that the role was of catalytic nature.

In considering the position of copper in the animal kingdom it is necessary to distinguish the situation in marine animals, such as the snail, octopus, etc., from that in higher animals. This distinction is advisable because the nature of the problem and the development of research has been quite different in the two cases. Whereas there has long been a great interest in the copper content of the blood of marine animals, it is only comparatively recently that much serious attention has been given to the copper content of higher animal tissues and blood. Although Devergie and Hervy (1840) demonstrated its presence in the tissues of man over a century ago, and a few years later Deschamps (1848) found copper in human blood, the amounts involved were very small and did not create much interest. Subsequent workers were inclined to believe that the small amounts of copper were of accidental origin, and it was not until about 1921

that copper was accepted as a definite constituent of all animal tissues.

A marked revival of interest in the copper content of higher animal tissues has developed during the past two decades as the result of studies on nutritional anemia. These studies, initiated in 1924 in the laboratories of the University of Wisconsin, have revealed that copper plays an important role in the formation of hemoglobin. Although the mechanism of this copper function is not yet clear, it appears that copper does not increase the absorption of ingested iron, but rather functions in the conversion of iron into hemoglobin and a series of such physiologically active compounds after the iron has been absorbed. These findings have led naturally to a close examination of the distribution of copper in different animal tissues, in plants, milk and other foods, to the effect of copper on iron storage and utilization, to following changes in the copper content of blood, and to studying the relationship of copper to certain enzyme systems. The work in this field has been reviewed by Elvehjem (1935) and more recently by Schultze (1940).

As previously inferred, the development of interest and research on the copper content of the blood of marine animals came about much earlier than in the case of higher animals. This was most likely due to the fact that relatively large amounts of copper were found in the blood of marine animals, and furthermore a correlation between copper content and function was evident very early. Likewise there was no question of the copper being of accidental origin, for its concentration in the blood of certain marine animals was found to be many times that in sea water. The pioneer observations in this field were made by Harless (1847) when he detected copper in the blue blood of snails and showed that the copper did not exist as a free salt but in combination with the blood proteins. Marine animals and invertebrates possessing red blood were found to contain much smaller amounts of copper. Bert (1867) observed that *Sepia* blood on exposure to air turned from colorless to a deep blue, and he suggested therefore that the blue component of the blood of molluscs and crustaceans should be considered as a definite respiratory pigment. This suggestion was firmly established by Fredericq (1878) when he isolated the copper-containing protein of octopus (*Octopus vulgaris*) blood and showed that it behaved with oxygen in a manner similar to that of the respiratory pigment hemoglobin. He gave the copper-containing protein the name hemocyanin and called its blue, easily dissociable compound with oxygen, oxyhemocyanin. These observations by Fredericq caused great interest in the hemocyanins; hence many investigators turned their attention to the respiratory function, copper content, and chemical nature of hemocyanins from various species of marine animals.

In quite recent years this interest in copper-containing proteins has been

greatly enhanced by the high degree of purification of several new copper proteins from both plant and higher animal sources. These include three enzymes having oxidase function, *i.e.*, tyrosinase (polyphenol oxidase), laccase, and ascorbic acid oxidase, all of plant origin, and several copper proteins of yet unestablished function which have been purified from higher animal tissues and fluids, *i.e.*, hemocuprein, hepatocuprein, milk copper protein, etc. It is the purpose of this review to present the recent work on hemocyanins and the copper proteins mentioned above.

II. THE HEMOCYANINS

For a complete presentation of our knowledge of hemocyanins as of ten years ago, the reader is referred to the review by Redfield (1934). Since that time extensive contributions have modified and extended our concept of the nature of these complex proteins. But before reviewing this later work in some detail, it is advisable to recall the following rather well established facts.

The hemocyanins are proteins occurring in solution in the blood of certain invertebrates, including species of molluscs and arthropods. They are conjugated proteins with copper as an essential part of the molecule, and have the property of forming a loose compound with molecular oxygen which dissociates at lower oxygen tensions. Thus the hemocyanins function in the transport of oxygen by the blood in a manner similar to the hemoglobins. The oxygenated compound (oxyhemocyanin) is blue, and when the oxygen has been removed the hemocyanin is colorless or perhaps slightly yellow.

Although hemocyanins have been found only in the blood and body fluids of invertebrates, such as crabs, snails, lobsters, octopi, scorpions, etc., it must not be inferred that the hemocyanins are the respiratory pigments peculiar to animals of inferior organization. It is well known that certain worms, which are placed lower on the evolutionary scale than the above animals, have as respiratory pigments iron-containing proteins such as hemoglobin, chlorocruorin, and hemerythrin. Furthermore, related species may have hemoglobin as the blood respiratory pigment in one case and hemocyanin in the other. In addition, the environment of the organism does not appear to control the type of pigment, since animals of both categories may live side by side. Likewise it is recognized that the hemocyanin blood of certain invertebrates has a higher capacity for transporting oxygen than the blood of some invertebrates containing hemoglobin. However, the majority of the hemoglobin bloods have a greater oxygen capacity than any of the bloods containing hemocyanin. The fact that the oxygen capacity of the blood of animals containing hemocyanin is on the average considerably below that of animals provided with hemoglobin appears

to be due primarily to the following fact. Hemoglobin is circulated in suspended cells or corpuscles whereas the hemocyanin is dissolved in the blood.[1] For this reason factors such as solubility and viscosity which control the effective concentration of the hemocyanin in the blood are not as important factors in hemoglobin blood.

Because of the similar functions of the two respiratory pigments, there has been a strong tendency to use the same methods of approach in research with the hemocyanins as proved successful with the hemoglobins. Such studies, however, have revealed certain striking differences between the two classes of pigments. Whereas the hemoglobins from different sources are always similar in molecular size,[1] a great variation from species to species is observed with the hemocyanins. The molecular weights vary from 350,000 for lobster hemocyanin to over 5,000,000 for snail hemocyanin. Furthermore, evolutionary developments affecting the respiratory process in hemoglobin and hemocyanin animals have been along different lines. In the case of animals containing hemoglobin blood, evolution has resulted in the development of a great variety of respiratory organs for these more active animals rather than in modification of the respiratory pigment. With animals containing hemocyanin blood, however, the reverse appears to be true.

It has recently been reported (Ball and Meyerhof, 1940) that animals provided with hemocyanin as the blood respiratory pigment also contain small amounts of iron porphyrin compounds. This fact suggests that the complete respiratory chain resembles that of the mammals except that hemocyanin replaces hemoglobin. The species investigated were *Limulus polyphemus, Busycon canaliculatum, Homarus americanus,* and *Loligo pealei* of which all showed the presence in various parts of the animals of one or more of myoglobin, cytochrome oxidase, the cytochromes, and succinic dehydrogenase. In addition it has been reported (Bing, 1938) that mammalian organs perfused in the Lindberg apparatus with an artificial fluid containing hemocyanin as the respiratory pigment, were able to reduce oxyhemocyanin rapidly. This demonstration at least supports the conclusion that hemocyanin is capable of transferring oxygen to iron-porphyrin derivatives farther along the respiratory chain. It has also been suggested by Ranzi (1938) that hemocyanin is not present during the early growth stages of the embyro of *Sepia officinalis,* but that copper is absorbed from sea water, and the hemocyanin is formed between Stages X and XVI in the development of the embryo.

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[1] See the discussion of the molecular weight data for the endocellular and plasma pigments in the subsection on Molecular Dimensions (page 196).

1. *Preparation*

The occurrence of hemocyanin has been observed in the blood of a number of invertebrates mostly classified as *Cephalopoda*, *Gastropoda*, *Crustacea*, and *Xiphosura*. This classification includes various species of crabs, lobsters, snails, squid, and octopi. In addition, hemocyanin definitely occurs in certain scorpions (Svedberg and Hedenius, 1933) and may be present in some spiders (Wilson 1901). The quahog (*Venus mercenaria*) is a doubtful source (Ball and Meyerhof, 1940). For a list of animals known to contain hemocyanin the reader is referred to Table III.

Because the hemocyanins are among the most easily crystallized proteins, they have often been obtained in crystalline form. This ease of crystallization appears to be due in part to the fact that they occur in fairly high concentration in the native blood, accompanied by moderately small amounts of other materials. Furthermore, Rawlinson (1943) suggests that the direct attachment of the heavy metal to the protein moiety of the hemocyanin molecule exerts forces tending to increase the ease of crystallization.

Many of the methods applied to the purification of other proteins have been employed in the isolation of hemocyanins. Included among the most generally useful methods are salt precipitation, dilution, dialysis, electrodialysis, pH shift, and occasionally ultracentrifugation. The early work on crystallization was reviewed by Dhéré (1919), who has provided details and historical information. The first hemocyanin to be crystallized was that from octopus. Henze (1901) added acetic acid to a saturated solution of the hemocyanin in strong ammonium sulfate, whereupon the hemocyanin became less soluble and crystallized. The hemocyanin of *Loligo* (squid) was crystallized in the same way. Octopus hemocyanin has also been crystallized by repeated ammonium sulfate precipitation and dialysis (Kubowitz, 1938). It has been observed by Montgomery (1930) that elevation of the temperature of the saturated hemocyanin solution also decreases the solubility. He pointed out that a combination of slightly increased temperature plus the addition of acetic acid gave maximum yields of crystalline hemocyanin without degradation of the protein. The hemocyanin of *Helix* (snail) is insoluble in the absence of electrolytes, and has been crystallized by prolonged dialysis. The crystallization may be brought about more rapidly by electrodialysis during which the crystals deposit at the positive pole. Recrystallization may be carried out by saturating a $N/500$ sodium sulfate solution with the hemocyanin, and on standing an abundant crystallization takes place (Redfield, 1934). The addition of papain to filtered *Helix* blood or to alkaline solutions of the hemocyanin results (Dhéré and Baumeler, 1929) in crystal formation after standing twenty-four hours. The possibility of modification of the hemocyanin by

this treatment has not been definitely excluded (Philippi and Hernler, 1930).

The hemocyanin of *Palinurus* has been obtained in crytsalline form by precipitating it by electrodialysis and then taking up the amorphous solid in just enough $N/5$ sodium chloride solution to dissolve it. On standing, crystals are formed (Redfield, 1934). Crystalline hemocyanin from the species *Jasus lalandii* (Australian crayfish) was obtained (Rawlinson, 1940) by dialyzing against cold water. Solutions of the hemocyanin of *Viviparus malleatus* (snail) yielded crystals (Cohen, 1942) on the addition of heparin. *Busycon* hemocyanin was precipitated, while the hemocyanin of *Limulus* was apparently unaffected by the addition of heparin. The pigments of other species have been crystallized in similar ways, but in some cases crystallization has not yet been accomplished. The hemocyanin of *Busycon canaliculatum* is an outstanding example of those not yet crystallized.

The crystal system to which a given hemocyanin belongs is uncertain, for different methods of preparation result in different crystalline forms. Since it is now recognized that crystalline form is not a good criterion of purity, the earlier emphasis on the crystallization of proteins has somewhat abated, and purification without the additional step of crystallization is now often employed for obtaining pure hemocyanins.

Such purification methods are very similar to those previously discussed for crystallizing the hemocyanins. For example, the hemocyanins of *Helix pomatia, Carcinus moenas, Octopus vulgaris*, and *Limulus polyphemus* were prepared by Roche *et al.* (1935) using dialysis against water at 0° C. A very small amount of an isoelectric acetate buffer or potassium phosphate buffer was added to hasten the precipitation. The first portion of the precipitate (extraneous matter) was discarded, and the subsequent hemocyanin precipitate was then reprecipitated (or recrystallized if crystallization happened to occur) from sodium chloride solution and dialyzed until salt-free. Purified *Helix pomatia* hemocyanin was also obtained (Putzeys and van de Walle, 1940) by precipitation from half saturated ammonium sulfate solution followed by dialysis and then by electrodialysis until the first solid appeared. The above solution on standing deposited non-hemocyanin material which was discarded, and the remaining solution contained the purified hemocyanin. By way of modification, Tiselius and Horsfall (1939b) prepared the hemocyanins of *Helix pomatia* and *Helix nemoralis* by dialysis against a primary-secondary phosphate buffer at an ionic strength of 0.1 and at 4° C. Such solutions when frozen and stored at −10° C. showed a single protein component by electrophoresis. When stored at +4° C. some change in the pigment took place, since insoluble matter was precipitated, and the remaining solution contained two electrophoretically distinguishable components.

As a final example of purification, Allison and Cole (1940) purified the hemocyanins of *Limulus polyphemus*, *Homarus americanus*, and *Cancer borealis* by isoelectric precipitation followed by electrodialysis. The hemocyanin was then dissolved in the minimum amount of 0.01N sodium hydroxide and reprecipitated by adjusting to the isoelectric point with 0.01N hydrochloric acid. This process was repeated three times and followed by electrodialysis against running distilled water. The hemocyanin precipitated on the membrane nearest the anode. It was found necessary to remove most of the salt before electrodialysis; otherwise denaturation occurred, probably caused by an excess of acid near the anode.

2. *Chemical Composition*

The elementary composition of the bloods and hemocyanins of many species has been reported. With the hemocyanins the main interest has centered on copper which, according to recent data, ranges from 0.15 per cent to 0.26 per cent on a dry weight basis. The more important data and particularly the more recent data on the composition of the purified hemocyanins are collected in Table I. From the data in this table it appears

TABLE I

Recent Data on the Elementary Composition of Various Hemocyanins

	C	H	N	S	Cu	References
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	
Mollusca						
Helix pomatia	53.4	6.9	15.15	0.76	0.24	(1)
			15.22			(2)
Busycon canaliculatum	53.5	6.65	15.9	1.23	0.245	(1)
Octopus vulgaris	53.4	6.95	15.9	1.04	0.25	(1)
			16.09			(2)
					0.25	(3)
Loligo pealei	52.75	6.8	15.75	1.19	0.26	(1)
Arthropoda						
Limulus polyphemus	53.4	6.9	16.9	1.10	0.173	(1)
			17.5	1.22		(4)
			16.10		0.173	(5)
Homarus americanus	53.07	6.85	16.78	0.90	0.187	(1)
			16.22		0.167	(5)
Carcinus*			16.83			(2)
Jasus lalandii					0.148	(6)
Cancer borealis			16.15		0.181	(5)

(1) Hernler and Philippi (1930 and 1933).
(2) Roche, *et al.* (1935).
(3) Kubowitz (1938).
(4) Mazur (1937).
(5) Allison and Cole (1940).
(6) Rawlinson (1940).
  * Not otherwise identified.

TABLE II

Recent Data on the Amino Acid Content of Various Hemocyanins

	Tryptophan	Tyrosine	Cystine	Arginine	Histidine	Lysine	Leucine	Valine	Alanine	References
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
Helix pomatia	5.90	4.59	2.05	5.26	5.81	7.46				(1)
							9.17	7.64	3.82	(2)
Helix aspersa	5.81	4.42	1.91	5.18	6.15	6.91				(1)
Octopus vulgaris	4.86	4.59	1.95	5.19	5.98	8.63				(1)
							10.93	6.37	7.54	(2)
Eledone moschata	5.68	3.89	2.08	5.44	6.11	8.47				(1)
							11.6	3.6	5.88	(2)
Sepia officinalis	5.55	4.82	2.02	5.62	5.92	7.90				(1)
							7.04	6.25	3.58	(2)
Carcinus moenas	5.21	4.82	1.99	6.65	8.89	7.97				(1)
							15.0	4.2	7.6	(2)
Dromia vulgaris	5.15	4.13	1.52	5.72	7.12	9.61				(1)
Maia squinado	4.44	4.27	1.88	5.87						(1)
Limulus polyphemus	4.52	4.50	2.26	6.85	9.08	8.10				(1)
							7.7	6.7	4.8	(2)
				6.37	4.52	8.92				(3)

(1) Roche and Jean (1934a)   (2) Roche and Morugue (1941)   (3) Mazur (1937)

that the hemocyanins of the *Mollusca* contain more copper than the hemocyanins of the *Arthropoda*. On the other hand, the hemocyanins of the *Arthropoda* are evidently slightly richer in nitrogen. The table shows that the hemocyanins of different species of *Mollusca* (or *Arthropoda*) cannot be differentiated on the basis of elementary analysis and suggests the same hemocyanin for all species within the group. However, studies of the physico-chemical properties of the hemocyanins have revealed striking dissimilarities.

Data indicating the distribution of nitrogen, shown by the amino acid content of hemocyanins from different species, is given in Table II. Roche and Jean (1934b) have claimed that a differentiation of the hemocyanins of the *Mollusca* from those of the *Arthropoda* can be made on the basis of the basic amino acid content. However, this conclusion is not self-evident from the data on arginine, histidine, and lysine shown in the table. Furthermore, such a conclusion seems premature in view of the marked disagreement in the histidine content of *Limulus polyphemus*. Mazur (1937) reported that of the 1.22 per cent sulfur in the hemocyanin of *Limulus polyphemus*, 49 per cent was found as methionine, 47.5 per cent as cystine, and 3.5 per cent was converted into hydrogen sulfide by reduction with hypophosphite in hydriodic acid. Finally, Gatterer and Philippi (1933), analyzing spectrographically the hemocyanin of *Busycon canaliculatum*, as well as hemocyanins of other species, were unable to find heavy metals other than copper in experimentally significant amounts. Particular attention was paid to zinc, iron and manganese.

3. *The Prosthetic Group Problem*

As pointed out above, copper is an integral part of the hemocyanin molecule, and this fact has been known for a long time. However, the manner in which the copper is bound to the protein moiety of the hemocyanin is still very much in doubt. Is copper directly attached to the protein, or is it attached indirectly through a prosthetic group analogous to that found with iron in the hemoglobin molecule? In other words, does copper in itself constitute the entire prosthetic group, or is it merely an essential part of the prosthetic group? Numerous investigators have searched for the answers to these questions.

Much of the research on the hemocyanins has been planned with the nature of the hemoglobins in mind. This has led first, to a search for a porphyrin derivative in the hemocyanin molecule, and then later to a search for any sort of a non-protein fragment which might function as a prosthetic group. A porphyrin has not been found, though there have been occasional reports of the presence of pyrrole nuclei in some degradation

products of hemocyanin. Although there has been some support for the presence of a definite organic prosthetic group[2], other investigators have disputed such findings. In the opinion of the present authors, the available evidence supporting either the presence or the absence of such a prosthetic group is inconclusive. Before considering the evidence which has accumulated from degradative studies, it seems advisable to discuss the absorption of oxygen by the hemocyanin molecule and the role of copper in this process.

The Role and State of Copper. Although the evidence is not extensive, the copper in the hemocyanin molecule is probably in the cuprous state. Kubowitz (1938), working with *Octopus* hemocyanin, observed that the addition of hydrogen cyanide followed by dialysis at pH 7.4 split copper out of the hemocyanin molecule and reduced the copper content to 0.044 per cent. He further noted the disappearance of the spectral band characteristic of the oxyhemocyanin. (See this section, Optical Properties.) After the removal of the copper as described above, the hemocyanin molecule was resynthesized by the addition of cuprous chloride under an inert atmosphere. Then, on reoxygenation, the spectral band of the oxyhemocyanin reappeared to the extent of 50 per cent of the original intensity, indicating the restoration of cuprous copper in one-half of the original hemocyanin. This result is the best evidence so far obtained that copper is present in the cuprous valence state in the unoxygenated hemocyanin molecule.

An earlier attempt by Conant, Chow, and Schoenbach (1933) to establish the valence state of the copper was based on the oxidation of hemocyanin with potassium permanganate and potassium molybdicyanide. The titration curves obtained revealed an equivalence of the oxidizing agent with the quantity of copper known to be present. Conant *et al.* called their oxidized hemocyanin, methemocyanin, and reduced it to a product which at least superficially resembled the original hemocyanin. They presumed, therefore, that the copper had been oxidized from the cuprous to the cupric state, since the expected amount of oxidizing agent was involved, and the oxidation was apparently reversible. However, Rawlinson (1941) maintained that Conant and coworkers were misled in their conclusions, since he was unable to find a change, by magnetic susceptibility measurements, in the valence state of copper while treating hemocyanin as described by Conant, *et al.* Although such measurements are capable of detecting a change in the valence state of the copper atom,

[2] The term "organic prosthetic group" is used throughout this discussion to designate a non-protein, metal-free organic fragment or complex as exemplified by the porphyrin part of the prosthetic group of hemoglobin. There is no intention to imply that the protein moiety of the hemocyanin molecule is not organic.

it is known that they cannot establish the actual valence of that atom in any given state. For this reason, Rawlinson's work casts doubt on the conclusions of Conant and coworkers, but neither adds to nor detracts from the evidence supplied by Kubowitz.

Bert (1867) observed a change in the color of the blood of cephalopods as it passed through the gills of these animals. The subsequent establishment of the physiological function of the hemocyanins as oxygen carriers was based on this observation. Analysis of the oxygen and carbon dioxide content of the arterial and venous bloods of the *Octopus* (Winterstein, 1909) and of squid (Redfield and Goodkind, 1929) showed that a very large proportion of the combined oxygen is utilized with each complete circuit of the blood. The hemocyanin bloods of the highest oxygen capacity are those of the cephalopods which are the most active of the large marine invertebrates. On the other hand, the bloods of the gastropods and of *Limulus* are saturated at lower oxygen pressures than the bloods of the cephalopods, and hence the former, though limited in activity, are fitted for life where oxygen is relatively scarce. The squid (a cephalopod) is capable of great activity in the presence of a plentiful supply of oxygen, but is extremely sensitive to the lack of oxygen. Even the active *Octopus* (also a cephalopod) has a better chance for survival than the squid, since the hemocyanin of the former is oxygenated at lower pressures than the hemocyanin of the squid (Wolvekamp, 1938b). The hemocyanins of the crustaceans are in a somewhat intermediate position. One of them, the hemocyanin of *Jasus lalandii*, has been reported (Rawlinson, 1940) to have an oxygen capacity of about one-fifth that of hemoglobin on a weight basis.

It has been well established by the data of Redfield, Coolidge, and Montgomery (1928) and Guillemet and Gosselin (1932) that during the oxygenation of hemocyanin, one molecule of oxygen combines with the quantity of hemocyanin containing two atoms of copper. This result was reported for the hemocyanins of all thirteen species tested, and was interpreted as strong evidence of a direct union of the oxygen with the copper. Recently it has been speculated (Rawlinson, 1941) that the orientation of groups within the protein portion of the hemocyanin molecule confers on the copper atom the power of combining directly with oxygen. Since such a combination results in the formation of oxyhemocyanin with a characteristic blue color, it was further suggested that this color might be due to a resonating structure formed by linking each oxygen molecule between two atoms of copper. Rawlinson pointed out that such an assumption makes the presence of a separate organic prosthetic group quite unnecessary.

A very important part of the study of the hemocyanins has been concerned with the combination of oxygen and the hemocyanin molecule. These studies have involved investigations of the oxygenation reaction of both hemocyanin bloods and the purified hemocyanins, and have yielded data on the kinetics of the reaction, the heat of the reaction, and particularly the equilibrium conditions. Practically all of this information was accumulated prior to 1934, largely as the result of the efforts of Redfield and coworkers, and was therefore surveyed in detail in his review (Redfield, 1934). For this reason, and the fact that in this review emphasis has been placed on the nature rather than the function of the copper proteins, the oxygenation reaction will not be discussed to any extent. It should be pointed out, however, that some of Redfield's (1934) interpretations of the oxygenation reaction were based on the belief, prevalent at that time, that the hemocyanin molecule contained a definite organic prosthetic group.[2]

The interaction of hemoglobin with carbon dioxide and the formation of compounds with carbon monoxide and cyanide have led many investigators to look for analogies with the hemocyanins. Because of their role in respiration, the nature of the interaction of hemocyanins with carbon dioxide has long been of interest. The early investigations (Redfield, 1934) revealed that, whereas carbon dioxide deoxygenates oxyhemocyanin, no evidence of any definite compound formation was obtained. However, it was recognized that the exchange of carbon dioxide for oxygen in the tissues, and the reverse exchange in the respiratory organs of marine animals, was dependent on hemocyanin. In more recent years, Wolvekamp (1938a) has found that a small amount of carbon dioxide greatly increases the dissociation of the oxyhemocyanins of *Sepia officinalis* (lobster) and *Octopus vulgaris*. About the same time, Wolvekamp (1938b) reported the presence of carbonic anhydrase in the gills of *Loligo* (squid). Later, however, he could not detect the presence of this enzyme in *Helix pomatia*, *Cancer pagarus*, and *Homarus vulgaris*, and found that the carbon dioxide in the bloods of these animals was present only as bicarbonate.

Cyanide decolorizes oxyhemocyanin (Kobert, 1903) to form a compound called cyanhemocyanin. This reaction has been made the basis of methods for estimating the amount of oxygen combined with the hemocyanins (Redfield, 1934). Cyanhemocyanin does not combine with oxygen to form a compound containing both oxygen and cyanide (Cook, 1928). Rather, it has been suggested that in the absence of excess cyanide, oxygen displaces cyanide from cyanhemocyanin (Craifaleanu, 1919). Pearson (1936) working with *Limulus* hemocyanin followed the decomposition of oxyhemo-

[2] See footnote on page 189.

cyanin on addition of cyanide. Based on the disappearance of the characteristic color of oxyhemocyanin, his data indicated that in the displacement reaction two cyanide groups combined with each copper atom. Although in certain experiments Pearson used only enough cyanide to partially decolorize the oxyhemocyanin present, he was not able to duplicate the results of Craifaleanu even with increased oxygen pressures. However, alkaline dialysis for three weeks was found to restore 60 per cent of the oxyhemocyanin previously decolorized. In addition, Pearson also observed that solutions of cyanhemocyanin contain free cyanide which is apparently in equilibrium with the cyanhemocyanin.

The literature relating to the formation of a definite compound between hemocyanin and carbon monoxide is contradictory. Certain investigators have been of the opinion that a definite compound is formed (Craifaleanu, 1919; Root, 1934; Roche, 1936). On the other hand, other workers have failed to obtain evidence of such compound formation (Dhéré and Schneider, 1922; Roche and Dubouloz, 1933a). Likewise, Rawlinson (1940) has recently reported that the hemocyanin of *Jasus lalandii* does not combine with carbon monoxide, the amount of carbon monoxide actually present being no more than could be accounted for by physical solution. Although there has been some tendency to discount the experiments of Root because they were carried out using *Limulus* serum rather than the purified hemocyanin, it should be pointed out that Allison and Cole (1940) have demonstrated that the carefully prepared sera of *Limulus polyphemus, Cancer borealis*, and *Homarus americanus* contain no proteins other than hemocyanin.

Degradation Studies. Quite a number of degradation studies have been carried out in the attempt to find and isolate the prosthetic group of hemocyanin. Various hemocyanins have been used. However, an examination of the contradictory results and opinions leads to the conclusion that a definite decision regarding the presence or absence of a prosthetic group[2] cannot be made at this time. As pointed out earlier, probably the greatest single factor contributing to this condition has been the tendency to view the hemocyanins in the reflected light of the hemoglobins. Because the work does not divide itself chronologically, those studies supporting the existence of a definite prosthetic group will be presented first.

Philippi (1919) obtained a dark green product by treatment of *Helix* hemocyanin with strong alkali. This material was 7 per cent copper and gave an intense pine splint test for pyrrole. The latter fact aroused much interest since pyrrole derivatives were known to be the fundamental units of the porphyrin group of hemoglobin. However, Dhéré and Baumeler

[2] See footnote on page 189.

(1928, 1929) were doubtful of the presence of porphyrins in Philippi's product for they were unable to detect the characteristic red fluorescence of these compounds. About the same time Schmitz (1930,1931) obtained by alkaline degradation a product from *Octopus* hemocyan in which he named hemocuprin. This material had the composition C 45.04, H 6.99, N 12.58, and Cu 6.27 per cent, and Schmitz believed it to be a tetrabasic organic acid with the copper bound as a complex. Furthermore, he found no evidence of a porphyrin structure.

Conant and Humphrey (1930) and Conant, Dersch, and Mydans (1934) using the strong alkali procedure of Philippi, prepared a material from *Limulus* hemocyanin which upon further treatment yielded a black insoluble powder containing practically all the copper of the original hemocyanin. They reported its composition to be: Cu 21.5, N 9.2, C 39.5, H 5.6, and S 8.0 per cent. They believed their product was a complex copper salt of a polypeptide containing leucine, tyrosine, and serine and that the sulfur was not present as cystine, cysteine, or methionine, and probably not as taurine. Since ammonia removed both copper and sulfur from the black powder, Conant, *et al.* suggested that the copper was attached as a complex to an unknown sulfur compound as well as to the polypeptide. In attempting to demonstrate that the black product was not formed by the recombination of degradation products, they showed that the addition of copper sulfate to the original *Limulus* blood did not greatly increase the amount of the black pigment formed. A molecular formula was proposed, and copper was reported (Conant, Dersch, and Mydans, 1934) to be present in the cupric state. Although Conant, *et al.* were unable to obtain hemocuprin from *Limulus* blood by the method of Schmitz, they reported that their black powder could be converted into a material similar to hemocuprin by prolonged treatment with alkali.

Laporta (1932) treated *Octopus* hemocyanin with acetone and acid, and obtained a product which he considered to be identical with Schmitz' hemocuprin. As a matter of fact, Roche and Dubouloz (1933a, b) observed the same ultraviolet spectrum for the products of both workers and concluded that they were identical. Laporta stated that his material gave a positive pyrrole test. In their study, Roche and Dubouloz duplicated the degradations of Schmitz and Laporta with the bloods of snail, octopus, and crab, and concluded that the copper complexes obtained were the prosthetic groups of the hemocyanins. They found, however, no evidence of a porphyrin structure.

Using the hemocyanin of snail, Florkin and Toussaint (1939) prepared the product first described by Philippi (1919). They then demonstrated that this material could be changed into the products described by both Schmitz and by Conant, *et al.*, thereby indicating a relationship between

the three copper-containing materials. With Conant's "compound" they obtained a positive orcinol test, indicating the presence of pentose, but the test was negative on Schmitz' hemocuprin.

As previously inferred, many workers have been of the opinion that the hemocyanins have no prosthetic group other than copper. Among the first to take this view was Henze (1901) who believed that the hemocyanin of *Octopus* contained nothing comparable with the porphyrin of hemoglobin. The ease of removing the copper from the hemocyanin by a mild acid treatment led him to propose a copper-albuminate type of linkage. This view was supported by Kobert (1903), Alsberg and Clark (1910), and by Roche (1930). Somewhat later Roche and Dubouloz (1936) degraded the hemocyanins of *Helix pomatia* and *Cancer pagarus* with acetone and hydrogen chloride and obtained a product similar to the hemocuprin of Schmitz. Hydrolysis of this material yielded leucine, tyrosine and good evidence for the presence of arginine. Because of the difference in amino acids, they pointed out that their degradation product was different from that of Conant *et al.* Roche and Dubouloz concluded that the copper was attached to a polypeptide, but felt that the polypeptide was merely a fragment of the protein molecule rather than a stable prosthetic group.

As the result of more recent experiments, Rawlinson (1940) came to the conclusion that the degradation product of the crystalline hemocyanin of *Jasus lalandii* was nothing more than basic copper sulfide contaminated or combined with protein break-down products which possessed peptizing properties. He claimed that much of the controversy over the nature of the copper degradation product was probably due to the fact that in certain cases workers had used whole bloods rather than purified hemocyanins. He pointed out that the whole blood of *Jasus* contained many things other than hemocyanin and emphasized that the use of such blood would increase the opportunities for contamination of the copper compounds with protein fragments.

In criticizing Conant's work, Rawlinson (1941) maintained that the copper was almost certainly detached from the protein as soon as the hemocyanin solution was made strongly alkaline. For this reason, he considered it very unlikely that the product of Conant *et al.* had its copper combined in the same manner and to the same groups as in the native protein. It is to be noted, however, that Rawlinson apparently disregarded the experiment of Conant, Dersch, and Mydans (1934) designed to show that their copper-containing degradation product did not arise through recombination of ionic copper and protein fragments.

The foregoing discussion makes it apparent that the problem of whether or not the hemocyanin molecule contains a prosthetic group in addition to copper, is a difficult one and one that has not yet been satisfactorily

answered. The complexity of the problem is further emphasized by the fact that certain investigators of long experience in the field have at one time or another supported each side of the controversy.

Methemocyanin. Conant, Chow, and Schoenbach (1933) investigated the effect of oxidizing agents on the hemocyanins of *Limulus polyphemus* and *Homarus americanus.* Only the very strong oxidizing agents, potassium permanganate and potassium molybdicyanide, appeared to alter the hemocyanin significantly in neutral solution. They believed that in the absence of oxygen the reaction resulted in the formation of a new compound which they called methemocyanin (to correspond with methemoglobin). This compound, which was colorless, became greenish-blue when shaken with air, and then colorless again when placed under vacuum. They interpreted this color change as being due to a reversible combination of colorless methemocyanin with oxygen to form greenish-blue oxymethemocyanin. By following the potassium permanganate (or potassium molybdicyanide) oxidation of hemocyanin either electrometrically or by indicator change, Conant *et al.* obtained data indicating reduction of one equivalent of oxidizing agent for each atom of copper. Hence they assumed that the reaction involved the oxidation of cuprous copper to the cupric state. The oxymethemocyanin was purified by ammonium sulfate precipitation, and during this process a brownish solid was observed which was reported as probably being manganese dioxide. The purification process led to a product that had a color distinctly different than that of oxyhemocyanin but of about the same intensity. Furthermore, chemical reduction led to a product that resembled the original hemocyanin, especially in the case of reoxygenation. The oxidizing power of the methemocyanin itself was found to be equivalent to a change of one electron for the amount of copper present.

As pointed out previously, Rawlinson (1941), as the result of magnetic susceptibility measurements, found no change in the valence state of copper in the presence of strong oxidizing agents and, therefore, criticized the above conclusions of Conant and coworkers. Rawlinson believes that Conant, *et al.* were misled by a "series of accidents," and he offered the following explanation of their findings. The very strong oxidizing agents partly oxidized some portions of the protein moiety rather than the cuprous copper, and the portion affected was not concerned with the oxygenation reaction of the hemocyanin molecule. During the oxidation at pH7 the oxidizing agent was reduced only to an intermediate valence state, and thus the resulting mixture contained oxidized protein plus manganese dioxide in the case of potassium permanganate. The manganese dioxide was highly peptized by the protein and could not be completely removed. This manganese dioxide protein mixture had a modified color and had the

properties ascribed by Conant to a new compound, oxymethemocyanin. Finally, Rawlinson noted that the apparent dependence of the oxidation on the amount of copper was fortuitous, and probably due to the fact that Conant's group used the same concentration of protein in each experiment. This would result in all experiments in the peptization of an amount of manganese dioxide always proportional to the amount of protein used and, thereby, proportional to the copper content.

4. *The Protein Moiety*

All the hemocyanins appear to be globulins and as such are soluble in dilute salt solutions (Quagliariello, 1924). This characteristic solubility fits the hemocyanins for circulation in body fluids which contain dissolved electrolytes. Furthermore, the hemocyanin molecules are very large, and hence are retained within the walls of the fluid spaces in which they circulate. Finally, as protein ampholytes the hemocyanins function as buffers, take part in the transport of carbon dioxide, and influence the equilibrium of electrolytes across membranes bathed by the blood. These last properties are all shared by many other blood proteins.

The existing knowledge of the detailed structure of the hemocyanins has been previously discussed in terms of the elementary composition and the amino acid content. However, such data contribute nothing regarding the presence or arrangement of larger structural units in the hemocyanin molecule. In fact, information on this important subject has been obtained only recently as the result of the investigations of Trurnit and Berghold (1942). These authors conducted interferometric studies of monomolecular films of the hemocyanin of *Helix pomatia*. They concluded that this molecule consists of eight rods arranged in two parallel sheets of four rods each. In addition, the coherence between the rods within a sheet seems greater than the coherence between the two sheets. Such observations are in agreement with the phenomenon of dissociation which will be discussed later.

Molecular Dimensions. The molecular dimensions of the hemocyanins have been widely investigated by a number of independent methods. The majority of the data has been obtained with the ultracentrifuge (often in conjunction with diffusion measurements), and such data are perhaps the most reliable. Although confirmation of the results from the ultracentrifuge has been generally obtained with other methods of measurement, the occasional disagreements have stimulated research on new and old methods alike.

The hemocyanins possess the largest known minimum molecular weights based on analytical data only. Assuming one atom of copper per molecule,

the molecular weights of the hemocyanins of the *Mollusca* are about 25,000. The hemocyanins of the *Arthropoda* have a lower copper content, and hence, the minimum molecular weights are somewhat higher, 37,000. Actually, these minimum molecular weights probably should be doubled for, as indicated previously, the quantity of hemocyanin that combines with a single oxygen molecule contains two atoms of copper. Cohn (1925) by ultrafiltration of *Limulus* hemocyanin estimated the molecular weight to be 68,100. However, shortly thereafter, Dhéré and Baumeler (1926) observed that hemoglobin would pass through collodion membranes which retained hemocyanin. They, therefore, believed hemocyanin molecules to be larger than those of hemoglobin. In addition, the oxygen equilibria of certain native bloods could be accounted for only by assuming a minimum unit large enough to combine with four molecules of oxygen. This requirement indicated a minimum weight of 200,000 to 300,000 (Redfield, 1934). Finally molecular weights, running from about 300,000 upward to several millions, were calculated by Svedberg and his collaborators from data obtained by using the ultracentrifugal methods of sedimentation velocity and sedimentation equilibrium. The endocellular pigments, such as the hemoglobins and hemerythrins, are comparatively small molecules with weights ranging from 17,000 to 68,000, while the plasma pigments (hemocyanins, erythrocruorins, and chlorocruorins) are very large, ranging from 300,000 upward (Roche, 1936). A single exception is the plasma erythrocruorin of *Chironomus* with a probable molecular weight of either 17,000 or 34,000.

Table III, showing molecular weights and related data for various hemocyanins, is largely adapted from Ericksson-Quensel and Svedberg (1936) and Svedberg (1937, 1939). For a more extensive list of sedimentation constants the reader is referred to Svedberg and Hedenius (1934). The molecular weight data in this table are based on studies made several years ago and therefore are probably subject to upward revision. As knowledge of the phenomenon of protein dissociation increases and its influence on molecular weight determinations is better understood, the reported molecular weights become larger. For instance, a comparison of the molecular weights in Table III with those reported by Svedberg and Chirnoaga (1928) and Svedberg and Ericksson (1932) a few years earlier, *i.e.*, *Helix* 50, *Octopus* 20, *Limulus* 13, *Homarus* 6.4, and *Palinurus* 3.6, all times 10^5, illustrates the tendency towards larger estimates of the molecular weight. Additional evidence of this upward trend is to be found in the report of Brohult (1940) that the hemocyanin from the fresh blood of *Helix pomatia* had a molecular weight of 89.1×10^5 for the main component. Ekwall (1942) reported a molecular weight of 87×10^5 in the pH range of 4.0 to 7.4 for the hemocyanin of *Paludina vivipara*. It should be pointed out,

TABLE III

Molecular Weight and Related Data for the Hemocyanins of Various Species

Species	pH	S_{20}	D_{20} $\times 10^7$	M_S $\times 10^{-5}$	M_e $\times 10^{-5}$	f/f_0	Isoelectric Point	References
Pandalus borealis	6.8	17.4			3.97	1.1		(1)
Palinurus vulgaris	6.8	16.4	3.4	4.46	4.47	1.2	4.6	(1, 2)
Helix pomatia (dissoc. comp.)	9.7	12.1	2.23	5.02		1.5	5.05	(1, 2, 6)
Busycon canaliculatum (dissoc. comp.)	9.6	13.5	3.29	3.79		1.4	4.49	(1, 2, 3)
Eledone moschata (dissoc. comp.)	11.6	10.6	2.16	4.57		1.9	4.6	(1, 2, 3)
Nephrops norvegicus	6.8	24.5	2.79	8.12		1.2	4.64	(1, 2, 3)
Homarus vulgaris	6.8	22.6	2.78	7.52	8.03	1.3	4.95	(1, 2, 3)
Helix pomatia (dissoc. comp.)	8.6	16.0	2.06	7.19	7.97	1.9	5.05	(1, 2, 7)
Helix nemoralis (dissoc. comp.)	8.6	16.6	1.92	7.99		1.8	4.63	(1, 2, 7)
Calcaris macandrae	6.8	34.0			13.29	1.2		(1, 4)
Octopus vulgaris	6.8	49.3	1.65	27.85		1.4	4.7, 4.8	(1, 5, 8, 9)
Eledone moschata	6.8	49.1	1.64	27.91		1.4	4.6	(1, 2, 3)
Rossea owenii	6.8	56.2	1.58	33.16		1.4		(1, 2)
Helix pomatia (main comp.)	6.8	98.9	1.38	66.30	66.80	1.2	5.06; 5.05; 5.3	(1, 2, 6, 10, 11, 12)
Busycon canaliculatum (main comp.)	6.8	101.7	1.38	68.14		1.2	4.49	(1, 3)
Busycon canaliculatum (aggreg. comp.)	6.8	130.4	1.17	99.80		1.2	4.40	(1, 3)

S_{20}=sedimentation constant at 20° C. expressed in Svedberg units, $S = 10^{-13}$.

D_{20}=diffusion constant at 20° C.

M_S=molecular weight based on sedimentation velocity measurements.

M_e=molecular weight based on sedimentation equilibrium measurements.

f/f_0=molecular frictional factor.

however, that at least a part of the upward trend in molecular weights must be due to improved methods and apparatus particularly as applied to diffusion constants, since an early report by Svedberg and Ericksson (1932) clearly showed recognition of the presence of two components in the hemocyanin of *Octopus vulgaris* with the larger one having a reported weight of 20.5×10^5.

Osmotic pressure data have been obtained for the estimation of the molecular weights of hemocyanins. Unfortunately, the osmotic methods yield a number average molecular weight in which the small particles exert a much greater effect than an equal weight of large particles. Thus, in measurements made on mixtures, which is usually the case with the hemocyanins, due to dissociation, the actual osmotic pressure is less than that exerted by a pure small component but greater than that exerted by a pure large component. Furthermore, with very large particles the pressures are low and difficult to measure. This is especially true since deviations from ideal solutions require measurements on rather dilute solutions followed by extrapolation to zero concentration. Hence, errors in the molecular weights based on osmotic data are often quite serious, but in any case this method, like the ultracentrifuge, indicates great molecular size for all the hemocyanins tested.

Roche, *et al.* (1935) reported minimum molecular weights based on osmotic pressure measurement for the hemocyanins: *Helix* 18×10^5, *Octopus* 7.1×10^5, and *Carcinus* 5.5×10^5. The actual values are probably much higher for *Helix* and *Octopus* hemocyanins (see Table III). Osmotic pressure data taken on *Limulus* hemocyanin in an aqueous buffer solution yielded an estimated average molecular weight of 5.65×10^5 (Burk, 1940). In 6.66 M urea solution, where dissociation into smaller components was expected, osmotic pressure measurements indicated a molecular weight of 1.42×10^5. During a three months' estivation of the animal, the hemocyanin of *Helix pisana* decreased in molecular weight by about a factor of two as evidenced by a doubling in the osmotic pressure of solutions of the hemocyanin (Roche and Roche, 1935). A similar result was observed for the hemocyanin of *Helix pomatia* after a hibernation period of five months. This observation that the size of the hemocyanin molecule may vary in

References for Table III

(1) Eriksson-Quensel and Svedberg (1936).
(2) Polson (unpublished). See Svedberg (1939).
(3) Pedersen (unpublished). See Svedberg (1939).
(4) Svedberg and Hedenius (1934).
(5) Polson (1936).
(6) Tiselius (1930).
(7) Pedersen (1933).
(8) Quagliariello (1920).
(9) Roche (1930).
(10) Stedman and Stedman (1927).
(11) Putzeys and van de Walle (1939).
(12) Putzeys and van de Walle (1940).

the animal from time to time by virtue of physiological changes, may in part explain some of the discrepancies in molecular weight data.

Using amandin with a molecular weight of 3.30×10^5 as a standard, Putzeys and Brosteaux (1941) determined the molecular weights of various hemocyanins by the method of light scattering. Their results are: *Palinurus* 4.62, *Homarus* 7.1, *Sepia* 32, and *Helix* 63.4, all times 10^5. These data are in fair agreement with the ultracentrifugal data of Table III, and the light scattering method appears to be more suitable than osmotic pressure measurement since small quantities of a small component have much less influence on the average molecular weight. Furthermore, the great particle size of the hemocyanin imposes no restriction on the accuracy of the measurement of the scattered light as it does on the measurement of the osmotic pressure. The molecular weights of the hemocyanins of a few species have also been obtained from viscosity and diffusion relationships (see Table IV).

TABLE IV

Molecular Weights of Three Hemocyanins

From Svedberg, The. (1939)

| Source | Viscosity and Diffusion Data | | | Ultracentrifugal Data |
	(1)	(2)	(3)	(4)
	$\times 10^5$	$\times 10^5$	$\times 10^5$	$\times 10^5$
Homarus	5.3	3.93	7.93	7.77
Octopus	19.52	13.37	27.8	27.8
Helix pomatia	42.5	31.01	63.5	66.5

(1) Kuhn (1932).
(2) Burgers (1938).
(3) Polson (1936).
4) Taken from Table III.

Tiselius and Gross (1934) reported a diffusion constant of 1.05×10^{-7} cm.²/sec. for *Helix pomatia* hemocyanin calculated to water at 20° C.

Estimates of the molecular diameters of hemocyanins have been based on ultracentrifugal and ultrafiltration data and quite recently on electron micrographs. Svedberg and Chirnoaga (1928) assumed spherical molecules of 50×10^5 weight for the hemocyanin of *Helix pomatia* and estimated a diameter of 24 m μ. Ultrafiltration studies led Elford and Ferry (1936) and Grabar (1936) to estimate respectively diameters of 18 to 28 m μ and 29 m μ for the same hemocyanin. Ekwall (1942) estimated the length of the hemocyanin of *Paludina vivipara* to be 80 to 100 m μ with a molecular weight of 87×10^5. Svedberg (1930) calculated a diameter of 9 m μ for *Limulus* hemocyanin.

Electron micrographs of the hemocyanin of *Limulus polyphemus* indi-

cated spherical molecules with an average diameter of 20 m μ (Stanley and Anderson, 1942). In the ultracentrifuge this hemocyanin sample contained two components. The first represented 10 per cent of the total with a sedimentation constant of 87.3S, and the second with a constant of 59.9S made up 90 per cent of the total hemocyanin. The latter component calculated as a sphere would have a diameter of 19 m μ. These same investigators, using the hemocyanin of *Viviparus malleatus*, found a single major component by means of the ultracentrifuge and estimated the average diameter to be 29 m μ from electron micrographs. Low contrast on the photographic plates indicated assymmetry, probably as discs or platelets. Calculated from a sedimentation constant of 95.0S assuming spherical symmetry, the average diameter of this molecule would be 24 m μ. *Busycon canaliculatum* also appeared to be assymmetrical, but the presence of three components in similar quantities made the interpretation of the electron micrographs somewhat uncertain (Stanley and Anderson, 1942). However, a diameter for the smallest component of about 10 m μ seemed reasonably certain, and the assymmetry of the large components was probably of the plate type.

Clark, Quaife, and Baylor (1943) reported a statistically determined average molecular diameter of 8.0 m μ for single molecules of the hemocyanin of *Loligo pealei* as measured from electron micrographs. The molecules seemed to be spherical, and possessed an average molecular weight (ultracentrifuge) of 2.16×10^6. X-ray diffraction patterns taken on the same substance indicated a diameter of 10 m μ. The diameter of *Limulus polyphemus* hemocyanin, also measured from electron micrographs, was found to be 17 m μ, a value in good agreement with that reported by Stanley and Anderson (1942).

Dissociation. Nearly all the data on such chemical and physical properties of the hemocyanins as solubility, viscosity, partial specific volume, acid-base titration, etc. were obtained prior to 1934, and were therefore discussed by Redfield (1934) in his review of the hemocyanins. At that time the unusual ability of the hemocyanin molecule to dissociate reversibly into smaller units was just becoming well recognized. As a consequence, much of the more recent research on the hemocyanins has been concerned with this phenomenon.

Among the first to suggest that reversible dissociation of the hemocyanins was probable were Svedberg and Heyroth (1929) who strongly suspected that the hemocyanin of *Helix pomatia* dissociated into smaller particles when dissolved at a pH removed from the isoelectric point. Likewise, the hemocyanin of *Limulus polyphemus* was also thought to be dissociated by high dilution. In both cases abnormally low sedimentation constants were observed. As an explanation for a report by Stedman and Stedman (1927)

that a change in viscosity of a hemocyanin solution took place during oxygenation, Svedberg and Heyroth suggested that dissociation was taking place. Another attempt to correlate viscosity and dissociation has been made more recently by Treffers (1940), who observed a linear variation of the fluidity (the reciprocal of viscosity) with the concentration of *Octopus* and *Homarus* hemocyanins. This finding was somewhat surprising to Treffers for he had expected that dissociation on dilution would cause a deviation from linearity, since he felt that dissociation would alter the volume fraction or, more especially, the effective shape of the particles. But, although a strict linearity in the fluidity-concentration relationship prevailed, Treffers hesitated to state definitely that no dissociation took place.

Returning to the evidence supporting the concept of dissociation, Svedberg and Eriksson (1932) reported the presence of two components in the hemocyanin of *Octopus vulgaris*. The change from one component to the other was found to be reversible and was produced by a change in the pH. Both components, however, existed simultaneously at certain hydrogen ion concentrations. The molecular weight of the large component (20×10^5) was found to be independent of pH over the range in which it existed but the size of the small component varied with pH.

In his review of animal pigments, Roche (1936) proposed that the existence of hemocyanins in different micellar states in the blood might be explained by other means than dissociation. Roche speculated that the protein micelles remained unchanged once formed by the animal, but that they could be rapidly replaced in the blood by newly synthesized micelles of variable size. He believed that the size of the newly formed micelle varied with the requirements of the animal, and in support of this view, he referred to the work of Roche and Roche (1935) showing that the average size of the hemocyanin molecule in the blood differed during estivation from that present while the animal was more active.

Eriksson-Quensel and Svedberg (1936) published results on stability studies of the hemocyanins of *Pandalus borealis*, *Limulus polyphemus*, *Palinurus vulgaris*, *Nephrops norvegicus*, *Homarus vulgaris*, *Cancer pagarus*, *Carcinus moenas*, and fourteen other species of hemocyanin-bearing animals of all classes. The data was presented in the form of pH-stability diagrams. That of the hemocyanin of *Busycon canaliculatum* is a typical example (see Fig. 1). These investigators concluded from their data that at the isoelectric point and in the pH ranges where associations or dissociations were reversible the resultant hemocyanin components were homogeneous with regard to their molecular weights. The stable zone for the component of highest molecular weight for a given hemocyanin always included the isoelectric point. The dissociation reactions were reversible
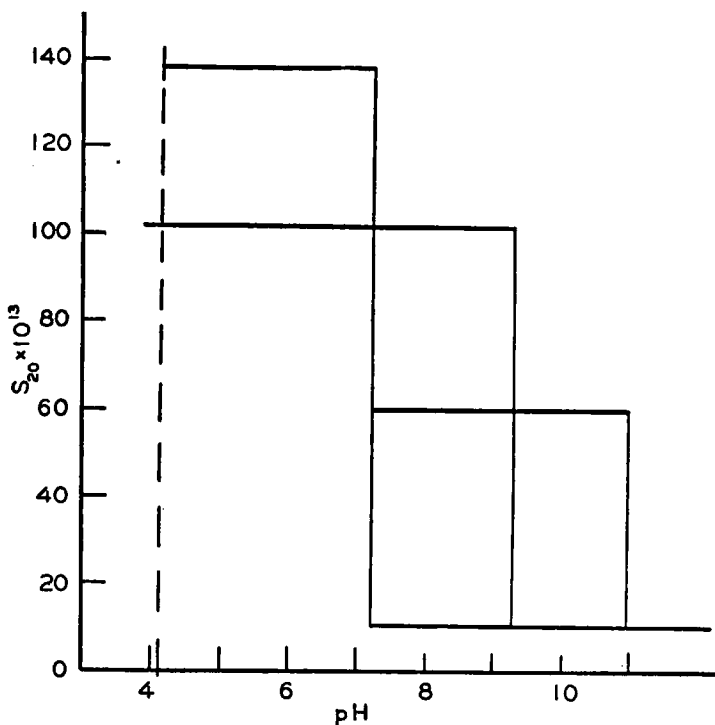
Fig. 1. pH-Stability diagram for the hemocyanin of *Busycon canaliculatum*. Adapted from Eriksson-Quensel and Svedberg (1936). Data plotted appear in the original paper as points grouped along the heavy lines. The abscissa represents the sedimentation constant of the components in solution at a given pH calculated to 20° C.

except at rather low and rather high pH values. Finally, the hemocyanins of related species were found to have related properties as evidenced by their stability diagrams.

Sedimentation constant data (Svedberg, 1937) on the hemocyanin of *Limulus polyphemus* indicated the presence at pH 6.8 of four homogeneous components with sedimentation constants of 5.9, 16.1, 34.6, and 56.6S. The presence of a small amount of a fifth component, intermediate in size between the largest components, was suggested by the sedimentation diagram given (see also Svedberg, 1939). An ultracentrifugal study by Brohult (1937) of a solution of *Helix pomatia* hemocyanin before and after subjecting the solution to sonic waves of a frequency of 250,000 vibrations per second, indicated an extensive irreversible dissociation of the hemocyanin into $\frac{1}{2}$ and $\frac{1}{8}$ molecules. At pH values where partial dissociation had already occurred, further dissociation took place during

the sonic treatment. Brohult stated that a heating effect was not the cause of this phenomenon and that the electrophoretic properties of the hemocyanin were unchanged by such dissociation. Brosteaux (1937) found the component in *Helix pomatia* hemocyanin of molecular weight 66×10^5 to be stable from about pH 4 to a little above pH 7 with dissociation above and below this range. The small components formed below pH 4 and above pH 8 were apparently ⅛ molecules. He also noted that the presence of calcium or magnesium ions extended the stability of the large component to above pH 9.

However, Svedberg and Brohult (1938) reported that the hemocyanin of *Helix pomatia* existed at pH 6.2 as half molecules only. At pH 7.4 half, as well as smaller molecules, were formed. They considered that the weakest link was that binding together the two halves of the main component. Further splitting then required a little more drastic treatment resulting in an increase in the number of ionized groups. Splitting by irradiation was effective only with light of wave length 280 m μ or shorter. Light corresponding to the oxyhemocyanin band, 320 to 370 m μ had no effect.

Tiselius and Horsfall (1939a) using the purified hemocyanins of *Helix pomatia*, *Helix nemoralis*, and *Littorina littorea*, examined them electrophoretically at pH 6.85 and found them to contain single electrophoretic components with evidence for a little of a second component in the last of the above hemocyanins. These three hemocyanins were chosen because they had been reported to have similar molecular weights but different electrophoretic properties. Likewise, all were known to dissociate when the pH of their solutions was altered. The hemocyanins of *Helix pomatia* and *Helix nemoralis* were mixed after adjustment of their solutions to pH 3.8 to cause partial dissociation into half molecules. After mixing, the pH was readjusted to 6.85, the value used during the examination of the individual hemocyanins. Electrophoresis revealed the presence of two components corresponding in mobility to pure *Helix pomatia* hemocyanin and pure *Helix nemoralis* hemocyanin. However, a third component was also found, and this new material had a mobility intermediate between the mobilities of the pure hemocyanins. In fact the rate of migration in the electric field could be estimated for this third component by assuming that it was formed from one-half molecule of each of the pure hemocyanins.

Using mixtures of the hemocyanins of *Helix pomatia* and *Littorina littorea*, Tiselius and Horsfall by similar experiments found that dissociation at pH 8.5 into half and eighth molecules followed by reassociation at pH 6.85 led to the formation of *Helix pomatia* hemocyanin and three mixed molecules. Repeated electrophoresis experiments revealed only these same four components although ultracentrifugal analysis revealed

quite a number of different molecular weights. With the two *Helix* hemocyanins split at pH 8.5 into eighth molecules and then reassociated, no evidence of individual components could be obtained, but the reassociation led to what appeared to be a number of closely related molecules that could not be resolved electrophoretically. It was suggested that all possible recombinations of *Helix pomatia* and *Littorina littorea* hemocyanins had not taken place, for some reassociations were blocked by generic differences in the molecules. Finally, Tiselius and Horsfall reported that the stabilities of the new components appeared to equal the stability of the original hemocyanins, and they concluded from the symmetry and constancy of the moving boundaries that no equilibrium existed between the components.

Roche and Darien (1939) studied the solubility diagrams of the hemocyanins of several species in ammonium sulfate. They deduced that dissociation occurred in the hemocyanins of those species of *Crustacea* studied, but reported probable homogeneity for the hemocyanins of the *Mollusca* investigated. Brohult and Claesson (1939) dissociated the hemocyanin of *Helix pomatia* in solutions of various concentrations by the addition of numerous substances. Electrolytes were quite effective, with variations partially attributed to differences in charge type. A minimum concentration of solute was necessary to cause any dissociation, and beyond a limiting value further increase in concentration caused no further dissociation. Calcium chloride caused the formation of eighth and sixteenth molecules though other salts resulted only in half molecules. The same investigators reported that the dissociation was only partially reversible when the products were smaller than half molecules and that glucose, glycerine, and urea all induced dissociation but to a much less extent than the electrolytes.

The latest and largest reported molecular weight for the hemocyanin of *Helix pomatia* is that of Brohult (1940) who used fresh blood and obtained a value of 89.1×10^5 by means of the ultracentrifuge. He observed that dissociation occurred into half molecules of weight 43.1×10^5, into eighth molecules of weight 10.3×10^5 and into intermediate molecules. In line with his findings of the previous year (see above) the presence of electrolytes and non-electrolytes increased the dissociation. Irradiation with ultra-violet light caused dissociation even at liquid air temperatures and X-rays had a similar effect. Both of these means of dissociation caused degradation of the polypeptide chains.

Paludina vivipara hemocyanin (molecular weight 87×10^5) dissociated below pH 3.7 into smaller units and below pH 1.9 into still smaller units according to Ekwall (1942). In the range of pH 7.6 to 8.0 whole molecules, half molecules, and eighth molecules existed together, with the half molecules apparently somewhat unstable. Between pH 8 and 10 only eighth

molecules (molecular weight 11.3×10^5) were found, and at higher pH values these broke up still further. Using molecular frictional coefficients, Ekwall calculated the lengths of the whole hemocyanin molecule and the half molecule and found them to be the same (80 to 100 mμ). He concluded, therefore, that the splitting into half molecules must have occurred along the long axis of the molecule. Finally, he found a dependence of dissociation on the ionic concentration, and in phosphate buffers detected the presence of particles approaching in size the much larger virus proteins.

Electrophoretic Properties. Redfield's (1934) review includes a table of the isoelectric points of the hemocyanins from fifteen species of animals. Table III presents some of the same data and also some more recent results. Finally, data, not to be found in either of these places, will be mentioned in the following brief discussion.

Yasuzumi, *et al.* (1937) reported that the hemocyanins of *Cristaria plicata* and *Palinurus japonica* possessed isoelectric points between pH values of 4.5 and 4.9. Electrophoretic studies of the hemocyanin of *Helix pomatia* were carried out by Putzeys and van de Walle (1939) and they observed that the shape of the mobility −pH curves were significantly dependent on the ionic strengths of added electrolytes and particularly on calcium ion. Calculations correcting for the ionic effects of the electrolytes brought all curves to practical coincidence, with the isoelectric point falling just below a pH of 5. The following year Putzeys and van de Walle (1940) reported an isoelectric point of 5.06 for *Helix pomatia* hemocyanin with no change caused by adding sodium chloride in different ionic strengths. On the other hand, they found that magnesium, calcium, and barium ions caused an apparent upward shift in the isoelectric point. Rawlinson (1940) reported an isoelectric point of 4.60 for the hemocyanin of *Jasus lalandii*.

Tiselius and Horsfall (1939b) applied the scale method of boundary analysis to the electrophoresis of the hemocyanins of *Helix pomatia* and *Helix nemoralis* (see previous sub-section) and found an abnormal boundary spreading during the electrophoresis. However, after reversing the direction of the current for a time equal to the period of the original electrophoresis, the boundaries returned to their original positions and shapes. The abnormal boundary spreading was explained as being due to the simultaneous existence either of several stable individual substances of the same weight but slightly different electrochemical properties, or of several interchangeable forms closely related electrochemically and in equilibrium with each other.

Optical Properties. Investigations of the spectra of the hemocyanins have been made by various workers. The characteristic blue color of oxyhemocyanin appears to be due to an absorption band in the visible region of the spectrum. Svedberg and Hedenius (1933) reported that the

position of the band depended on the species. For example, the maximum absorption in the visible band by the oxyhemocyanin of *Helix* was found at 550 mμ, and the corresponding position for *Paludina* oxyhemocyanin was 600 mμ. Hence, Svedberg and Hedenius suggested that the chemical constitution of the active groups of these molecules might differ. In addition to this band which disappeared on deoxygenation (Roche and Dubouloz, 1933a, b) two other absorption bands occur in the ultraviolet region of the spectrum. One of these, having a maximum absorption at 346 mμ, was attributed to the prosthetic group by Dhéré (1920). This band also disappeared on deoxygenation and was replaced by a progressive absorption between 300 and 380 mμ according to Roche and Dubouloz. The other ultraviolet absorption band occurs at 278 mμ and is probably due to the protein moiety since a similar band has been reported for other proteins.

Rawlinson (1940) reported the existence of all three bands for the hemocyanin of *Jasus lalandii* with the oxyhemocyanin bands located at 558 and 335 mμ. The latter was found to decrease with decreasing pH and disappeared below pH 4. There has been some disagreement as to whether or not the absorption of hemocyanin solutions follows Beer's law (Quagliariello, 1922; Svedberg and Heyroth, 1929; Redfield, 1930).

Optical properties other than the absorption of light have also been of interest to various investigators. Using the hemocyanins of five species, Quagliariello (1920), Redfield (1934) and Rawlinson (1940) studied the relationship between the hemocyanin concentration and the refractive index of the solutions.

The crystals of *Octopus* hemocyanin were found (Henze, 1901) to be doubly refractive, and Snellman and Björnstahl (1941) reported a strongly positive streaming double refraction with the hemocyanin of *Helix pomatia*. This streaming double refraction was increased by increasing the viscosity. A shift in pH had little effect although ultracentrifugal analysis indicated a very marked dissociation.

Rawlinson (1940) observed that the hemocyanin of *Jasus lalandii* in 2.01 per cent solution at pH 7.0 and 20° C. had a specific rotation of −42.5° using the green line of the mercury arc.

Immunological and Enzymatic Properties. In recent years some interest in the immunological properties of hemocyanins has developed. This interest has resulted from the theory that the amount of antibody in the precipitin reaction is dependent on the molecular weight of the antigen. According to the proponents of this theory, *viz.*, Hooker and Boyd (1935, 1936, 1942), the molecular weight of the antigen is inversely proportional to the ratio of antibody to antigen in precipitates at the equivalence point. The high molecular weights of the hemocyanins have led to their use in

the examination of this theory. The above mentioned investigators, as well as Youmans and Colwell (1943), found that the injection of hemocyanin led to the production of antibodies in experimental animals. The combination of antibody with antigen (hemocyanin) resulted in precipitates in which the ratio of antibody to antigen was very low when compared to ratios observed with antigens of low molecular weight. Seastone, Loring, and Chester (1937) were able to sensitize guinea pigs to *Limulus polyphemus* hemocyanin with the production of anaphylactic shock on reinjection of the hemocyanin. Hooker (1938), using *Limulus* hemocyanin, reported that the formation of a precipitate with the antibody was greatly delayed over certain concentration ranges. He suggested the presence of dissociation components as a possible cause of this phenomenon.

Bhagvat and Richter (1938) reported that the bloods of *Cancer, Homarus, Loligo* and *Helix* contained a thermolabile constituent which caused the rapid aerobic oxidation of catechol and homocatechol. Attempts to purify the active principle led to the preparation of hemocyanins and of copper complexes derived from the hemocyanins. The activity of these products was higher than that of the unpurified blood proteins and was not reduced by recrystallization. Furthermore, they possessed a greater activity than equivalent quantities of inorganic copper. Bhagvat and Richter concluded that the hemocyanins of these species acted as pseudo-phenolases, but found no reason to believe that this pseudo-activity had any physiological significance.

III. The Oxidases

Within the past decade copper has been shown to be an essential part of the enzyme molecule in the case of three plant oxidases, *i.e.*, tyrosinase (polyphenol oxidase), laccase and ascorbic acid oxidase. All three of these enzymes have in common the property of catalyzing the direct oxidation of their respective substrates by atmospheric oxygen according to the general equation:

$$BH_2 + \tfrac{1}{2}O_2 \xrightarrow{\text{enzyme}} B + H_2O$$
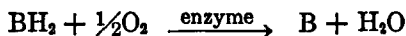
They do not function anaerobically, that is, they will not bring about the oxidation of their substrates by means of methylene blue or similar dyes, and their action does not result in the formation of hydrogen peroxide at least within the range of concentration that can now be experimentally measured.[3] Likewise, they are sensitive to cyanide. For these reasons they

[3] The fact that these systems do not produce hydrogen peroxide may be of primary biological importance. The problem has been investigated experimentally in the case of tyrosinase (Dawson and Ludwig, 1938) and ascorbic acid oxidase (Steinman and Dawson, 1942). There are also theoretical considerations that support the view that metallo-enzyme systems of this type do not produce hydrogen peroxide.

are classified as oxidases rather than dehydrogenases as the equation might suggest.

Two of these enzymes, tyrosinase and laccase, have been known since the last century, and many workers have investigated their properties and speculated as to their role in respiratory processes. On the other hand, ascorbic acid oxidase has been known for only a comparatively short time, being first described by Szent-Györgyi (1930) who called it "hexoxidase." Although it has been definitely established only recently (Boswell and Whiting, 1938; Baker and Nelson, 1943a) in the case of tyrosinase (polyphenol oxidase), it seems probable that all three of these oxidases function as respiratory enzymes in the life processes of certain plants.

Because of the early interest and rapid development of research in the field of the hemocyanin respiratory pigments, it may at first thought seem somewhat surprising that the essential role of copper in the above oxidases was not established until less than a decade ago. The reason for the slower progress in the case of the oxidases is apparent, however, when one considers the problem more closely. In the case of the hemocyanins, relatively large amounts of homogeneous or nearly homogeneous material were available early for study without very great effort. Their high concentration in the blue blood and body fluids of easily accessible marine animals made their isolation and purification a relatively simple problem. In several cases they were crystallized with ease. Because of these facts, knowledge of the physico-chemical properties of the hemocyanin copper proteins became relatively extensive, and to-day our knowledge is on as firm a basis as with any other class of proteins (see Section II).

In the case of the above oxidases, however, the situation is almost the reverse. As a corollary of their high catalytic activity, the oxidases occur in the plant tissues and fluids in exceedingly low concentration. For this reason, their isolation, concentration and purification has been a tedious and difficult task, leading ultimately, when successful, to only a few milligrams or less of highly active protein of questionable homogeneity. Many steps are involved in the purification process, and thus many chances exist for protein denaturation with concurrent loss in enzyme activity and homogeneity. Furthermore with each step there is the danger of contamination with copper.

Because of the difficulty of obtaining more than just a few milligrams of purified enzyme, our knowledge of the nature of these copper protein oxidases is very meager. No studies at all comparable to those described for the hemocyanin copper proteins (see Section II) have as yet been possible. As a consequence, little or nothing is known about the size, shape, and other physical properties of the oxidase molecules. Nothing is known about their amino acid content, and only in the case of laccase

is there a suggestion as to the nature of the prosthetic group. Although many investigators have worked with these oxidases, most of the studies have been concerned with the activity of the enzymes, and their function in natural respiratory processes. Such studies require only minute amounts of the enzymes, and frequently only crude or partially purified preparations have been used.

Thus for all three oxidases the literature contains results of numerous investigations pertaining to their substrate specificity and classification, to their conditions of optimum activity and methods of assay, to the mechanisms of the oxidative reactions which they catalyze, to the nature and effect of various inhibitors, to theories regarding their catalytic function, etc. It is not, however, within the scope or purpose of this volume to include a complete survey of such studies. Rather, it is the intention in this section to discuss the work bearing primarily on the nature of these oxidases as copper proteins. For a more extensive review of their enzymatic character, the reader is referred to Kastle (1910), Sutter (1936), Elvehjem, et al. (1939), Green (1940), Nelson and Dawson (1944), Tauber (1938).

1. Laccase

Yoshida (1883) reported that the latex of the Japanese lacquer tree (Rhus vernicifera) contained a thermolabile nitrogenous substance that caused the darkening and hardening of the latex in air. A few years later Bertrand (1894, 1895a) using the latex of the Indo-Chinese lacquer tree, isolated an enzyme which he called laccase, and showed that the darkening and hardening of the latex in air was due to the enzymatic oxidation of the polyphenolic bodies, urushiol and laccol, present in the latex. The structures of urushiol and laccol as proposed by Majima, et al. (1922) are given below:

OH

OH—OH
—$C_{15} H_{27}$
Urushiol

OH

OH—OH
—$C_{17} H_{31}$
Laccol
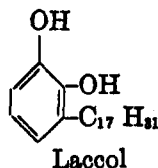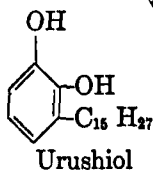
Bertrand observed that the enzyme was capable of catalyzing the aerobic oxidation of various polyhydric phenols and related compounds, such as hydroquinone, pyrogallol, guaiacum, etc., and used the color development with guaiacum as a means of demonstrating the presence of laccase in many plants (Bertrand, 1895b) and a large number of species of mushrooms (Bertrand and Bourquelot, 1895, 1896).

It was during this same period that Bourquelot and Bertrand (1895, 1896) and Bertrand (1896a) described the properties of another oxidase,

tyrosinase (see next section), which differed from laccase in that it catalyzed the aerobic oxidation of the amino acid tyrosine and certain other monophenols. Likewise tyrosinase was found to have no action on hydroquinone. Bertrand (1896b) observed that both laccase and tyrosinase were frequently found in the same plants and he used the coloration development during the oxidation of tyrosine and hydroquinone as a means of distinguishing the two oxidases. He also reported that they could be separated by taking advantage of the greater stability of laccase towards heat and alcohol.

During the course of his investigations Bertrand (1897a) observed that laccase preparations always contained manganese, and he believed that the oxidizing power of the laccase was proportional to the amount of manganese present. Furthermore he found that oxidations accomplished by laccase were greatly accelerated by small amounts of manganous salts, and that no other metal was capable of accelerating the oxidation brought about by laccase. Bertrand (1897b) was disposed therefore to regard manganese as a coenzyme of laccase. These findings and views of Bertrand stimulated interest in the oxidases, and many workers at the turn of the century concerned themselves with the role and nature of the oxygen catalysts involved in biological oxidations (Kastle, 1910). In more recent years, several investigators (Brooks, 1934; Suminokura, 1936; Brooks, 1937; Graubard, 1939; Gregg and Miller, 1940) have studied the activity of laccase from different sources on a wide variety of substrates. Such studies have made possible a comparison of laccase activity with that of other oxidases on the basis of substrate specificity and conditions for measurement of optimum activity.

With the development of better methods of purification, the role of manganese with laccase became doubtful. Suminokura (1930) reported that manganous salts had no effect on the action of laccase from Japanese lac and concluded that manganese was therefore not a coenzyme of laccase. Fleury and Campora (1934) purified laccase by several methods and found such a very low content of manganese and iron in their highly active purified preparations that an essential role of these metals in the enzyme seemed doubtful. The final answer to the problem, in reference to the metal component, however, came five years later.

Keilin and Mann (1939a) isolated from the latex of the Indo-Chinese lacquer tree (*Rhus succedanea*) a highly purified blue copper protein that had all the properties of the laccase in the crude latex. The acetone-precipitated powder of the latex was extracted with water, and the laccase was isolated, purified and concentrated by a method similar to that previously used by the same investigators in the purification of polyphenol oxidase from the cultivated mushroom (Keilin and Mann 1938a). The best laccase preparation had a copper content of 0.154 per cent and contained no

hematin, iron or manganese. The copper was present in non-dialyzable form and was essential to the enzyme activity as shown by the fact that the activities of different preparations were proportional to their copper content. Furthermore, the activity was strongly inhibited by potassium cyanide, hydrogen sulfide, sodium azide and diethyldithiocarbamate. However, carbon monoxide was without effect on the enzyme. Keilin and Mann reported that this enzyme preparation contained about 55 per cent polysaccharide on a dry weight basis, which was in agreement with a nitrogen content corresponding to 45 per cent protein, *i.e.*, 6.45 per cent nitrogen. They pointed out that if the polysaccharide was assumed to be an impurity, the copper content of the homogeneous laccase protein would then be about 0.34 per cent, a value identical to that reported for crystalline hemocuprein (Keilin and Mann, 1938b).

Keilin and Mann (1939a) compared the properties of this laccase preparation with those of the polyphenol oxidase which they had previously isolated in purified form from the cultivated mushroom and had shown to be a copper protein. Several marked differences were immediately apparent. The laccase oxidized *p*-phenylenediamine better than catechol, whereas polyphenol oxidase had little or no action on *p*-phenylenediamine and showed maximum activity with catechol. The laccase oxidized hydroquinone but had no action on the monophenols, *p*-cresol and tyrosine, whereas the polyphenol oxidase did not oxidize hydroquinone and slowly oxidized the monophenols after a lag period. Crude laccase preparations were found to oxidize ascorbic acid directly, but on purification of the enzyme the ability to oxidize ascorbic acid became progressively weaker. They reported that the ability to oxidize ascorbic acid could be restored to purified preparations by the addition of *p*-phenylenediamine but not by catechol. On the other hand, the polyphenol oxidase oxidized ascorbic acid only through a carrier such as catechol. The laccase was found to be much less active than the mushroom polyphenol oxidase as evidenced by a comparison of the Q_{O_2} values at 20° using *p*-phenylenediamine and catechol as substrates respectively, *i.e.*, 40,000 for laccase and 1,160,000 for polyphenol oxidase. The laccase Q_{O_2} value, obtained by making allowance for the polysaccharide as an impurity, is about the same as that reported for the potato polyphenol oxidase (Kubowitz, 1937, 1938). Of particular interest is the fact that whereas carbon monoxide strongly inhibits polyphenolase activity it was found to have no effect on the activity of laccase.

Highly active solutions of the purified laccase were found to possess a distinct blue color. For example, Keilin and Mann (1939a) reported that a laccase solution containing 0.015 mg. of enzyme per cc. had a blue color equivalent to that of a 7 per cent copper sulfate solution. This color disappeared on the addition of substrate such as hydroquinone or ascorbic

acid and was restored on shaking the leuco-enzyme with air. At first, by analogy with hemocuprein and the hemocyanins, it was believed that the blue color was in some way a property of the copper prosthetic group of the enzyme. However, as the result of a more recent investigation, Keilin and Mann (1940a) have concluded that such is not the case. In this later investigation it was found possible to separate the blue pigment from laccase in a condition free of copper and protein but always associated with carbohydrate material.

In order to further check the relationship between the color and the enzyme, Keilin and Mann (1940a) also isolated and purified laccase from other sources, i.e., the Japanese and Burmese lacquer trees (*Rhus vernicifera* and *Melanorrha usitata*). In all cases they found the purified and active enzyme to be a blue copper protein, the color of which was not due to copper but to a closely associated blue pigment. The copper content of their best preparation was 0.24 per cent. As previously stated, the blue pigment could be removed in a condition free of copper and protein but always containing carbohydrate. The separated pigment could be reversibly reduced to the leuco compound, but was irreversibly decolorized by strong acids and by boiling. The fact that in no case were they able to obtain an active enzyme free of the blue pigment led them to conclude that the pigment was in some way connected with the normal activity of the enzyme. A connection between the enzyme and pigment was also suggested by the fact that the blue pigment of the active enzyme was more sensitive to acids and to heat than the separated pigment.

No statements were made by Keilin and Mann as to whether or not it was possible to recombine the protein, copper and blue pigment to yield an active enzyme. At the present time the molecular weight and other physico-chemical properties of the protein moiety of the enzyme are unknown. Likewise the chemical nature of the blue pigment is unknown, except that it appears to be carbohydrate in nature. Further investigations must be completed before it will be possible to say definitely whether the pigment is intimately bound to the enzyme as an additional prosthetic group, or merely acts as a carrier between the enzyme and certain substrates.

Kidney "Laccase." Keilin and Hartree (1935) reported that fresh kidney tissue, when ground with sand and suspended in buffer solution (pH 7.8), possessed the properties of a polyphenol oxidase in that it promoted the aerobic oxidation of polyphenols such as dihydroxy phenylalanine (dopa), catechol, pyrogallol and adrenaline, but had no action on monophenols such as phenol, p-cresol and tyrosine. The oxidation reaction, which was inhibited by cyanide, led to the formation of colored pigments. Others,

including Bing, Zucker, and Perkins (1941) have also presented evidence for the existence of an aerobic polyphenolase in kidney extracts.

Cadden and Dill (1942) described a cell-free kidney extract that possessed the ability to catalyze the aerobic oxidation of hydroquinone but not of monophenols. The active principle appeared to be enzymatic in nature in that it was partially inactivated by heat, precipitated by alcohol or acetone, and was not dialyzable. Because of these properties Cadden and Dill suggested that the active constituent might be a polyphenol oxidase of the laccase type.

Following up this suggestion, Baker and Nelson (1943b) reexamined the laccase nature of the active principle after further purification. They obtained a preparation containing 15.6 per cent nitrogen and 0.03 per cent copper in non-dialyzable form, indicating that it was a crude copper-bearing protein. The copper content increased as the purification progressed. In addition to hydroquinone, the kidney preparation catalyzed the aerobic oxidation of catechol, homocatechol, and adrenaline, although its activity towards these substances was less than towards hydroquinone. It showed no activity towards ascorbic acid, p-phenylenediamine, dihydroxymaleic acid, tyramine, p-cresol, pyrogallol, homogentisic acid, or 2, 3-dihydroxy naphthalene. The activity towards hydroquinone was seriously inhibited by small amounts of potassium cyanide and sodium azide, and prolonged heating or the addition of acid caused irreversible loss in activity. After examining the oxidation of hydroquinone, Baker and Nelson concluded that "the enzyme can hardly be classed as a true oxidase, $i.e.$, belonging to the same class as cytochrome oxidase, tyrosinase, laccase, sweet potato catecholase, ascorbic acid oxidase, etc., because it brings about only an incomplete reduction of the oxygen molecule resulting in the formation of hydrogen peroxide."

2. *Tyrosinase — Polyphenol Oxidase*

As previously pointed out, tyrosinase is a phenol oxdizing enzyme that was first recognized by Bourquelot and Bertrand (1895) while they were investigating various plants for the presence of laccase. They first found it in the fungus *Russula nigricans* to which they were attracted by the fact that its tissue on injury showed rapid discoloration. The following year Bertrand (1896a) identified the substrate in the fungus as the amino acid tyrosine and therefore called the enzyme tyrosinase. Subsequent studies by Bertrand and many other workers have revealed that the enzyme occurs widely spread in nature. The presence of phenolases of the tyrosinase type has been recognized for a long time in most of the plants and vegetables which show the darkening reaction on injury of the tissue, $i.e.$, potato, mushrooms, banana, apple, pear, etc. In more recent years, interest

has developed in the role of such phenolases in the respiration of other plants such as tea (Roberts, 1942) and tobacco (Smirnov and Pshennova, 1941). The presence of tyrosinase in insects and other invertebrates was established early (Kastle, 1910). Although its presence in higher animals has been suggested numerous times, confirmation of such claims has been difficult.

Hegeboom and Adams (1942) demonstrated that mouse melanoma tumor was a relatively rich source of tyrosinase and dopa (dihydroxyphenylalanine) oxidase. The presence of the latter enzyme in skin was first suggested by Bloch (1929). More recently the dopa oxidase activity of skin extracts has been studied by Ginsberg (1944). Charles and Rawles (1940) reported that extracts of black chicken feather germs showed a cyanide sensitive heat labile tyrosinase activity.

The enzyme tyrosinase, as isolated from the mushroom or potato, is characterized by the fact that it possesses the ability to catalyze the aerobic oxidation of certain monophenols as well as o-dihydric phenols. In this respect it differs from laccase which has no action on monophenols. Whereas tyrosinase catalyses best the oxidation of o-dihydric phenols such as catechol and is without action on p-dihydric phenols such as hydroquinone, laccase catalyses the oxidation of both types of dihydric phenols with preference for the hydroquinone type. These specificity differences between the two enzymes were recognized by Bertrand (1896b) and numerous later workers, who showed that crude tyrosinase preparations possess the ability to oxidize many other phenols besides tyrosine. These include monohydric phenols such as phenol, p-cresol, p-hydroxyphenylacetic acid, p-hydroxyphenylamine and derivatives, etc., and o-dihydric phenols such as catechol, pyrogallol, 4, 5-dihydroxyphenylalanine, protocatechuic acid, adrenaline, etc. As previously stated, the enzyme is without action on p-dihydric phenols, and the same is true of m-dihydric phenols such as resorcinol.

Because of its ability to catalyze the aerobic oxidation of both monohydric and o-dihydric phenols, by apparently different mechanisms, there has been considerable controversy over naming and classifying this oxidase. In recent years several investigators have observed that during the isolation and purification of the enzyme, the ratio of the o-dihydric phenolase activity to the monophenolase activity can be greatly increased over that existent in the expressed juice of the plant. This apparent separation of the two activities has led certain workers to abandon the name tyrosinase and introduce the name polyphenol oxidase. This group attributes the monophenolase activity to some unknown and easily lost factor. However, during the past five years considerable evidence that the two activities belong to the same enzyme protein complex has been accumulated in these

laboratories. Thus the substitution of the name polyphenol oxidase for the earlier name tyrosinase cannot be accepted without challenge. A recent review of the work on tyrosinase (Nelson and Dawson, 1944) presents the evidence pertaining to this controversy. For reasons outlined there, the present authors feel that until more data are obtained, the original name, tyrosinase, is to be preferred.

Two plants which are particularly rich in the enzyme and which, therefore, serve as good starting materials for its isolation and purification are the common, edible mushroom, *Psalliota campestris,* and the ordinary white potato, *Solanum tuberosum.* From a preparational view-point the mushroom is the better of these two sources; since aqueous extracts of the potato, besides containing large amounts of inert globulin-like proteins which are difficult to remove from the enzyme, also contain relatively large amounts of another enzyme, *i.e.,* peroxidase. The methods employed for isolating and purifying the enzyme from the crude expressed juices or aqueous extracts involve operations such as fractional salt precipitations, fractional precipitations with alcohol or acetone, adsorption to agents such as alumina and calcium phosphate gel, elution, precipitation with lead acetate, dialysis, etc. Throughout these operations the enzyme behaves in all respects like a water soluble protein. It is very sensitive to elevated temperatures and low pH, and all operations must be carried out above pH 5 and in the vicinity of room temperature or lower to insure a respectable yield of the enzyme. Due to the presence of a natural substrate along with the enzyme, the expressed juices of the mushroom or potato darken very rapidly on exposure to air. During this process, enzyme activity is frequently lost and it is very difficult to remove the color in subsequent purification operations. It is advisable, therefore, to remove as much as possible of the natural phenolic substrate (or substrates) at an early stage in the purification. Experience has shown that a cold acetone wash (dry-ice chilled) of the ground tissue before extraction with water or dilute salt solution helps to yield colorless enzyme preparations. Highly purified preparations in concentrated solution have a light yellow-green color.

Interest in the mode of action of tyrosinase and the nature of its reaction products has been high since the outstanding work of Raper (1928) in connection with the enzymatic conversion of tyrosine into melanin. Numerous workers since that time have investigated the reactions of tyrosinase with other phenols, and have attempted to throw light on the role of tyrosinase in natural pigmentation and respiratory processes. Such studies, concerned primarily with the enzymatic functions of tyrosinase, are not, however, within the scope of this review. They have been recently reviewed elsewhere (Nelson and Dawson, 1944). Only those studies of tyrosinase or

polyphenol oxidase pertaining to its nature as a copper protein will be discussed here.

The first experimental evidence that copper was an essential part of the phenol oxidase of the potato was provided by Kubowitz (1937, 1938), who found a linear relationship between the copper content of his preparations and the catalytic activity (toward catechol) of the enzyme. The activity of his best enzyme preparation, which had a copper content of 0.2 per cent, was inhibited by agents which chemically combine with copper, such as cyanide, diethyldithiocarbamate, salicylaldoxime, and carbon monoxide. He proved that the copper was essential to the activity of the enzyme by removing it and demonstrating that the copper-free protein no longer possessed the ability to catalyze the oxidation. He found that the copper could be removed by a treatment similar to that which removes copper from the hemocyanin molecule, i.e., treatment with cyanide followed by dialysis, and just as in the case of the hemocyanin copper protein, the resolution was reversible. Practically complete enzyme activity could be restored by adding excess copper ion to the inactive protein. However, to obtain complete restoration of the activity, Kubowitz found that he had to add to the copper-free protein about ten times the theoretical amount of copper ion. He interpreted this fact to mean that only part of the copper combined with the protein at the "catalytic point of attachment" and the rest was bound by groups not concerned with the enzymatic activity. Other divalent metals such as iron, cobalt, nickel, manganese, and zinc were found to be ineffective in restoring the enzyme activity when added to the copper-free protein.[4]

As the result of carbon monoxide inhibition studies, Kubowitz concluded that the copper in the enzyme, as isolated, is in the cupric state. He postulated that in its reaction with catechol, the enzyme copper is reduced to the cuprous state, which in turn is reoxidized by molecular oxygen. The two stage reaction may be represented as:

$$4 \text{ Cu}^{++} \text{ (enzyme)} + 2 \text{ catechol} \longrightarrow 4 \text{ Cu}^{+} \text{ (enzyme)} + 2 \text{ } o\text{-quinone} + 4\text{H}^{+}$$
$$4 \text{ Cu}^{+} \text{ (enzyme)} + 4\text{H}^{+} + \text{O}_2 \longrightarrow 4 \text{ Cu}^{++} \text{ (enzyme)} + 2\text{H}_2\text{O}$$
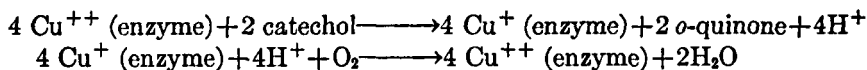
Following Kubowitz' work on the oxidase of the potato, Keilin and Mann (1938a) found that the phenol oxidase of the common, edible mushroom was also a copper protein compound. They used the cultivated mushroom (*Agaricus campestris*) as starting material and isolated about 10 mg. of the enzyme in highly purified form. Keilin and Mann observed that the

[4]Tenenbaum and Jensen (1943) have recently reported that under certain conditions salts of iron, cobalt, and manganese, as well as those of copper, can "reactivate" mushroom tyrosinase which has been "inactivated" by potassium cyanide, sodium diethyldithiocarbamate, or potassium ethyl xanthate.

activity of the enzyme preparation did not become proportional to its copper content until after several steps in the purification process. On the basis of its copper content, as compared with that of various crystalline hemocyanins, they considered their best preparation (0.3 per cent copper) to be pure. This preparation was found to be completely free from hematin, and all of its oxidation reactions were strongly inhibited by potassium cyanide, hydrogen sulfide and carbon monoxide. On a copper basis, their mushroom preparation was about twenty times as active as the potato oxidase described by Kubowitz.

Keilin and Mann emphasized that whereas their crude mushroom oxidase catalyzed the aerobic oxidation of monophenols, this property was rapidly lost during the purification process. As a consequence, they found the purified enzyme to be specific for the oxidation of o-dihydric phenols such as catechol and pyrogallol. They chose, therefore, to call the enzyme polyphenol oxidase, and dismissed the monophenolase activity with the statement that it "probably requires the presence or the gradual formation of an additional factor, the nature of which remains still to be determined."

For reasons which have been discussed elsewhere (Nelson and Dawson, 1944), it has seemed desirable to workers in the authors' laboratory to retain the name tyrosinase even for purified enzyme preparations possessing marked activity towards catechol with little activity towards monophenols such as p-cresol and tyrosine. Such preparations have, therefore, been called high catecholase preparations of tyrosinase. Ludwig and Nelson (1939) described several preparations of this type from the common mushroom (*Psalliota campestris*), and found, as had Keilin and Mann (1938a), that the catecholase activity became proportional to the copper content of the enzyme after several steps in the purification. No proportionality between the copper content and monophenolase (cresolase) activity existed, for the ratio of catecholase to cresolase activity varied from one preparation to another. Judging from the copper contents and the Q_{O_2} values, their best preparations were about one-third as pure as the best preparation of Keilin and Mann (0.3 per cent copper, $Q_{O_2} = 1,160,000$).

The following year Parkinson and Nelson (1940) reported that, by changing the purification procedure somewhat, it was possible to obtain from the same source, the common mushroom, purified enzyme which was relatively high in monophenolase (cresolase) activity, i.e., the ratio of the two activities in the purified enzyme was comparable to that found in the expressed juice or crude extract of the mushroom. They referred to such preparations, therefore, as high cresolase preparations of tyrosinase. Parkinson and Nelson made a large number of preparations of this type and showed that both activities were proportional to the copper content of the purified enzyme. Their best preparations contained about 0.1 per

cent copper. These findings led Parkinson and Nelson to conclude that "tyrosinase as it exists in the common mushroom is one enzyme complex rather than two separate independent factors."

None of the above workers, using the common mushroom as a source of the enzyme, succeeded in obtaining enough purified enzyme to attempt an investigation of the chemical nature of the prosthetic group, or to study the physico-chemical properties of the protein moiety of the tyrosinase molecule. The latter course of study has received some attention more recently in the authors' laboratory. Before describing these experiments, however, it seems advisable to discuss the tyrosinase from the wild mushroom.

Dalton and Nelson (1939) isolated from the wild mushroom (*Lactarius piperatus*) a highly purified tyrosinase preparation that possessed as its most striking characteristic a high monophenolase activity (*p*-cresol) as compared with its activity towards catechol. The ratio of the two activities remained constant throughout the isolation and purification process and both activities became proportional to the copper content after several purification steps. Their best preparation contained 0.23 per cent copper and no iron. Concentrated solutions were practically colorless, but two absorption bands were observable in the ultraviolet region of the spectrum; a pronounced band due to protein at 273 mμ and a weak band at about 330 mμ probably due to copper.

By using a procedure similar to that usually employed for crystallizing hemocyanins, colorless, well shaped crystals belonging to the isometric system were obtained from rather crude as well as purified preparations of the enzyme. In no case, however, was more than 10 per cent of the dry weight of a purified preparation obtainable in crystalline form. The crystals contained 0.25 per cent copper, 13.6 per cent nitrogen and in solution showed the same ultraviolet absorption as did the purified enzyme solution. They were insoluble in water, dilute acids, and salt solutions, but when freshly prepared were soluble in dilute alkali (secondary sodium phosphate solution, pH 8). Solutions of the crystals were only slightly active towards *p*-cresol and catechol; but on standing such solutions showed a tendency to increase in activity, and the ratio of the two activities remained the same as that of the uncrystallized enzyme. Dalton and Nelson concluded that the enzyme and the crystals were very closely related: "Either the enzyme becomes, for some unknown reason, inactive when it is crystallized, or an inactive form, such as a proenzyme, accompanies the enzyme as it is purified."

As previously stated, an attempt has been made recently in the authors' laboratory to gain some information about the physico-chemical properties of the protein moiety of the tyrosinase molecule (Mallette, Ames, and

Dawson, 1945). These studies have been directed towards determining if possible whether or not the two enzymatic activities of mushroom tyrosinase are properties of a single copper protein. Electrophoresis experiments with crude preparations have been carried out using a large-capacity electrophoresis cell (Tiselius type) which permitted division of the cell contents at the completion of the experiment into several fractions large enough to allow for enzyme and protein assay. Parallel experiments involving highly purified tyrosinase have been carried out using a small capacity Tiselius apparatus equipped to analyze the protein boundaries optically.[5] These experiments revealed that with crude preparations the ratio of the two activities varied irregularly from one section of the cell to another, whereas, with highly purified tyrosinase preparations, the ratio remained relatively constant throughout the cell. This would suggest that in the case of the crude preparations the activity ratio as measured was markedly influenced by accompanying inert proteins. In agreement with this interpretation is the fact, observed by many workers, that the ratio of the two activities is most easily changed during the early stages of the purification process.

The variation with pH of the electrophoretic mobility of the main component of a purified high catecholase preparation (C172-5AB) is shown in Table V. The mobility μ is that of the descending boundary, and the

TABLE V

Mobility of Main Component of Purified High Catecholase Preparation

pH	mobility, μ cm.^{2}/sec./volt	Homogeneity per cent
4.97	2.4×10^{-5}	100
7.58	4.6×10^{-5}	93
8.90	7.9×10^{-5}	90

homogeneity is expressed as the per cent of the total protein represented by the active component, as determined by optical analysis of the boundary during electrophoresis at the pH indicated. The results show that the isoelectric point of the active component is considerably below pH 5. Kubowitz (1937) reported that his purified potato tyrosinase had an isoelectric point of pH 5.4. The enzyme loses its copper in solutions buffered below pH 5, and for this reason electrophoretic mobility or homogeneity studies are impossible at a lower pH value.

[5] All electrophoresis runs in the small capacity Tiselius apparatus and all ultracentrifuge studies were made by Dr. D. H. Moore, College of Physicians and Surgeons, Columbia University, New York, New York.

TABLE VI

*Activity and Electrophoretic Data for Three Purified Tyrosinase Preparations of the High Catecholase Type**

Enzyme Preparation	Cu.	Activity/γ Cu.		Activity/mg. dry wt.		Ratio	Electrophoresis Data		
		Cat.	Cres.	Cat.	Cres.	Cat./Cres.	μ	pH	Homogeneity
	per cent								per cent
C211-228F2	0.21	2130	48	4400	95	48	6.310^{-5}	7.58	100
C175-BI	0.10	2100	104	2150	107	20	5.310^{-5}	7.71	95
C172-5AB	0.10	2300	137	2270	135	17	4.610^{-5}	7.58	93

* Data for preparations from the mushroom, *P. campestris* (Mallette, Ames and Dawson, 1945). The catecholase activity units (= *Cat.*) are those of the chronometric method (Miller, Mallette, Roth, and Dawson, 1944). The cresolase activity units (= *Cres.*) are those of Gregg and Nelson (1940). The activity values per mg. dry weight when multiplied by 600 give Qo_2 values.

Some of the electrophoresis results obtained with three mushroom tyrosinase preparations of the high catecholase type are shown in Table VI. It can be seen that with preparations of this type, where the ratio of catecholase activity to cresolase activity was high, only the catecholase activity was proportional to the copper content. Likewise, it is to be noted that the electrophoretic mobility of the enzyme increased as the ratio of the catecholase activity to the cresolase activity increased. In other words the surface character of the molecule, upon which the mobility was mainly dependent, varied with the ratio of the two activities. These results agreed with those obtained using a preparation having a low ratio of catecholase to cresolase activity, *i.e.*, a high cresolase preparation. The preparation had an activity ratio ,of 1.6, and its main component, constituting 70–80 per cent of the total protein as determined electrophoretically, had a mobility of 2.9×10^{-5} cm.²/sec./volt at pH 7.71. A sample of this component was found to have the same activity ratio as the original preparation. The faster moving component, constituting 20–30 per cent of the total protein, had no activity.

It should be pointed out that the most highly purified preparation (C211-228F2) of those shown in the table, using the activity and copper content as the basis of purity, was found to be only about 85 per cent homogeneous in the ultracentrifuge, and the light-weight heterogeneous material (about 15 per cent of the total protein) was found to possess no enzymatic activity. Although more than one explanation of these data may be possible, the following explanation has been suggested by Nelson and Dawson (1944).

The activity, copper, and homogeneity data of Table VI can be correlated in terms of differences in molecular weights of the copper protein enzyme. The active component of C211-228F2 was found to have a

molecular weight of about 100,000 using the ultracentrifuge. Since the preparation contained 0.21 per cent copper, and the active component constituted about 85 per cent of the total protein, it might be inferred that the pure active component of the preparation contained about 0.25 per cent copper. This copper content corresponds to four atoms of copper per enzyme molecule having a molecular weight of 100,000. It follows that a four copper atom enzyme molecule having a molecular weight of about 200,000 would contain about 0.13 per cent copper. Unfortunately, it was not possible to determine in the ultracentrifuge the homogeneity and molecular weight of the two preparations, containing 0.1 per cent copper, shown in the table. However, a molecular weight of 200,000 for mushroom tyrosinase had previously been reported by Tenenbaum (1940), who used the diffusion method of Northrop and Anson (1928–29). Although the copper content of the preparation used by Tenenbaum was not reported, the fact that the preparation apparently had a considerably lower catecholase to cresolase activity ratio than C211-228F2 is of interest.

As a result of these and other data Nelson and Dawson (1944) concluded that tyrosinase is one copper protein entity or complex possessing two enzymatic activities. The ratio of these activities depends on factors such as the size, shape, surface characteristics, etc., of the protein complex. A change in ratio during preparative stages would thus be interpreted as being due to chemical or physical modification or fragmentation of the protein complex.

Recently Sreerangachar (1944) purified the phenol oxidase of tea leaf and found it contained 0.08 per cent copper. His best preparation which had been concentrated about 800 times was completely free from iron, and he reported that it had an activity about one-tenth of that found for the potato oxidase by Kubowitz (1938). It will be recalled that Keilin and Mann (1938a) reported that on a copper basis, their mushroom preparation was about twenty times as active as the potato oxidase of Kubowitz. It might be noted that on the same basis the preparation C211-228F2 of Table VI is about three times as active as that of Keilin and Mann. Such comparisons of the activity of an enzyme from different sources should, however, be made with considerable caution. Not only does the reported activity depend very markedly on the way it was measured but also on the extent of the alteration and chemical breakdown of the enzyme protein that may have occurred during the isolation and purification.

Protyrosinase. Bodine, *et al.*, have published during the past few years a series of papers dealing with their studies of an inactive form of tyrosinase, which they have termed protyrosinase (Bodine and Boell, 1935; Bodine, Allen, and Boell, 1937; Bodine and Allen, 1938; Allen and Bodine, 1941a). This material occurs in the eggs of grasshoppers, *Melanoplus differentialis,*

and in the larvae of the meal worm, *Tenebrio molitor*. In the case of the grasshopper, Allen, Ray, and Bodine (1938) found that the protyrosinase begins to form in the egg immediately following the diapause period according to a simple autocatalytic reaction. To obtain the protyrosinase from grasshoppers, the diapause eggs were triturated in 0.9 per cent sodium chloride solution, centrifuged, the lipoidal layer removed, and the fluid brought to pH 6.8. After fractionating with ammonium sulfate, a precipitate was obtained which was dissolved in 0.9 per cent sodium chloride and dialyzed against the same concentration of sodium chloride. The resulting solution contained the inactive form of tyrosinase but not active tyrosinase.

On treatment of the saline solution of protyrosinase with detergents, such as sodium oleate, aerosol OT (dioctyl sodium sulfosuccinate), or duponol (sodium dodecyl sulfate, etc.), active tyrosinase results. Several substances such as chloroform, acetone, urethane and urea, as well as heating the saline solution to 60–70°C. were also found to activate the protyrosinase (Bodine and Allen, 1941). It was also found (Allen, Otis, and Bodine, 1943) that vigorous shaking of the saline solution of protyrosinase resulted in irreversibly changing the latter into definite proportions of protyrosinase, active tyrosinase, and inactive products. Bodine and Tahmisian (1943) reported that salts of mercury, gold, platinum, and palladium tend, when present within definite concentrations, to change protyrosinase into the active enzyme. Higher concentrations, however, proved toxic to the active enzyme but not to the protyrosinase.

The common meal worm, *Tenebrio molitor*, has long been recognized as a good source of tyrosinase (Raper, 1928, 1932). Bodine and Allen (1941), however, found that the oxidase occurs in the worm entirely in the inactive protyrosinase form. The reason that the worms serve as a good source for active tyrosinase appears to be due to the fact that in extracting the oxidase the worms are usually ground with water saturated with chloroform. As mentioned above chloroform has been shown to convert protyrosinase into the active tyrosinase. Bodine and Allen (1941) also found that protyrosinase occurs together with active tyrosinase in the serum of the crayfish. No protyrosinase could be found in the common mushroom and potato, the usual sources of the active enzyme.

Protyrosinase appears to be more sensitive to pH changes than tyrosinase. It shows instability at pH 7.3 and at pH 9.3 is changed irreversibly into a mixture of tyrosinase and an inactive product. It is also less stable than tyrosinase at pH 4.8 (Allen, Otis, and Bodine, 1942).

Allen and Bodine (1941b) have demonstrated that copper is an essential constituent of protyrosinase from grasshopper eggs. They were led to investigate the role of copper in the protyrosinase by the fact that the proenzyme yielded an active tyrosinase which was inhibited by cyanide,

diethyldithiocarbamate, and carbon monoxide. They found that protyrosinase from which the copper had been removed by the potassium cyanide method of Kubowitz (1938) could no longer be converted into active tyrosinase by means of aerosol. However, treatment of the copper-free protyrosinase with excess copper sulfate in 0.9 per cent sodium chloride solution yielded protyrosinase as evidenced by the fact that treatment with aerosol produced 98 per cent of the expected tyrosinase activity. Just as found by Kubowitz (1938), salts of iron, cobalt, nickel, manganese, and zinc were unable to replace those of copper.

3. *Ascorbic Acid Oxidase*

The first isolation of vitamin C in crystalline form came about as the result of the interest of Szent-Györgyi in the mechanism of biological oxidation. From adrenal cortex and from orange juice Szent-Györgyi (1928) isolated crystals of a substance of carbohydrate nature which he called "hexuronic acid." This crystalline acid, which had remarkable reducing properties, and which was readily oxidized by peroxidase in the presence of a phenol and hydrogen peroxide, was later shown to be identical with crystalline vitamin C (King and Waugh, 1932). The name, ascorbic acid, was suggested by Szent-Györgyi and Haworth (1933) during the course of studies leading to the establishment of its chemical structure and synthesis (see review by King, 1936).

Szent-Györgyi (1930, 1931) prepared from cabbage leaf an enzyme "hexoxidase" which was relatively specific in its ability to catalyze the direct aerobic oxidation of "hexuronic acid." The enzyme was highly active and because of the reversible nature of the oxidation (involving the removal of two hydrogen atoms from hexuronic acid), Szent-Györgyi believed that the reaction occupied an important position in the normal respiration of many plants. The enzyme had no action on phenols or polyphenols and did not function under anerobic conditions with dyes as hydrogen acceptors.

Following this description of "hexoxidase" from cabbage by Szent-Györgyi, several investigators reported the existence of an ascorbic acid oxidizing enzyme in other plants. Zilva (1934) reported the presence of a similar enzyme in seedling apples, and Tauber *et al.* (1935a, b) isolated from Hubbard squash an oxidase which they felt was different than that of Szent-Györgyi. They suggested the name "ascorbic acid oxidase" for their preparation. Srinivasan (1936) prepared the enzyme from the pods of the drumstick tree (*Moringa pterygosperma*), and Hopkins and Morgan (1936) reported its presence in cauliflower juice. Among the first to supply evidence of the widespread occurrence of this type of an enzyme were Kertesz, Dearborn, and Mack (1936) who demonstrated the presence of a

heat labile ascorbic acid oxidase in cabbage, squash, peas, pumpkin, string beans, lima beans, sweet corn, Swiss chard, carrots, parsnips, and spinach. Stone (1937) added banana, cucumber, marrow (vegetable) and potato to this list but failed to find the enzyme in cantaloupe, green pea, lettuce, lucerne, onion, spinach, and watermelon. Silverblatt and King (1938) demonstrated that an adequate supply of air was necessary while testing for the "oxidase" activity in plant juices. Using an aeration technique, they observed a rapid oxidation of vitamin C in the presence of cantaloupe juice, and suggested that Stone's (1937) failure to detect the enzyme in cantaloupe and lettuce was probably due to insufficient aeration during the measurement.

During this period the inhibitory effect of certain substances (cyanide, sulfide, carbon monoxide, diethyldithiocarbamate, etc.) on the activity of ascorbic acid oxidase preparations from a variety of sources was noted by a number of investigators. (See reviews by Tauber, 1938; and Elvehjem, et al., 1939). Many of these earlier investigations were primarily concerned with the mechanism and kinetics of the enzymatic oxidation of vitamin C. However, as inferred previously, the prime concern of this paper is to review the work on ascorbic acid oxidase that pertains to its copper protein nature.

The catalytic effect of copper salts on the aerobic oxidation of vitamin C was observed by Hess and Unger (1921) and was studied more extensively by Barron, DeMeio and Klemperer (1936). Barron, Barron and Klemperer (1936) questioned the existence of a specific oxidase in plant juices and suggested that the destruction of vitamin C in biological fluids was brought about mainly by the catalytic action of ionic copper and hemochromogen catalysts. Using inhibitors that were more specific in their action on copper than those used by Barron et al., Stotz, Harrer, and King (1937) clearly demonstrated the dependence of the catalytic activity of squash and cauliflower juices on their copper content. They believed that the catalytic effect of copper in such plant juices was enhanced by its combination with protein but argued that the name "ascorbic acid oxidase" should   applied to this "enzyme" because of its unspecific character.
        this view, they found that a mixture of copper and albumin
        any of the characteristic properties of the "enzyme," such as
        of heating, the effect of pH, and the effect of several copper
        including diethyldithiocarbamate and cyanide. In a subsequent
        McCarthy, Green, and King (1939) compared the substrate speci-
        and inhibition characterisitics of the copper protein "oxidases" from
        cumber and potato. Whereas, both "oxidases" were inhibited by spe-
cific copper inhibitors, they found that the cucumber oxidase was without action on catechol, and confirmed Kubowitz' (1938) observation that the

potato oxidase did not catalyze the direct oxidation of ascorbic acid. In support of the suggestion that something other than copper, such as protein, was involved in the "oxidase" action of certain plant juices, Straub (1938) found that the ash of cucumber juice was 5 to 10 times less active than the whole juice in catalyzing the autoxidation of ascorbic acid. Also Spruyt and Vogelsang (1938) concluded that "ascorbic acid oxidation and copper do not go hand in hand, and the oxidation is certainly not exclusively to be ascribed to the presence of copper."

Although there was little direct evidence at that time, Tauber (1938) and Graubard (1939) were among those who supported the view that the biological oxidation of ascorbic acid was due to a specific copper protein enzyme which they termed "ascorbic acid oxidase." Matsukawa (1940) studied the oxidation of ascorbic acid by copper-albumin mixtures and found that the rate of oxidation was dependent on the ratio of albumin to copper in the reaction system. This observation was also made by Steinman and Dawson (1941) who found that with small amounts of inert protein, the catalytic effect of copper on the oxidation of ascorbic acid was enhanced. However, with much larger amounts of inert protein for a constant amount of copper, the rate of oxidation was greatly inhibited over that observed with copper alone. It is clear, therefore, that comparisons of the catalytic activity of ascorbic acid oxidase with the activity of synthetic copper-inert-protein mixtures must be made with caution.

The first definite evidence of the specific copper protein nature of ascorbic acid oxidase came about only when the enzyme had been prepared in a high state of purity. At least three groups of workers independently approached the problem from this viewpoint at about the same time. Ramasarma, et al. (1940) appear to have been the first to report a rough parallelism between ascorbic acid oxidase activity and copper content throughout several stages of purification of the enzyme. However, their best preparations (from drumstick and cucumber) contained less than 0.03 per cent copper and had a very low specific activity. Within a few months both Stotz (1940) and Lovett-Janison and Nelson (1940) reported preparations containing 0.25 and 0.15 per cent copper from cucumber and summer crook-neck squash respectively. The squash en activity of 630 units per milligram of dry weight whereas th by Stotz from the cucumber possessed an activity of 850 unit gram dry weight. At first glance it would appear that this pr was considerably more pure than that of Lovett-Janison and However, the activity unit used by Stotz was smaller by a factor and a comparison of the preparations on a Q_{O_2} basis shows the Lov Janison and Nelson preparation to be over four times more active per milligram dry weight than the preparation described by Stotz. Thus the

high copper content reported by Stotz would suggest that the cucumber preparation either contained considerable non-enzyme copper (inactivated copper), or the proportionality factor between enzyme activity and copper is very much lower for cucumber oxidase than for the squash enzyme.

Although Stotz (1940) in his brief report did not mention any correlation between enzyme activity and the copper content during the purification, he did point out that the purified enzyme was inhibited by low concentrations of sodium diethyldithiocarbamate and cyanide. Lovett-Janison and Nelson (1940) compared the copper content and enzyme activity throughout a preparative procedure involving a large number of operations. They found that after the preliminary stages of purification, the enzymatic activity became proportional to the copper content and further purification failed to change the activity per microgram of copper. Their best preparation, containing 0.15 per cent copper, was free of manganese and had a Q_{O_2} of about 350,000. It was also free of peroxidase activity and was inactive toward phenols such as catechol, p-cresol, and hydroquinone.

These findings established beyond question the specific copper protein nature of ascorbic acid oxidase, and Lovett-Janison and Nelson (1940) emphasized the fact that their preparation catalyzed the aerobic oxidation of ascorbic acid over 1000 times more effectively than an equivalent amount of ionic copper.[6]

The following year Meiklejohn and Stewart (1941) using cucumber juice reported that the ascorbic acid oxidase activity was proportional to the copper content after all the free copper had been removed by prolonged dialysis. They found also that the activity of the juice was much greater than that of ionic copper or copper in the form of a "loose nonspecific combination with protein." Complete removal of the copper by dialysis against sodium cyanide inactivated the juice, but the activity was restored on the addition of excess copper sulfate. The restored activity was greater than that of the amount of copper sulfate used for the restoration.

Usi⸌⸍ ⸌modification of the method employed by Lovett-Janison and
⸍), Powers, Lewis and Dawson (1944) prepared from summer
squash (*C. pepo condensa*) an ascorbic acid oxidase containing
⸍ent copper. Using comparable activity measurements the oxi-
⸍a specific activity ($Q_{O_2} = 600,000$) over one and a half times greater

⸍ should be pointed out that whereas the enzyme activity is largely independent
⸍ the buffer system used, the rate of the reaction catalyzed by ionic copper is markedly influenced by the buffer system employed (Steinman and Dawson, 1942). Thus such comparisons can only be evaluated with full knowledge of the buffer systems employed.

than that of the preparation of Lovett-Janison and Nelson. However, the activity per microgram of copper was the same.

Powers, Lewis, and Dawson observed that their purified enzyme rapidly lost its activity when highly diluted. They found, however, that when the dilution was made with a dilute inert protein solution, the enzyme was much more stable. Using this procedure in preparing the enzyme dilutions for activity measurements, their best preparation was found to have a Q_{O_2} of about 1,000,000.

In concentrated solutions the enzyme was blue. Thus a solution containing 1970 units of enzyme activity per ml., and about 4.5 micrograms of copper per ml., had a color comparable to that of a copper nitrate solution containing 2500 micrograms of cupric ion per ml. Such concentrated solutions, when dialyzed salt free, precipitate the activity in the form of an amorphous greenish-blue protein. This protein readily dissolved in 0.1 molar secondary sodium phosphate without loss in activity, and such solutions were stable for several months when the concentration exceeded 0.1 per cent protein and the solution was stored at refrigerator temperatures. In solutions buffered below pH 4 the enzyme rapidly lost its activity presumably due to dissociation of the copper from the enzyme molecule. Ames and Dawson (1945) reported that dialysis of such an ascorbic acid oxidase preparation against dilute acid resulted in loss of copper.

Both the preparations described by Lovett-Janison and Nelson (1940) and Powers, Lewis, and Dawson (1944) were free of peroxidase activity. This point is of interest in connection with the theory postulated by Huszak (1937) concerning the role of ascorbic acid oxidase as related to peroxidase, in plant respiration. The essential feature of this theory, which was accepted by other workers in the field (Szent-Györgyi, 1939; Roberts, 1939, Jayle, 1939; Ebihara, 1939) was the assumption that hydrogen peroxide was produced during the oxidation of ascorbic acid by ascorbic acid oxidase. The hydrogen peroxide thus produced was presumed to be utilized in the oxidation of flavone substances by means of peroxidase. Using a peroxidase-free ascorbic acid oxidase preparation of the type described above, Steinman and Dawson (1942) failed to detect hydrogen peroxide as a primary product of the enzymatic oxidation of . by ascorbic acid oxidase and concluded that "there appears reason at present for supporting the view that hydrogen per reaction product of the enzymatic oxidation of ascorbic acid, a capable of entering into secondary reactions." Of interest is the fi extensive studies of the mechanism of the oxidation of ascorbic ac catalyzed by cupric ion have revealed that hydrogen peroxide is one of tu. products formed (Lyman, Schultze, and King, 1937; Dekker and Dickinson, 1940; Hand and Greisen, 1942; Steinman and Dawson, 1942; Silver-

blatt, Robinson, and King, 1943; Peterson and Walton, 1943; Weissberger, Lu Valle, and Thomas, 1943; Weissberger and Lu Valle, 1944).

An extensive series of studies on the chemical nature of ascorbic acid oxidase has been carried out by Tadokoro and associates (1941). In one of the papers in this series Tadokoro and Takasugi (1939) report the isolation of a crystalline material from pumpkin juice and state that the crystals possessed strong ascorbic acid oxidase activity and showed the properties of albumins. As a result of this report, Sumner and Somers (1943) have credited Tadokoro and Takasugi with the crystallization of ascorbic acid oxidase. In view of the method of preparation and the low activity of their crystals, relative to some of the preparations described above, and the fact that the identity of the crystals with the enzyme was not definitely proved, it seems advisable to withhold judgment as to whether or not ascorbic acid oxidase has yet been obtained in crystalline form.

IV. Other Copper Proteins

During the past decade certain other copper-bearing proteins have been isolated, but as yet little is known of their physiological significance. During this same period there has been some controversy concerning the presence of copper in certain well known proteins. For example, Agner (1938) intimated that a copper compound was associated with the activity of purified horse liver catalase. On the other hand, Dounce and Frampton (1939) and Sumner and Dounce (1939) found that both horse and beef liver crystalline catalase of high activity were essentially free from copper. During the same year Agner (1939) found that the copper in his preparations could be removed without loss of activity. At the present time it appears, therefore, that copper is not essential to the activity of the catalase molecule. However, it has been established by Schultze and Kuiken (1941) that copper has an essential role in the formation of the catalase found in the liver, kidney, and blood of rats.

It has also been suggested that copper may be a structural component of cytochrome oxidase (Keilin and Hartree, 1938; Graubard, 1941). Although there is as yet little direct evidence in favor of this supposition, the indirect evidence summarized by Keilin and Hartree is of interest. In addition to the fact that copper salts are the only known simple metal salts which rapidly oxidize all the components of cytochrome, and the fact that cytochrome oxidase and the copper protein polyphenol oxidase of plants are quite similar in many of their actions, it has been known for some time that the addition of copper to copper-deficient diets for rats produces an increase in the cytochrome content and the cytochrome oxidase activity of various tissues (Cohen and Elvehjem, 1934). More recently Schultze (1940, 1941) has found a similar effect of copper feeding on the

cytochrome oxidase content of the bone marrow. The fact that cytochrome oxidase is very intimately bound to the insoluble material of the cell has thus far made it impossible to evaluate the role of copper by the direct and usual methods involving isolation and purification.

With soluble proteins, the difficulty involved in identifying copper as an essential part of the molecule is not very great when the protein has a physiological activity that can be easily measured. However, in the case of proteins having no known activity, the problem is much more difficult, for copper has the ability to form, with many proteins, complexes of variable stability (Macheboeuf and Viscontini, 1943).

1. *Hemocuprein and Hepatocuprein*

As pointed out in the introductory section of this review, the presence of copper in animal tissues and blood was first recognized about a century ago. Since that time many workers have wondered about the state of the copper in red blood corpuscles and serum. After a number of investigators (Warburg and Krebs, 1927; Abderhalden and Moller, 1928; Tompsett, 1934; Eisler, Rosdahl and Theorell, 1936; Boyden and Potter, 1937) as the result of dialysis, acid extraction and electrophoresis experiments, had suggested that the copper in serum was present in a more or less loose combination with an organic compound, probably protein in character, Mann and Keilin (1938) succeeded in isolating such a copper-protein compound, in crystalline form, from red blood corpuscles of the ox, sheep and horse. They named the blue crystalline copper protein, *hemocuprein*, and stated that it was obtained also from horse serum.[7]

The crystalline hemocuprein of Mann and Keilin contained 14.35 per cent nitrogen, 1.12 per cent sulfur and 0.34 per cent copper. Solutions of the material appeared to be homogeneous in the ultracentrifuge, and a sedimentation constant was obtained indicating a molecular weight not far from 35,000. This value is in agreement with a copper content of two atoms per protein molecule. The copper was found to be *only loosely* bound to the protein, which appeared to belong to the class of albumins. Thus treatment with a dilute solution of trichloracetic acid precipitated a colorless protein which was devoid of copper. Fresh preparations of the crystalline protein contained no free copper, but on long standing, or when damaged by heat or acid treatment, free copper appeared.

The pronounced blue color of hemocuprein solutions was found to be much stronger than that of an equivalent amount of copper salt in alkaline solution. The blue color disappeared when hemocuprein solutions were treated with sodium hyposulfite, and the reduction was irreversible as

[7] Cohn, *et al.* (1940) reported that crystalline hemocuprein was obtained from horse plasma by ammonium sulfate fractionation.

evidenced by the fact that the color was not revived by shaking in the presence of air or by treatment with dilute hydrogen peroxide.

Mann and Keilin (1938) also isolated from ox liver another copper protein, *hepatocuprein*, which like hemocuprein possessed a copper content of 0.34 per cent. Gruzewska and Roussel (1937) had reported the year before that the copper in liver was present in combination with an organic non-dialyzable compound such as a protein, and they described liver protein preparations containing 0.155 per cent copper (lamb) and 0.21 per cent copper (calf liver). Although Mann and Keilin pointed out that in nature the blood and liver copper proteins might be intimately connected, or even interconvertible, they chose to distinguish them by name since the hepatocuprein was practically colorless and showed no tendency to crystallize.

Mann and Keilin were unable to find any direct biological or catalytic function for hemocuprein or hepatocuprein. The purified copper proteins did not combine with oxygen, as does hemocyanin, nor did they catalyze directly phosphorylation reactions or any of the reactions catalyzed by polyphenol oxidase, cytochrome oxidase, peroxidase, catalase or carbonic anhydrase. Nevertheless, it would seem likely that hemocuprein and hepatocuprein represent important steps in copper metabolism, and may directly or indirectly be concerned with the role of copper in the synthesis of hemoglobin, cytochrome, and cytochrome oxidase in higher animals.

2. *Iron-Copper Nucleoprotein*

In connection with the role of copper in the synthesis of heme compounds, the work of Saha and Guha (1941) is of interest. As the result of their study of the availability of iron in fish and other animal tissues, Saha and Guha claimed that 30–40 per cent of the total non-hemin iron in several animal tissues consisted of an iron-copper-nucleoprotein complex. The complex was isolated in a fairly pure state, and the copper was shown to be loosely combined, being removed by trichloracetic acid and also by alkali. Experiments with anemic rats indicated that the complex (or its breakdown products after absorption) might be a precursor for the formation of hemoglobin. Previous elimination of the copper from the complex diminished its hemopoietic power, and quantities of iron and copper, corresponding in amounts to a given quantity of the complex, were found in feeding experiments to be considerably less potent than the complex in regard to hemoglobin formation.

In a more detailed account of his studies on the nature of the nonhemin iron in fish tissue, Saha (1941) reported that the iron-copper-nucleoprotein substance contained 0.0432 per cent Fe, 0.0072 per cent Cu, 1.95 per cent P, 10.1 per cent N, and gave positive tests for protein, carbohydrate, and

purine material. It was insoluble in hot or cold water, organic solvents, dilute or strong mineral acids, ammonia, sodium carbonate solutions, but was soluble in dilute cold sodium hydroxide solutions. Precipitation with acid of the alkali-dissolved material gave the same Fe : P : N ratio as the original material. All of the copper was extractable with trichloracetic acid, and the iron could be removed by dialysis against sodium pyrophosophate.

3. Milk Copper Protein

It has been known for a relatively long time that cow's milk contains a small amount of copper. When Hart, Steenbock, Waddell, and Elvehjem (1928) demonstrated that copper was a necessary supplement to iron in the prevention of anemia in rats fed on a diet of whole cow's milk, interest in the copper content of milk was naturally aroused. The following year Elvehjem, Steenbock, and Hart (1929) found that the earlier values for the copper content were too high, and reported that cow's milk normally contains about 0.15 mg. of copper per liter. Sylvester and Lampitt (1935) determined the copper content of sixteen samples of milk drawn from cows of different breeds and found the copper content to range from 0.09 to 0.17 mg. per liter with a mean of 0.12.

Following McIlroy's (1936) report that the copper in milk was almost entirely in combination with protein, traces only being found in the fat, Dills and Nelson (1942) attempted the isolation of a copper-bearing protein from raw cow's milk. Using a procedure similar to that which has been employed in the same laboratory for purifying other copper proteins in a native condition, such as tyrosinase from mushrooms and from potatoes, and ascorbic acid oxidase from squash, they obtained from 40 liters of unpasteurized skimmed milk about 9 mg. of protein material containing 0.19 per cent copper and 15 per cent nitrogen. The copper was nonionic and could not be removed by dialysis at pH 6.5. However, dialysis against a dilute hydrochloric acid solution of pH 3.5 resulted in a rapid loss in copper. The loss of copper in dilute acid solutions is a well known property of copper proteins (Kubowitz, 1938). Dialysis experiments at pH 6.5 were also used to demonstrate that the copper protein in the presence of copper salts did not combine with more copper.

Dills and Nelson were unable to find any chemical reactions which were catalyzed by their milk copper protein, and they stressed the fact that the protein showed no polyphenolase or ascorbic acid oxidase activity. The latter point is of interest in connection with the fact that milk shows a loss in ascorbic acid on standing (Tauber, 1936).

4. Copper in Virus Protein and in the Cytoplasmic Granules of Various Cells

During the purification of the elementary body of vaccinia, Hoagland,

et al. (1941) found a certain degree of parallelism between the extent of the virus purification and the copper content. Spectroscopic analysis revealed that all other metallic constituents present during the early stages of the virus purification were lost as the purification process progressed. The copper-containing constituent proved to be different from any of the known copper protein enzymes, as evidenced by its lack of reactivity with appropriate substrates, and no biological role could be ascribed to it. The copper in a sample of dried purified virus amounted to 0.05 per cent, and it could not be removed from the virus by repeated washing, ultrafiltration, dialysis against 0.1 molar potassium cyanide, or by electrodialysis over a pH range which did not inactivate the virus.

Cells contain granules of various sizes which by appropriate means may be extracted from the cells. Claude (1941) reported that the final chemical composition and appearance of the purified granules depended markedly on the procedure used for their extraction and purification. The centrifugal fractionation of an extract of dried yeast cells that had been incubated three hours at 40° C. resulted in a material whose blue color and copper content resembled that of the hemocyanins. The chemical composition of the pigmented granules was found to be as follows: C, 53.15; H, 7.80; N, 11.73; P, 0.30; ash, 1.65; and copper, 0.116 per cent.

Claude (1941) found that granules isolated from other cells also contained copper but in smaller amounts. Thus small particles isolated from the malignant cells of a mouse leukemia were found to contain 0.023 per cent copper, and two types of granules isolated from guinea pig liver contained 0.034 and 0.013 per cent copper, respectively. Claude felt that the copper present in the cytoplasmic granules was "associated with the protein" and suggested the possibility that the copper compound might "play the role of a respiratory pigment in the cell."

V. Proteins Containing Other Metals

A survey of the literature reveals that most of the research up to the present time on metal proteins has been on the iron porphyrin, magnesium porphyrin, and copper proteins. The iron proteins are to be reviewed in a subsequent volume of "Advances in Protein Chemistry" and it is not within the scope of this review to survey the extensive studies on chlorophyll.

Many studies have demonstrated the essential role of a variety of metals in the physiological processes of both plants and animals. The various investigators have approached the problem by studying the dietary requirements, the analysis of tissues and organs, and the catalytic effect of metals in vitro on physiologically important reactions. A partial list of the metals studied in one or more of these ways includes zinc, vanadium, cobalt, nickel, molybdenum, manganese, magnesium, etc. The results of such

TABLE VII

Some Metal-Enzyme Systems Involved in Carbohydrate Metabolism

Enzyme	Activating Metal*	Substrate Reaction	References
Phosphoglucomutase	Mg, Mn, Co	glucose-1-phosphate $\rightleftharpoons$ glucose-6-phosphate	(1, 2)
Hexokinase	Mg	hexose + ATP → hexose-6-phosphate + ADP	(3)
Triose mutase (Phosphoglyceromutase)	Mg	3-phosphoglycerate $\rightleftharpoons$ 2-phosphoglycerate	(4)
Enolase	Mg, Mn, Zn	2-phosphoglycerate $\rightleftharpoons$ phosphoenolpyruvate	(5, 6)
Phosphopyruvate phosphatase	Mg	phosphoenolpyruvate + ADP → pyruvate + ATP	(7, 8, 9)
Hexodiphosphatase	Mg	fructose-1, 6-diphosphate + ADP $\rightleftharpoons$ fructose-6-phosphate + ATP	(10)
Bone phosphatase	Mn	hexosemonophosphate → hexose + phosphate	(11)
"Alkaline" kidney phosphatase	Mg, Mn, Co	β-glycerophosphate → glycerol + phosphate	(12)
Intestinal epithelium phosphatase (Phytase)	Mg	partial hydrolysis of inositolhexaphosphoric acid (Phytin)	(13)
Adenylpyrophosphatase	Ca, Mn, Mg, Ba	ATP → ADP	(14, 15, 16)
Yeast phosphatase ("Alkaline" kidney phosphatase?)	Mg, Mn, Co, Fe, Ni	β-glycerophosphate → glycerol + phosphate	(12)
Isocitric acid dehydrogenase	Mn, Mg	isocitric acid + CoII → a-keto-β-carboxyglutaric acid + H$_2$CoII	(17)
Carboxylase	Mg, Mn	pyruvic acid → acetaldehyde + carbon dioxide	(18, 19)
Pyruvic dehydrogenase	Mg, Mn, Co	2 pyruvic acid → acetic acid + lactic acid + carbon dioxide	(20, 21)

* Activating metals all present as divalent ions.

ADP = adenosinediphosphate.   ATP = adenosinetriphosphate.   CoII = coenzyme II.   H$_2$CoII = reduced coenzyme II.

investigations suggest that many metallo-organic compounds exist in living organisms, and some of these compounds undoubtedly are of protein character. Yet at this writing only in a very few cases has a metal other than iron or copper been shown to be a definite part of a naturally occurring protein molecule. In fact the only examples for which the data can be considered fairly conclusive are a few zinc and magnesium proteins.

As the result of studies since the turn of the century by a large number of workers in the field of carbohydrate metabolism, the complex mechanisms of glycolysis and alcoholic fermentation are today better understood than most comparable biological processes. Such studies which have been the subject of numerous reviews (Lipmann, 1939; Cori, 1939; Green, 1940; Lipmann, 1941; Meyer, 1943; Krebs, 1943; Barron, 1943; Sumner and Somers, 1943; Potter, 1944. See also "A Symposium on Respiratory Enzymes," the University of Wisconsin Press, 1942; and recent volumes of Annual Reviews of Biochemistry) have revealed that many enzymatic equilibria are involved in each process, and much of our knowledge has been gained by isolating these equilibria, and studying them in vitro. All of the reactions are catalyzed by specific proteins, and it has been found in numerous cases that certain metals and other factors such as coenzyme 1 or 2, diphosphothiamine, adenosinetriphosphate, etc., must also be present.

In most of the cases the metal requirement has been established by studying the effect of a variety of metallic ions on the activity of the system. In certain instances there is no activity in the absence of appropriate metallic ions, but in the majority of cases the metallic ions merely enhance the activity. In systems where more than one metallic ion is effective, it is generally found that an order of effectiveness can be established. The fact that magnesium has an important role and most of the other metals are also divalent and of the type that do not readily undergo change in valence, has led to the suggestion that "the metal acts as a chemical link between the protein and the prosthetic group and has purely structural significance" (Green, 1941). Some of the systems and activating metallic ions that have

References for Table VII

(1) Cori, Colowick, and Cori (1937).
(2) Cori, Colowick, and Cori (1938).
(3) Lutwak-Mann and Mann (1935).
(4) Meyerhof and Kiessling (1934).
(5) Lohmann and Meyerhof (1934).
(6) Warburg and Christian (1941).
(7) Lehmann (1935).
(8) Parnas and Ostern (1935).
(9) Cohen and Gerard (1937).
(10) Lohmann (1933).
(11) Combs, Norris, and Heusser (1942).
(12) Massart and Vandenriessche (1940).
(13) Patwardhan (1937).
(14) Szent-Györgyi and Banga (1941).
(15) Needham (1942).
(16) Bailey (1942).
(17) Adler, Euler, Gunther, and Plass (1939).
(18) Green, Herbert, and Subrahmanyan (1941b).
(19) Kubowitz and Lüttgens (1941).
(20) Lipmann (1939).
(21) Still (1941).

been investigated in connection with the studies of glycolysis, alcoholic fermentation, and related processes are shown in Table VII.

1. *Carboxylase*

Of the enzyme systems shown in Table VII the role of the metal is best understood in the case of the carboxylase system. This enzyme which is found in yeast, bacteria, fungi, and higher plant and animal tissues, catalyzes the decarboxylation of pyruvic acid and certain other α-ketocarboxylic acids (Green, *et al.* 1941a; Sumner and Somers, 1943). Auhagen (1932) observed that magnesium ions and a coenzyme "cocarboxylase" were necessary parts of the carboxylase system of yeast. Lohmann and Schuster (1937) crystallized cocarboxylase and found it to be the pyrophosphate of thiamine. In a study of the effect of metallic ions on the activity of the enzyme, the same investigators found that manganese was more effective than magnesium, but pointed out that magnesium was probably concerned physiologically since it was present in yeast in relatively high concentration whereas manganese occurred only in traces.

Green, *et al.* (1940, 1941b) prepared a highly purified and stable form of carboxylase from top brewer's yeast and reported that the enzyme was a diphosphothiamine magnesium protein. Their best preparation contained 0.46 per cent diphosphothiamine and 0.13 per cent magnesium. No other metal was found. The specific protein could be separated from the prosthetic group and the metal by precipitation three times with ammoniacal ammonium sulfate, and the original enzyme activity could be reconstituted by combining the protein with diphosphothiamine and magnesium. Other divalent metals such as manganese, iron, calcium, cadmium, zinc, and cobalt could be used instead of magnesium but with varying effectiveness. Monovalent and trivalent metals were inactive, and silver, copper, and mercury completely inhibited the activity in 0.0001 molar concentration. In high salt concentrations or at pH 6 the carboxylase was found to be a stable conjugated protein, whereas in dilute salt solutions or at pH 8 the enzyme was almost completely dissociated.

Green, *et al.* believed that the molar ratio of diphosphothiamine to protein to metal was 1 : 1 : 5. Kubowitz and Lüttgens (1941) found that their purest carboxylase contained 7 g. atoms of magnesium and 1 mole of diphosphothiamine to about 75,000 g. of protein. Melnick and Stern (1940) reported that purified yeast carboxylase has a molecular weight of about 141,000 and an isoelectric point at pH 5.1.

2. *Arginase*

Some of the most extensive work along the lines of metal activation has been carried out with the enzyme arginase. This enzyme is found in the

liver, kidney, and spleen of mammals as well as in certain plants, *e.g.*, the jack bean. In animals the enzyme is concerned in the formation of urea, splitting the amino acid *l*-arginine into urea and *l*-ornithine. Early studies of the enzyme activity revealed that the catalytic activity of the protein was low when the concentration of heavy metal was low. The addition of divalent manganese, cobalt, or nickel was found to increase the activity to a maximum, and further addition of the metal did not further increase the activity. The activation produced by manganous ion was greater than for nickel and generally greater than for cobaltous ion. In no case was the action of cobaltous ion found to be greater than that of manganous ion (Greenberg, 1944; Hunter and Downs, 1944). Facts such as these have led numerous investigators to postulate the presence of manganese in arginase. Although the enzyme has been purified by several workers, no one has demonstrated a parallelism between the enzymatic activity and the manganese content during several stages of purification. Richards and Hellerman (1940) partially purified the enzyme and examined the preparation spectrographically. They found neither cobalt nor nickel but did find manganese and iron. Ambiguous results from their activation studies with ferrous iron led them to imply that manganese was the only metal likely to be found in arginase. On the other hand, Rossi (1942) claimed that, although manganese accelerated the activity of arginase, it was not a constituent of the enzyme.

3. *Insulin*

Of the three known proteins containing zinc, insulin was the first in which the natural occurrence of zinc was fairly well established. This protein hormone was first crystallized by Abel (1926) who demonstrated the protein nature of the rhombohedral crystals and observed their characteristic physiological activity. A few years later Scott (1934) and Scott and Fisher (1935) prepared crystalline insulin by various methods and reported positive tests for zinc in all of their preparations. One preparation had a zinc content of 0.52 per cent. Subsequently Cohn *et al.* (1939, 1941) found, by the use of radioactive zinc that the zinc content of insulin crystals was dependent on the pH of the solution from which they were crystallized. In solutions below pH 5.5 the zinc content was always 0.34 per cent, but from solutions of higher pH the zinc content was higher. However, crystals with a high zinc content never contained more than two atoms (about 0.36 per cent) of the metal per protein molecule after they were equilibrated against conductivity water. Further evidence of the variability of the zinc content of insulin crystals was obtained by Sahyun (1941) who prepared crystallin insulin from pancreatic extract with the protein containing only 0.15 per cent of the metal.

Some knowledge of the amino acid content of the insulin molecule is available as the result of the studies of Nicolet and Shinn (1941) and Chibnall (1942). The former investigators reported a serine content of 3.57 per cent and a threonine content of 2.66 per cent. They pointed out, however, that any part of their serine value might actually be due to hydroxylysine. Chibnall (1942) in a summary of his studies reported amino acid contents as follows: arginine, 3.05 per cent; histidine, 10.7 per cent; lysine, 1.26 per cent; cystine, 12.5 per cent; tyrosine, 12.5 per cent; and an amide nitrogen content of 1.65 per cent. Chibnall also reported a study of the number of free amino groups and concluded that the insulin molecule might contain eighteen separate polypeptide chains.

Bath and Ellis (1941) approached the problem of the structure of the insulin molecule by means of infrared absorption spectroscopy. Their failure to find bands corresponding to OH groups led them to conclude that these groups were involved in structural linkages. On the other hand, Crammer and Neuberger (1943) examined the ultraviolet absorption of insulin and found that it was unnecessary to assume linkages of the phenolic groups in order to explain their results.

While investigating the molecular size of insulin by means of the ultracentrifuge, Sjögren and Svedberg (1931) observed a stable molecule from pH 4.5 to 7.0 with a molecular weight of 35,000. The particles appeared to be spherical, and had a radius of 2.18 m μ. At pH values below 4.5 and above 7.0 the molecule was apparently broken into smaller units. Recently, Miller and Andersson (1942) also using the ultracentrifuge, obtained an apparent molecular weight of 46,000 for a twice recrystallized sample of insulin.

Because insulin is rather easily crystallized, it is readily available in relatively large quantities and in a high state of purity. Such conditions have led to its use as a subject in the X-ray examination of the general structure of proteins. Since Crowfoot (1941) and Astbury (1943) have recently reviewed this work as applied to insulin, the details will not be presented here. It is sufficient to point out that Bernal (1939), using the X-ray data of Crowfoot and Riley (1939) as a basis, suggested that the insulin molecule might contain eighteen subunits. It will be recalled that this is in agreement with the suggestion made somewhat later by Chibnall (1942) on the basis of free amino nitrogen data. Astbury (1943) warned, however, that the data did not permit one to assume a dependence of the X-ray observations on the number of peptide chains.

Though it seems probable that zinc is an essential part of insulin, it must be kept in mind that such has not yet been conclusively proved. In the first place the actual zinc content has been difficult to ascertain, since, as pointed out earlier, the conditions of purification appear to exert a

pronounced effect. Furthermore, it is well known that amorphous zinc-free insulin has a physiological activity nearly as great as that of the crystalline zinc-containing material. This latter fact might be explained, however, on the basis that the zinc-free insulin combines with the metal in the body of the experimental animal. It is quite possible that the zinc content of insulin crystallized from pancreatic extracts arises during the isolation and purification process, *i.e.*, the zinc is removed from some other substance and combines with the protein portion of the insulin molecule. It may be argued, however, that such a possibility is rather unlikely, for many different methods of preparation yield a zinc-containing molecule. It is also known that cobalt and cadmium induce the crystallization of insulin (Scott and Fisher, 1935).

4. *Carbonic Anhydrase*

The enzyme which catalyzes both phases of the reaction was first demon-

$$H_2CO_3 \rightleftharpoons CO_2 + H_2O$$
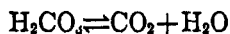
strated to be present in the erythrocytes of cow blood by Meldrum and Roughton (1932) who called it "carbonic anhydrase." Following a series of investigations by Roughton and associates and other groups of workers in reference to the occurrence, purification, and catalytic properties of the enzyme, Keilin and Mann (1939b, 1940b) reported that highly purified preparations from the erythrocytes of ox blood had a zinc content ranging from 0.31 to 0.33 per cent. Magnesium, manganese, iron, copper, and lead were present only in traces. During the process of preparation, the enzymatic activity, measured by the evolution of carbon dioxide from sodium bicarbonate, was found to be proportional to the zinc content. In addition all of the zinc present in red blood cells was believed to be contained in the enzyme. Keilin and Mann reported that their preparation of carbonic anhydrase was apparently pure as judged by cataphoretic and ultracentrifuge studies. The latter studies were made by Eirich and Rideal (1940) who reported a sedimentation constant of $S_{25} = 3.80$ S. Hove, *et al.* (1940) reported that they had confirmed the results of Keilin and Mann in reference to the zinc content of the enzyme, and the fact that all of the zinc in the erythrocytes was located in the enzyme.

Scott and Mendive (1941) reported that they had prepared a carbonic anhydrase with a purification factor (based on activity per unit weight) greater than that obtained by Keilin and Mann. In spite of the greater purification, Scott and Mendive found only 0.15 per cent zinc in their different preparations. This led them at first to doubt that zinc was an integral part of the enzyme molecule. However, on repeated attempts at rther purification by electrophoresis and adsorption, they were unable to

reduce the zinc content any further. Their best preparation had a total nitrogen content of 15.9 per cent and contained 1.3 per cent cystine and 4.1 per cent tyrosine.

The following year Scott (1942) succeeded in crystallizing carbonic anhydrase and reported that the crystals, which were highly active, had a total nitrogen content of 16.5 per cent and a zinc content of about 0.2 per cent (Scott and Fisher, 1942). The purified enzyme was found to rapidly lose its activity in dilute solutions, such as those used for activity measurements. The addition of peptone to such solutions "protected"[8] the enzyme, as evidenced by the fact that a greater activity was measured in the presence of peptone. A specimen of the enzyme prepared by Scott and Fisher was studied by Petermann and Hakala (1942) in the ultracentrifuge. The material was estimated to be 85 per cent pure with a sedimentation constant of 2.8 S and a molecular weight of about 30,000. The isoelectric point was found to be about pH 5.3.

Although it seems clear that carbonic anhydrase is a zinc-protein enzyme, it is evident that there is a considerable disagreement between the two main groups of workers as to the zinc content and the specific activity of the enzyme. There also appears to be an important difference in opinion as to the sedimentation constant of the enzyme molecule.

Recently Keilin and Mann (1944) suggested that the low zinc values reported by Scott and Fisher might be due to their method of zinc analysis, and they offered an explanation to account for the higher activities measured by Scott and coworkers. Shortly thereafter Scott and Fisher (1944) defended their method of zinc analysis and pointed out that a polarographic analysis of their enzyme by an independent investigator had showed a zinc content of 0.22 per cent, a value in good agreement with theirs. To explain their higher activity data, they reëmphasized the importance of using a protective agent such as peptone during the activity measurement of highly purified preparations. In other words, they inferred that Keilin and Mann were probably measuring only a fraction of the true enzymatic activity.

5. Uricase

The enzyme uricase catalyzes the oxidation of uric acid to allantoin accompanied by the reduction of oxygen to hydrogen peroxide. Holmberg (1939) obtained and purified the enzyme from pig liver, using the manometric uptake of oxygen as a measure of the activity. He found the enzyme to be insoluble except in solutions buffered near pH 10 or above. Noting that the activity was cyanide sensitive, Holmberg investigated the

[8] This phenomenon of an inert protein protecting an enzyme has also been observed in other systems (Nelson and Dawson, 1944; Powers, Lewis, and Dawson, 1944).

metal content of his preparation. He found 0.025 per cent iron, a probable zinc content of 0.13 per cent and no lead.

The results obtained somewhat later by Davidson (1942) supported Holmberg's suggestion that uricase might be a zinc protein. Using two different methods of preparation of the enzyme from pig liver, Davidson found in both cases fairly high iron and zinc contents. Only in the case of zinc, however, was there a parallelism between the activity and the metal content during the purification process. His best preparation showed a maximum zinc content of 0.09 per cent, and by spectrographic analysis contained, in addition to zinc and iron, traces of copper, lead, manganese, magnesium, aluminum, and tin.

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REFERENCES

Abderhalden, E., and Möller, P. (1928). *Z. physiol. Chem.* **176**, 95.

Abel, J. (1926). *Proc. Natl. Acad. Sci. U. S.* **12**, 132.

Adler, E., Euler, H. von, Gunther, G., and Plass, M. (1939). *Biochem. J.* **33**, 1028.

Agner, K. (1938). *Biochem. J.* **32**, 1702.

Agner, K. (1939). *Naturwissenschaften* **27**, 418.

Allen, T. H., and Bodine, J. H. (1941a). *Proc. Natl. Acad. Sci. U. S.* **27**, 269.

Allen, T. H., and Bodine, J. H. (1941b). *Science* **94**, 443.

Allen, T. H., Otis, A. B., and Bodine, J. H. (1942). *J. Gen Physiol.* **26**, 151.

Allen, T. H., Otis, A. B., and Bodine, J. H. (1943). *Arch. Biochem.* **1**, 357.

Allen, T. H., Ray, O. M., and Bodine, J. H. (1938). *Proc. Soc. Exptl. Biol. Med.* **39**, 549.

Allison, J., and Cole, W. (1940). *J. Biol. Chem.* **135**, 259.

Alsberg, C., and Clark, E. (1910). *J. Biol. Chem.* **8**, 1.

Ames, S. R., and Dawson, C. R. (1945). *Ind. Eng. Chem., Anal. Ed.* **17**, 249.

Astbury, W. (1943). *Advances in Enzymology* **3**, 63.

Auhagen, E. (1932). *Z. physiol. Chem.* **209**, 20.

Bailey, K. (1942). *Biochem. J.* **36**, 121.

Baker, D. L., and Nelson, J. M. (1943a). *J. Gen. Physiol.* **26**, 269.

Baker, D. L., and Nelson, J. M. (1943b). *J. Biol. Chem.* **147**, 341.

Ball, E., and Meyerhof, B. (1940). *J. Biol. Chem.* **134**, 483.

Barron, E. S. G. (1943). *Advances in Enzymology* **3**, 149.

Barron, E., Barron, A., and Klemperer, F. (1936). *J. Biol. Chem.* **116**, 563.

Barron, E., De Meio, R., and Klemperer, F. (1936). *J. Biol. Chem.* **112**, 625.

Bath, J., and Ellis, J. (1941). *J. Phys. Chem.* **45**, 204.

Bernal, J. (1939). *Proc. Roy. Soc. (London)* **A170**, 75.

Bert, P. (1867). *Compt. rend.* **65**, 300.

Bertrand, G. (1894). *Compt. rend.* **118**, 1215; *Bull. soc. chim.* [3] **11**, 717.

Bertrand, G. (1895a). *Compt. rend.* **120**, 266.

Bertrand, G. (1895b). *Compt. rend.* **121**, 166.

Bertrand, G. (1896a). *Compt. rend.* **122**, 1215.

Bertrand, G. (1896b). *Compt. rend.* **123**, 463.

Bertrand, G. (1897a). *Bull. soc. chim.* [3] 17, 753; *Compt. rend.* 124, 1355; *Compt. rend.* 124, 1032; *J. pharm. chim.* 5, 545.

Bertrand. G. (1897b). *Bull. soc. chim.* [3] 17, 577.

Bertrand, G. (1920). *Bull. soc. sci. hyg. aliment.* 8, 49.

Bertrand, G., and Bourquelot, E. (1895). *Compt rend. soc. biol.* 47, 579.

Bertrand, G., and Bourquelot, E. (1896). *Compt rend. soc. biol.* 43, 811.

Bhagvat, K., and Richter, D. (1938). *Biochem. J.* 32, 1397.

Bing, R. (1938). *Science* 87, 554.

Bing, R. J., Zucker, M. B., and Perkins, W. (1941). *Proc Soc. Exptl. Biol. Med.* 48, 372.

Bloch, B. (1929). *Am. J. Med. Sci* 177, 609.

Bodine, J. H., and Allen, T. H. (1938). *J. Cellular Comp. Physiol.* 11, 409; 12, 71.

Bodine, J. H., and Allen, T. H. (1941). *J. Cellular Comp. Physiol.* 18, 151.

Bodine, J. H., Allen, T. H., and Boell, E. J. (1937). *Proc. Soc. Exptl. Biol. Med.* 37, 450.

Bodine, J. H., and Boell, E. J. (1935). *J. Cellular Comp. Physiol.* 6, 263.

Bodine, J. H., and Tahmisian, T. N. (1943). *Arch. Biochem.* 2, 403.

Boswell, J. G., and Whiting, G. C. (1938). *Ann. Botany* 11, 847.

Bourquelot, E., and Bertrand, G. (1895). *Compt. rend. soc. biol.* 47, 582.

Bourquelot, E., and Bertrand, G. (1896). *J. pharm. chim.* 3, 177:

Boutigny (d'Evreux) (1833). *J. chim. med.* 9, 147.

Boyden, R., and Potter, V. R. (1937). *J. Biol. Chem.* 122, 285.

Brohult, S. (1937). *Nature* 140, 830.

Brohult, S. (1940). *Nova Acta Regiae Soc. Sci. Upsaliensis* [4] 12, No. 4, 7.

Brohult, S., and Claesson, S. (1939). *Nature* 144, 111.

Brooks, G. (1934). "Laque d'Indochine," Actualitées scientifiques et industrielles No. 94, 1.

Brooks, G. (1937). *La Nature* No. 3011, 359.

Brosteaux, J. (1937). *Naturwissenschaften* 25, 249.

Bucholz, C. F. (1816). *Report pharm.* 2, 253.

Burgers, J. M. (1938). "Second Report on Viscosity and Plasticity." Amsterdam.

Burk, N. F. (1940). *J. Biol. Chem.* 133, 511.

Cadden, J. F., and Dill, L. V. (1942). *J. Biol. Chem.* 143, 105.

Charles, D., and Rawles, M. (1940). *Proc. Soc. Exptl. Biol. Med.* 43, 55.

Chibnall, A. (1942). *Proc. Roy. Soc. (London)* B131, 136.

Clark, G., Quaife, M., and Baylor, M. (1943). *Biodynamica* 4, 153.

Claude, A. (1941). *Cold Spring Harbor Symposia Quant. Biol.* 9, 263.

Cohen, E., and Elvehjem, C. A. (1934). *J. Biol. Chem.* 107, 97.

Cohen, R. A., and Gerard, R. W. (1937). *J. Cellular Comp. Physiol.* 10, 223.

Cohen, S. S. (1942). *J. Biol. Chem.* 144, 353.

Cohn, E. (1925). *Physiol. Rev.* 5, 349.

Cohn, E., Ferry, J., Livingood, J., and Blanchard, M. (1941). *J. Am. Chem. Soc.* 63, 17.

Cohn, E., Ferry, J., Livingood, J., and Blanchard, M. (1939). *Science* 90, 183.

Cohn, E. J., McMeekin, T. L., Oncley, T. L., Newell, J. M., and Hughes, W. L. (1940). *J. Am. Chem. Soc.* 62, 3386.

Combs, G., Norris. L., and Heusser, O. (1942). *J. Nutrition* 23, 131.

Conant, J., Chow, B., and Schoenbach, E. (1933). *J. Biol. Chem.* 101, 463.

Conant, J., Dersch, F., and Mydans, W. (1934). *J. Biol. Chem.* 107, 755.

Conant, J., and Humphrey, W. (1930). *Proc. Natl. Acad. Sci. U. S.* 16, 543.

Cook, S. F. (1928). *J. Gen. Physiol.* 11, 339.

Cori, C. F. (1939). *Cold Spring Harbor Symposia Quant. Biol.* 7, 260.

Cori, C. F., Colowick, S. P., and Cori, G. T. (1937). *J. Biol. Chem.* 121, 465.

Cori, G. T., Colowick, S. P., and Cori, C. F. (1938). *J. Biol. Chem.* **123**, 375.

Craifaleanu, A. (1919) *Boll. Soc. Nat. Napoli Anno* **32**, 141. (Taken from Redfield, 1934.)

Crammer, J., and Neuberger, A. (1943). *J. Biol. Chem.* **37**, 302.

Crowfoot, D. (1941) *Chem. Rev.* **28**, 215.

Crowfoot, D., and Riley, D. (1939). *Nature* **144**, 1011.

Dalton, H. R., and Nelson, J. M. (1939). *J. Am. Chem. Soc.* **61**, 2946.

Davidson, J. (1942). *Biochem. J.* **36**, 252.

Dawson, C. R., and Ludwig, B. J. (1938). *J. Am. Chem. Soc.* **60**, 1617.

Dekker, A. O., and Dickinson, R. G. (1940). *J. Am. Chem. Soc.* **62**, 2165.

Deschamps, M. (1848). *J. pharm. chim.* [3] **13**, 91.

Devergie, and Hervy (1840). *Annales d'hygiene* 180.

Dhéré, C. (1915). *J. physiol. path gén.* **16**, 985.

Dhéré, C. (1919). *J. physiol. path. gén.* **18**, 503.

Dhéré, C. (1920). *J. physiol. path. gén.* **18**, 1081.

Dhéré, C., and Baumeler, C. (1926). *Compt. rend soc. biol.* **95**, 328.

Dhéré, C., and Baumeler, C. (1928). *Compt. rend. soc. biol.* **99**, 726.

Dhéré, C., and Baumeler, C. (1929). *Compt. rend. soc. biol.* **101**, 1071.

Dhéré, C., and Schneider, A. (1922) *J. physiol path. gén.* **20**, 34.

Dills, W. L., and Nelson, J. M. (1942). *J. Am. Chem. Soc.* **64**, 1616.

Dounce, A. L., and Frampton, V. L. (1939). *Science* **89**, 300.

Ebihara, T. (1939). *J. Biochem. (Japan)* **29**, 199.

Eirich, F., and Rideal, E. (1940). *Nature* **146**, 541.

Eisler, B., Rosdahl, K. G., and Theorell, H. (1936). *Biochem. Z.* **286**, 435.

Ekwall, P. (1942). *Finska Kemistamfundets Medd.* **51**, 67.

Elford, W., and Ferry, J. (1936). *Biochem. J.* **30**, 84.

Elvehjem, C. A. (1935). *Physiol. Rev.* **15**, 471.

Elvehjem, C. A. *et al.* (1939). "Respiratory Enzymes." Burgess Publishing Company, Minneapolis.

Elvehjem, C. A., Steenbock, H., and Hart, E. B. (1929). *J. Biol. Chem.* **83**, 27.

Eriksson-Quensel, I., and Svedberg, T. (1936). *Biol. Bull.* **71**, 498.

Fleurent, E., and Levi, L. (1920). *Bull. soc. chim.* **27**, 440.

Fleury, P., and Campora, C. (1934). *Bull. soc. chim. biol.* **16**, 1589.

Florkin, M., and Toussaint, C. (1939). *Compt. rend. soc. biol.* **132**, 45.

Fredericq, L. (1878). *Arc. zool. exptl. et gén.* **7**, 535. (Taken from Redfield, 1934.)

Gatterer, A., and Philippi, E. (1933). *Z. physiol. Chem.* **216**, 110.

Ginsberg, B. (1944). *Genetics* **29**, 176.

Grabar, P. (1936). *Compt. rend. soc. biol.* **121**, 1472.

Graubard, M. (1939). *Enzymologia* **6**, 332.

Graubard, M. (1941). *Am. J. Physiol.* **131**, 584.

Green, D. E. (1940). "Mechanisms of Biological Oxidations." University Press, Cambridge.

Green, D. E. (1941). *Advances in Enzymology* **1**, 177.

Green, D. E., Herbert, D., and Subrahmanyan, V. (1940). *J. Biol. Chem.* **135**, 795.

Green, D. E., Herbert, D., and Subrahmanyan, V. (1941b). *J. Biol. Chem.* **138**, 327.

Green, D. E., Westerfeld, W. W., Vennesland, B., and Knox, W. E. (1941a) *J. Biol. Chem.* **140**, 683.

Greenberg, D. M. (1944). Personal communication.

Gregg, D. C., and Miller, W. H. (1940). *J. Am. Chem. Soc.* **62**, 1374.

Gregg, D. C., and Nelson, J. M. (1940). *J. Am. Chem. Soc.* **62**, 2500.

Gruzewska, Z., and Roussel, G. (1937). *Compt. rend. soc. biol.* **125**, 957.

Guerithault, B. (1920). *Compt. rend.* **171**, 196.

Guillemet, R., and Gosselin, G. (1932). *Compt. rend. soc. biol.* **111**, 733.

Hand, D. B., and Greisen, E. C. (1942). *J. Am. Chem. Soc.* **64**, 358.

Harless, E. (1847). *Arch. Anat. Physiol.* p. 148.

Hart, E. B., Steenbock, H., Waddell, J., and Elvehjem, C. A. (1928). *J. Biol. Chem.* **77**, 797.

Hegeboom, G., and Adams, M. (1942). *J. Biol. Chem.* **145**, 273.

Henze, M. (1901). *Z. physiol. Chem.* **33**, 370.

Hernler, F., and Philippi, E. (1930). *Z. physiol. Chem.* **191**, 23.

Hernler, F., and Philippi, E. (1933). *Z. physiol. Chem.* **216**, 110.

Hess, A. F., and Unger, L. J. (1921). *Proc. Soc. Exptl. Biol. Med.* **19**, 119.

Hoagland, C. L., Ward, S. M., Smadel, J. E., and Rivers, T. M. (1941). *J. Exptl. Med.* **74**, 69.

Holmberg, C. (1939). *Biochem. J.* **33**, 1901.

Hooker, S. (1938). *Proc. Soc. Exptl. Biol. Med.* **38**, 911.

Hooker, S., and Boyd, W. (1935). *J. Bact.* **29**, 57.

Hooker, S., and Boyd, W. (1936). *J. Immunol.* **30**, 33.

Hooker, S., and Boyd, W. (1942). *Ann. N. Y. Acad. Sci.* **43**, Art. 2, 107.

Hopkins, F. G., and Morgan, E. J. (1936). *Biochem. J.* **30**, 1446.

Hove, E., Elvehjem, C., and Hart, E. (1940). *J. Biol. Chem.* **136**, 425.

Hunter, A., and Downs, C. (1944). *J. Biol. Chem.* **155**, 173.

Huszák, S. (19 7). *Z. physiol. Chem.* **247**, 239.

Jayle, M. F. (1939). *Bull. soc. chim. biol.* **21**, 14.

Kastle, J. H. (1910). "The Oxidases." Hygienic Lab. Bull. **59**, U. S. Treasury Dept., Washington, D. C.

Keilin, D., and Hartree, E. F. (1935). *Proc. Roy. Soc. (London)* **B119**, 114.

Keilin, D., and Hartree, E. F. (1938). *Nature* **141**, 870.

Keilin, D., and Mann, T. (1938a). *Proc. Roy. Soc. (London)* **B125**, 187.

Keilin, D., and Mann, T. (1938b). *Proc. Roy. Soc. (London)* **B126**, 303.

Keilin, D., and Mann, T. (1939a). *Nature* **143**, 23.

Keilin, D., and Mann, T. (1939b). *Nature* **144**, 442.

Keilin, D., and Mann, T. (1940a). *Nature* **145**, 304.

Keilin, D., and Mann, T. (1940b). *Biochem. J.* **34**, 1163.

Keilin, D., and Mann, T. (1944). *Nature* **153**, 107.

Kertesz, Z., Dearborn, R., and Mack, G. (1936). *J. Biol. Chem.* **116**, 717.

King, C. G. (1936). *Physiol. Rev.* **16**, 238.

King, C. G. and Waugh, W. A. (1932). *Science* **75**, 357.

Kobert, R. (1903). *Pflüger's Arch. ges. Physiol.* **98**, 411.

Krebs, H. A. (1943). *Advances in Enzymology* **3**, 191.

Kubowitz, F. (1937). *Biochem. Z.* **292**, 221.

Kubowitz, F. (1938). *Biochem. Z.* **299**, 32.

Kubowitz, F.. and Lüttgens, W. (1941). *Biochem. Z.* **307**, 170.

Kuhn, W. (1932). *Z. physiol. Chem.* A **161**, 1.

Laporta, M. (1932). *Boll. soc. ital. biol. sper.* **7**, 630.

Lehmann, H. (1935). *Biochem. Z.* **281**, 271.

Lehmann, K. B. (1895). *Arch. Hyg.* **24**, 18.

Lipmann, F. (1939). *Cold Spring Harbor Symposia Quant. Biol.* **7**, 248.

Lipmann, F. (1941). *Advances in Enzymology* **1**, 99.

Lohmann, K. (1933). *Biochem. Z.* **262**, 137.

Lohmann, K., and Meyerhof, O. (1934). *Biochem. Z.* **273**, 60.

Lohmann, K., and Schuster, P. (1937). *Biochem. Z.* **294**, 188; Naturwissenschaften **25**, 26.

Lovett-Janison, P. L., and Nelson, J. M. (1940). *J. Am. Chem. Soc.* **62**, 1409.

Ludwig, B. J., and Nelson, J. M. (1939). *J. Am. Chem. Soc.* **61**, 2601.

Lutwak-Mann, T., and Mann, T. (1935). *Biochem. Z.* **281**, 140.

Lyman, C. M., Schutze, M. O., and King, C. G. (1937). *J. Biol. Chem.* **118**, 757.

McCarthy, J. F., Green, L. F., and King, C. G. (1939). *J. Biol. Chem.* **128**, 455.

McIroy, R. J. (1936). *New Zealand J. Sci. Tech.* **17**, 710.

Machebocuf, M., and Viscontini, M. (1943). *Compt. rend.* **217**, 305.

Majima, R. *et al.* (1922). *Ber.* **55**, 172.

Mallette, M. F., Ames, S. R., and Dawson, C. R. (1945). To be published.

Mann, T., and Keilin, D. (1938). *Proc. Roy. Soc. (London)* B126, 308.

Maquenne, L., and Demoussy, E. (1920). *Compt. rend.* **170**, 87.

Massart, L., and Vandendriessche, L. (1940). *Naturwissenschaften* **28**, 143.

Matsukawa, D. (1940). *J. Biochem.* (Japan) **32**, 265.

Mazur, A. (1937). *J. Biol. Chem.* **118**, 631.

Meiklejohn, G., and Stewart, C. (1941). *Biochem. J.* **35**, 755.

Meissner, W. (1817). *Ann. chim. phys.* **4**, 106; *Deutsch. Jahr. Pharm.* **18**, 19.

Meldrum, N. V., and Roughton, F. J. W. (1932). *J. Physiol.* **75**, 15P.

Melnick, J. L., and Stern, K. G. (1940). *Enzymologia* **8**, 129.

Meyer, K. H. (1943). *Advances in Enzymology* **3**, 109.

Meyerhof, O., and Kiessling, W. (1934). *Naturwissenschaften* **22**, 838.

Miller, G., and Andersson, K. (1942). *J. Biol. Chem.* **144**, 45).

Miller, W. H., Mallette, M. F., Roth, L. J., and Dawson, C. R. (1944). *J. Am. Chem. Soc.* **66**, 514.

Montgomery, H. (1930). *Biol. Bull.* **58**, 18.

Needham, D. M. (1942). *Biochem. J.* **36**, 113.

Nelson, J. M., and Dawson, C. R. (1944). *Advances in Enzymology* **4**, 99.

Nicolet, B., and Shinn, L. (1941). *J. Am. Chem. Soc.* **63**, 1486.

Northrop, J. H., and Anson, M. L. (1928–29). *J. Gen. Physiol.* **12**, 543.

Parkinson, G. G., and Nelson, J. M. (1940). *J. Am. Chem. Soc.* **62**, 1693.

Parnas, J. K., and Ostern, P. (1935). *Biochem. Z.* **279**, 94.

Patwardhan, V. N. (1937). *Biochem. J.* **31**, 560.

Pearson, O. H. (1936). *J. Biol. Chem.* **115**, 171.

Pedersen, K. O. (1933). *Kolloid Z.* **63**, 268.

Pedersen, K. O. (Unpublished investigations). See Svedberg (1939).

Petermann, M., and Hakala, N. (1942). *J. Biol. Chem.* **145**, 701.

Peterson, R. W., and Walton, J. H. (1943). *J. Am. Chem. Soc.* **65**, 1212.

Philippi, E. (1919). *Z. physiol. Chem.* **104**, 88.

Philippi, E., and Hernler, F. (1930). *Z. physiol. Chem.* **191**, 28.

Polson, A. G. (1936). *Nature* **137**, 740; and unpublished investigations.

Polson, A. G. (Unpublished investigations). See Svedberg (1939).

Potter, V. R. (1944). *Advances in Enzymology* **4**, 201.

Powers, W. H., Lewis, S., and Dawson, C. R. (1944). *J. Gen. Physiol.* **27**, 167.

Putzeys, P., and Brosteaux, J. (1941). Meded. Kon. Vlaamsche Akad. Wetensch., Letteren Schoone Kunsten Belgie, Klass Wetensch. **3**, No. 1.

Putzeys, P., and van de Walle, P. (1939). *Bull. soc. chim. biol.* **21**, 185.

Putzeys, P., and van de Walle, P. (1940). *Trans. Faraday Soc.* **36**, 32.

Quagliariello, G. (1920). *Arch. sci. biol.* (Italy) **1**, 246.

Quagliariello, G. (1922). *Pubbl. staz. zool. Napoli*, **1**, 57.

Quagliariello, G. (1924). In Winterstein, H., "Handbuch der vergleichenden Physiologie" 1, 597, Jena.

Ramasarma, G., Datta, N., and Doctor, N. (1940). *Enzymologia* 8, 108.

Ranzi, S. (1938). *Ricerca sci.* 9, I, 631.

Raper, H. S. (1928). *Physiol. Rev.* 8, 244.

Raper, H. S. (1932). *Ergeb. Enzymforschung* 1, 270.

Rawlinson, W. A. (1940). *Australian J. Exptl. Biol. Med. Sci.* 18, 131.

Rawlinson, W. A. (1941) *Australian J. Exptl. Biol. Med. Sci.* 19, 137.

Rawlinson, W. A. (1943). *Australian Chem. Inst. J. Proc.* 10, 21.

Redfield, A. C. (1930). *Biol. Bull.* 58, 150.

Redfield, A. C. (1934). *Biol. Rev.* 9, 175.

Redfield, A. C., Coolidge, T., and Montgomery, H. (1928). *J. Biol. Chem.* 76, 197.

Redfield, A. C., and Goodkind, R. (1929). *Brit. J. Exptl. Biol.* 6, 340.

Richards, M., and Hellerman, L. (1940). *J. Biol. Chem.* 134, 237.

Roberts, E. A. H. (1939). *Biochem. J.* 33, 836.

Roberts, E. A. H. (1942). *Advances in Enzymology* 2, 113.

Roche, A., and Roche, J. (1935). *Compt. rend.* 201, 1522.

Roche, J. (1930). *Arch. phys. biol.* 7, 207.

Roche, J. (1936). *Ann. Rev. Biochem.* 5, 463.

Roche, J., and Darien, Y. (1939). *Compt. rend. soc. biol.* 131, 686.

Roche, J., and Dubouloz, P. (1933a). *Compt. rend.* 196, 646.

Roche, J., and Dubouloz, P. (1933b). *Bull. soc. chim. biol.* 15, 954.

Roche, J., and Dubouloz, P. (1936). *Compt. rend. soc. biol.* 122, 234.

Roche, J., and Jean, G. (1934a). *Bull. soc. chim. biol.* 16, 769.

Roche, J., and Jean, G. (1934b). *Compt. rend. soc. biol.* 115, 1645.

Roche, J., and Morugue, M. (1941). *Trav. members soc. chim. biol.* 23, 1329.

Roche, J., Roche, A., Adair, G., and Adair, M. (1935). *Biochem. J.* 29, 2576.

Root, R. (1934). *J. Biol. Chem.* 104, 239.

Rossi, A. (1942). *Arch. sci. biol. (Italy)* 28, 40.

Saha, K. C. (1941). *Ann. Biochem. Exptl. Med.* 1, 195.

Saha, K. C., and Guha, B. C. (1941). *Nature* 148, 595.

Sahyun, M. (1941). *J. Biol. Chem.* 138, 487.

Schmitz, A. (1930). *Naturwissenschaften*, 18, 798.

Schmitz, A. (1931). *Z. physiol. Chem.* 194, 232; 196, 71.

Schultze, M. O. (1940). *Physiol. Revs.* 20, 37.

Schultze, M. O. (1941). *J. Biol. Chem.* 138, 219.

Schultze, M. O., and Kuiken, K. A. (1941). *J. Biol. Chem.* 137, 727.

Scott, D. (1934). *Biochem. J.* 28, 1592.

Scott, D. (1942). *J. Biol. Chem.* 142, 959.

Scott, D., and Fisher, A. (1935). *Biochem. J.* 29, 1048.

Scott, D., and Fisher, A. (1942). *J. Biol. Chem.* 144, 371.

Scott, D., and Fisher, A. (1944). *Nature* 153, 711.

Scott, D., and Mendive, J. (1941). *J. Biol. Chem.* 139, 661; 140, 445.

Seastone, C., Loring, H., and Chester, K. (1937). *J. Immunol.* 33, 407.

Silverblatt, E., and King, C. G. (1938). *Enzymologia* 4, 222.

Silverblatt, E., Robinson, A. L., and King, C. G. (1943). *J. Am. Chem. Soc.* 65, 137.

Sjögren, B., and Svedberg, T. (1931). *J. Am. Chem. Soc.* 53, 2657.

Snellman, O., and Bjornstahl, Y. (1941). *Kolloid-Beihefte* 52, 403.

Smirnov, A., and Pshennova, K. (1941). *Biokhemiya* 6, 29.

Spruyt, J., and Vogelsang, G. (1938). *Arch. néerland physiol.* 23, 423.

Sreerangachar, H. (1944). *Biochem. J.* **37**, 653.
Srinivasan, M. (1936). *Biochem. J.* **30**, 2077.
Stanley, W., and Anderson, T. (1942). *J. Biol. Chem.* **146**, 25.
Stedman, E., and Stedman, E. (1927). *Biochem. J.* **21**, 533.
Steinman, H. G., and Dawson, C. R. (1941). Unpublished.
Steinman, H. G., and Dawson, C. R. (1942). *J. Am. Chem. Soc.* **64**, 1212.
Still, J. (1941). *Biochem. J.* **35**, 380.
Stone, W. (1937). *Biochem. J.* **31**, 508.
Stotz, E. (1940). *J. Biol. Chem.* **133**, C.
Stotz, E., Harrer, C. J., and King, C. G. (1937). *Science* **86**, 35; *J. Biol. Chem.* **119**, 511.
Straub, F. B. (1938). *Z. physiol. Chem.* **254**, 205.
Suminokura, K. (1930). *Biochem. Z.* **224**, 292.
Suminokura, K. (1936). *Bull. Chem. Soc. Japan* **11**, 299.
Sumner, J. B., and Dounce, A. L. (1939). *J. Biol. Chem.* **127**, 439.
Summer, J. B., and Somers, G. F. (1943). "Chemistry and Methods of Enzymes."
    Academic Press, New York.
Sutter, H. (1936). *Ergeb. Enzymforschung* **5**, 273.
Svedberg, T. (1930). *Kolloid Z.* **51**, 10.
Svedberg, T. (1937). *Nature* **139**, 1051.
Svedberg, T. (1939). *Proc. Roy. Soc. (London)* **B127**, 1.
Svedberg, T., and Brohult, S. (1938). *Nature* **142**, 830.
Svedberg, T., and Chirnoaga, E. (1928). *J. Am. Chem. Soc.* **50**, 1399.
Svedberg, T., and Eriksson, I. (1932). *J. Am. Chem. Soc.* **54**, 4730.
Svedberg, T., and Hedenius, A. (1933). *Nature* **131**, 325.
Svedberg, T., and Hedenius, A. (1934). *Biol. Bull.* **66**, 191.
Svedberg, T., and Heyroth, F. (1929). *J. Am. Chem. Soc.* **51**, 539; **51**, 550.
Sylvester, N. D., and Lampitt, L. H. (1935). *Analyst* **60**, 376.
Szent-Györgyi, A. (1928). *Biochem. J.* **22**, 1387.
Szent-Györgyi, A. (1930). *Science* **72**, 125.
Szent-Györgyi, A. (1931). *J. Biol. Chem.* **90**, 385.
Szent-Györgyi, A. (1939). "On Oxidation, Fermentation, Vitamins, Health, and
    Disease." Williams and Wilkins Co., Baltimore.
Szent-Györgyi, A., and Banga, I. (1941). *Science* **93**, 158.
Szent-Györgyi, A., and Haworth, W. N. (1933). *Nature* **131**, 24.
Tadokoro, T., and Takasugi, N. (1939). *J. Chem. Soc. Japan* **60**, 188.
Tadokoro, T., Takasugi, N., and Saito, T. (1941). *J. Chem. Soc. Japan* **62**, 419.
Tauber, H. (1936). *Proc. Soc. Exptl. Biol. Med.* **35**, 422.
Tauber, H. (1938). *Ergeb. Enzymforschung* **7**, 301.
Tauber, H., and Kleiner, I. S. (1935a). *Proc. Soc. Exptl. Biol. Med.* **32**, 577.
Tauber, H., Kleiner, I. S., and Mischkind, D. (1935b). *J. Biol. Chem.* **110**, 211.
Tenenbaum, L. E. (1940). Dissertation, Columbia University.
Tenenbaum, L. E., and Jensen, H. (1943). *J. Biol. Chem.* **147**, 27.
Tiselius, A. (1930). *Nova Acta Regiae Soc. Sci. Upsaliensis* **7**, No. 4.
Tiselius, A., and Gross, D. (1934). *Kolloid Z.* **66**, 11.
Tiselius, A., and Horsfall, F. (1939a). *J. Exptl. Med.* **69**, 83.
Tiselius, A., and Horsfall, F. (1939b). *Arkiv Kemi, Mineral., Geol.* **13 A**, No. 18.
Tompsett, S. L. (1934). *Biochem. J.* **28**, 1544.
Treffers, H. P. (1940). *J. Am. Chem. Soc.* **62**, 1405.
Trurnit, H., and Berghold, G. (1942). *Kolloid Z.* **100**, 172.
Warburg, O., and Christian, W. (1941). *Naturwissenschaften* **29**, 589.

Warburg, O., and Krebs, H. (1927). *Biochem. Z.* **190**, 143.
Weissberger, A., and LuValle, J. E. (1944). *J. Am. Chem. Soc.* **66**, 700.
Weissberger, A., LuValle, J. E., and Thomas, D. S. (1943). *J. Am. Chem. Soc.* **65**, 1934.
Wilson, W. H. (1901). Records of Egyptian Government School of Medicine. (Taken from Redfield, 1934.)
Winterstein, H. (1909). *Biochem. Z.* **19**, 384.
Wolvekamp, H. P. (1938a) *Z. vergleich. Physiol.* **25**, 541.
Wolvekamp, H. P. (1938b). *Natuurw. Tijdschr.* **20**, 256.
Yasuzumi, G., Miyamoto, K., Kurokarwa, K. (1937). *Folia Anat. Japon.* **15**, 545.
Yoshida, H. (1883). *J. Chem. Soc.* **43**, 472.
Youmans, G., and Colwell, C. (1943). *J. Immunol.* **46**, 217.
Zilva, S. S. (1934). *Biochem. J.* **28**, 663.

Mucoids and Glycoproteins

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CONTENTS

I. INTRODUCTION

Compared to the progress in the knowledge of chemistry, physiology, and pathology of the proteins proper, our knowledge of the mucoids and glycoproteins has remained rather incomplete. As evidence of this no comprehensive review of this field has appeared since the monograph of Levene (1925). Furthermore in textbooks of biochemistry, the field of carbohydrate-containing proteins and of mucopolysaccharides finds only incidental mention, or the data given are erroneous and antiquated to a surprising extent. Although practically nothing is known about the metabolism of these substances and little about their functions, enough data have been accumulated in the last twenty years to indicate rather important roles for some of these substances in biological processes.

II. DEFINITION AND CLASSIFICATION

A reviewer of mucopolysaccharides, mucoids, and glycoproteins is faced with the problem of giving his own definition and classification, since in this field, unlike the general field of proteins, there is no accepted ter-

minology. In this paper it is proposed to limit the discussion to substances which contain hexosamine. This excludes proteins containing nucleic acids or glycolipids. Aminosugars other than hexosamines or simple proteins containing hexoses in the absence of hexosamine have not been encountered in nature thus far.

We propose to classify the hexosamine-containing compounds into three main groups, the mucopolysaccharides, the mucoids, and the glycoproteins. As mucopolysaccharides we define polysaccharides which contain hexosamine as one component, whether they occur free or whether they be obtained by chemical manipulation from substances of higher molecular weight. As mucoids we define substances which contain a mucopolysaccharide in firm chemical union with a peptide, where the hexosamine content is greater than 4 per cent.[1] The group of proteins which contain less than 4 per cent hexosamine, classified as glycoproteins, embraces many proteins listed as albumins and globulins in the accepted classification of proteins. It obviously would be undesirable to change this accepted classification. It would, for example, not be advisable to separate the two components of crystalline serum albumin into two different classes, the albumin proper and the hexosamine-containing albumins. It therefore might suffice to designate the latter with the prefix glyco-. While some of these proteins are probably pure and single molecular species, many may be complexes of mucoid with simple protein. It might be pointed out here that the crystallographically most perfect proteins such as insulin, pepsin, chymotrypsin, or trypsin do not contain any carbohydrate.

In general this classification follows one proposed in an earlier review (Meyer, 1938). It is based on the nature of the carbohydrate moiety. We distinguish between simple and complex mucopolysaccharides. Simple mucopolysaccharides are those containing N-acylated hexosamine in combination with other carbohydrates or carbohydrate derivatives such as uronic or hexonic acids. Complex mucopolysaccharides contain in addition, either sulfuric or phosphoric acid groups or lipid in firm chemical union with the mucopolysaccharide.

The ease with which the mucopolysaccharides can be obtained free from their natural sources varies greatly. As a rule, mucopolysaccharides of acidic nature, in contrast to the electroneutral compounds, can be easily obtained free of protein by mild procedures. Neutral mucopolysaccharides from animal sources occur in firm chemical linkage with protein. In some

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[1] The distinction between mucoids and glycoproteins based on a hexosamine content of 4 per cent or over is arbitrary. This figure is chosen since compounds with such a hexosamine content possess the solubility properties of mucoids and remain soluble after precipitation by alcohol.

microörganisms, however, the neutral polysaccharides may occur not bound to protein.

Acid mucopolysaccharides as they occur for example in vitreous humor, synovial fluid, cornea, gastric juice, and cartilage are in part dissociated, since in the native fluid or neutral aqueous extracts they migrate in an electric field independently of the proteins present. On the other hand on acidification they form protein compounds of definite stoichiometric proportions (Meyer, Palmer, and Smyth, 1937). In the literature these protein compounds have been called mucins or mucoids, such as vitreous and synovial mucin, cornea and chondromucoid. It seems to us best not to use the latter terms. The term "mucin"[2] has only a physiological meaning, denoting a viscous secretion.

While the simple mucopolysaccharide acid, hyaluronic acid, in nature occurs entirely in the dissociated form, the complex mucopolysaccharide acids, especially the sulfuric acid esters, seem to be bound much more firmly to protein. This linkage in part at least is of polar nature. It is, however, not known whether the linkage is entirely polar. Another difference between hyaluronic acid and the sulfuric acid esters is the nature of the protein with which the carbohydrate is associated. Thus complexes formed on acidification of the hyaluronic acid — containing fluids like vitreous humor (Meyer and Palmer, 1936), synovial fluid (Meyer, Smyth, and Dawson, 1939), or some tumor fluids (Meyer and Chaffee, 1940a) precipitate with serum globulin fractions. On the other hand the sulfuric acid esters occurring as intercellular substances in the cornea or in cartilage probably exist in part as protein complexes with specific proteins. As a rule, some difficulty is encountered in bringing these complexes into solution, this difficulty varying with the tissues. Thus chondroitinsulfuric acid in very good yield can be extracted from defatted and finely ground cartilage by 10 per cent calcium chloride at a pH of about 9 (Meyer and Smyth, 1937), while umbilical cord (Meyer and Palmer, 1936) or skin (Meyer and Chaffee, 1941) seem to require more drastic treatment. The data available at present seem insufficient to designate as mucoids the complexes obtained from tissues by more or less drastic treatment followed by acidification.

However, there is no evidence that an ester linkage (Levene, 1925) is involved as has been claimed. Chondroitinsulfuric acid in hyaline cartilage

[2] The term "mucin" and "pseudomucin" as used by the pathologist does not seem to have a clear cut biochemical basis. As a rule acid mucopolysaccharides cause the typical "mucin" clots on acidification of cyst fluids. However, we have encountered such fluids which contained hyaluronic acid in high concentrations; on dilution and acidification these did not give such a clot, apparently because of the large excess of hyaluronic acid over protein. On the other hand, some ovarian cysts contain fluids which give a precipitate on dilution and acidification but yield only a neutral mucopolysaccharide.

may be a salt of the acid with a collagen, since the ratio of hexosamine to total nitrogen is similar to that of the artificial products prepared from gelatin and chondroitinsulfuric acid (Meyer, Palmer, and Smyth, 1937). The form in which chondroitinsulfuric acid is combined in the connective tissues like dermis (Meyer and Chaffee, 1941) or umbilical cord (Meyer and Palmer, 1936) is unknown.

Certain of the mucoids, for example ovomucoid, seromucoid, and gonadotropic hormones, are distinguished by great solubility in aqueous solutions. They are not readily precipitated by trichloroacetic, sulfosalicylic, picric, or flavianic acids. They are precipitated by alcohol or acetone without losing their solubility in aqueous media. No member of this group seems ever to have been crystallized. Some mucoids are almost completely insoluble. Among these little studied mucoids is the so-called ovomucin of eggwhite (for literature see Young, 1937) for which we proposed the term ovomucoid-β, the chalazae of eggshells, the so-called residual protein of vitreous humor and the capsule of the lens. All these substances seem to serve some architective function. Whether other histological structures such as limiting or basal membranes belong to this group is unknown.

The following scheme summarizes the data outlined above.

I. Mucopolysaccharides
    A. Neutral mucopolysaccharides (do not contain an acid group)
        1. Acetylglucosamine only
           Example: chitin
        2. Acetylglucosamine and galactose
           a. In ratio 1:1
               Example: neutral polysaccharide of pig gastric mucosa (Meyer, Smyth, and Palmer, 1937) (the main fraction is a mucoid, see below).
           b. In ratio 1:3
               Example: Type XIV pneumococcus polysaccharide (Goebel, Beeson, and Hoagland, 1939).
        3. Glucosamine, galactose, and rhamnose
           Example: Shiga-Kruse specific polysaccharide (Morgan, 1938; Morgan and Partridge, 1940, 1941).
    B. Acid Mucopolysaccharides
        1. Simple acid mucopolysaccharides
           a. Acetylglucosamine and glucuronic acid
               Example: hyaluronic acid (Meyer and Palmer, 1936).
        2. Complex acid mucopolysaccharides
           a. Sulfomucopolysaccharides
               α. Acetylglucosamine, glucuronic acid, and sulfuric acid
                   Example: hyaluronosulfate from cornea (Meyer and Chaffee, 1940b)
               β. Acetylglucosamine, sulfuric acid, and undetermined uronic acid
                   Examples: acid mucopolysaccharide of hog gastric mucosa (Meyer, Smyth, and Palmer, 1937); heparin (trisulfuric ester), (Jorpes and Bergstroem, 1937; Charles and Todd, 1940).

γ. Acetylgalactosamine, glucuronic acid, and sulfuric acid
Example: chondroitin sulfate (Levene, 1925).
   b. Phosphomucopolysaccharides
      a. Aminosugar, galactose, and phosphate
         Examples: C carbohydrate of pneumococcus (Goebel, *et al.*, 1943);
            C carbohydrate of streptococcus (?) (Zittle and Harris, 1942).

II. Mucoids or mucoproteins (contain more than 4 per cent aminosugar)
   A. Soluble neutral mucoids
      1. Acetylglucosamine and galactose in ratio 1:1
         Examples: mucoid of oviduct of the frog (Schulz and Becker, 1935); gonadotropic hormone of pregnancy urine (?) (Gurin, 1945); blood group A substance of gastric mucosa (Meyer, Smyth, and Palmer, 1937; Landsteiner and Harte, 1940) and saliva (Landsteiner, 1936a); mucoid from anthrax bacillus (Ivánovics, 1940).
      2. Acetylglucosamine and mannose
         Examples: ovomucoid (Levene and Mori, 1929); serum mucoid (?) (Rimington and Van der Ende, 1940); seroglycoid(?) (Hewitt, 1939).
   B. Insoluble mucoids (composition unknown)
      Examples: "ovomucin" or ovomucoid-β (Young, 1937; Meyer, 1938); chalazae (Young, 1937); residual protein of vitreous humor, lens capsule (may be glycoproteins).
   C. Acid Mucoids
      1. Acetylglucosamine and gluconic acid
         Example: submaxillary mucoid (see below).

III. Glycoproteins (contain less than 4 per cent hexosamine)
      1. Acetylglucosamine and mannose
         Examples: ovalbumin (Neuberger, 1938); serum albumin (Rimington, 1929, 1931).
      2. Composition and homogeneity unknown
         Examples: serum globulins; stromatin; and many others.

III. GENERAL METHODS OF PREPARATION
OF MUCOPOLYSACCHARIDES AND MUCOIDS

Most mucopolysaccharides and mucoids are characterized by a high solubility in water and a relative resistance towards denaturation by agents which denature proteins. Thus extraction with aqueous salt solutions in many instances suffices to obtain these compounds in solution. In microorganisms more drastic treatment has to be used to disintegrate the organism. In many instances, however, the polysaccharides are liberated during growth into the media or, as in the case of pneumococci, are easily obtained in solution by autolysis of the organisms. If the microörganisms do not autolyze, disintegration by ball mills or other bacterial mills (Booth and Green, 1938) or by ultrasonic vibration (Chambers and Flossdorf, 1936) has to be used. It has become a rule to employ procedures as mild as possible in order to avoid loss of biological properties, as for example antigenic-

ity. But in some instance rather drastic procedures, as the use of hot for-
mamide, hot acid or alkali, or extraction with 90 per cent phenol have been
employed for the liberation of mucopolysaccharides.

Acid mucopolysaccharides as a rule are far easier to obtain in pure form
than the neutral substances. In some instances advantage may be taken
of the salt formation of acid mucopolysaccharides with proteins. For this
purpose the extracts or fluids have to be diluted to obviate the dissociating
influence of salts. If the protein concentration is insufficient or if the pro-
tein has been digested as in commercial gastric mucin, a carbohydrate-free
protein may be added, such as edestin or gelatin. In some instances there
are also present enzymes which decrease the yield of polysaccharides. In
such cases extraction with concentrated urea solutions may be used.
Usually it is advantageous to reduce the bulk and simultaneously denature
some proteins by dehydration with acetone.

For the removal of proteins, digestion with pepsin and trypsin has
proven successful in some instances. Precautions have to be used with
pepsin, since commercial preparations contain rather high concentrations
of a mucopolysaccharide. It has to be noted too that many mucopoly-
saccharides and mucoids are quite effective inhibitors of proteolytic
enzymes. Protein is removed effectively by shaking with mixtures of
chloroform and butyl, amyl, or octyl alcohol. This procedure was appar-
ently first used by P. Plósz as quoted by Mörner (1895). The latter used
shaking with chloroform and with mixtures of chloroform and butyl alcohol
for the removal of protein in urine. The method was rediscovered by Sevag
(1934). Mechanical stirring by an efficient stirrer for one-half to one hour
is as effective as shaking for several hours as recommended by Sevag.
Previous freezing with liquid air as recommended by the latter is unneces-
sary. The denaturation requires salt. Usually a neutral or slightly alkaline
reaction is used. At acid reaction some mucoids may become insoluble
by this treatment. For the removal of nitrogenous impurities remaining
in solution after chloroform-amyl alcohol treatment, adsorption on zinc
hydroxide, cadmium hydroxide, or in acid solution by Lloyd's reagent has
been found useful.

The separation of mixtures of different polysaccharides may be very
difficult. In material from animal sources, glycogen usually accompanies
the mucopolysaccharides, since like the latter it is precipitated by alcohol
and glacial acetic acid. Digestion for a few hours with filtered saliva or
another good source of amylase, like clarase, in the presence of toluene will
suffice to remove it. In mixtures of acid and neutral mucopolysaccharides,
separation may be obtained by salt formation with protein of the acid
compounds. Mixtures of sulfomucopolysaccharides with neutral or simple
acid mucopolysaccharides may be separated by fractionation of the neutral

solution with alcohol in the presence of 10 per cent calcium or barium chloride. The sulfomucopolysaccharides require a lower concentration for precipitation than the simple polysaccharides. A very promising procedure for the separation of polysaccharides from peptides or amino acids was introduced by Neuberger (1938) for the isolation of the mucopolysaccharide component of crystalline egg albumin. The hydrolysis mixture after tryptic digestion was treated with ketene in alkaline reaction, giving N-acetyl compounds. The basic amino acids are removed by phosphotungstic acid and the remaining N-acetylated amino acids are removed, after acidification, by chloroform extraction. The water-soluble carbohydrates were acetylated at low temperature with acetic anhydride and pyridine. The resulting O-acetyl compounds were extracted with chloroform and saponified at about pH 9 with lithium hydroxide. The deacetylated polysaccharide containing N-acetylglucosamine was precipitated by alcohol.

The mucopolysaccharides and mucoids are, in general, amorphous substances of high molecular weight. Heparin seems to form a crystalline barium salt (Charles and Scott, 1936) in acetic acid solution, but the dried material appears amorphous. Among the mucoids, an eggwhite fraction having a high avidin potency has in a preliminary note (Pennington, Snell, and Eakin, 1942) been claimed to be crystalline. However, the biological potency of these crystalline preparations was considerably lower than amorphous preparations and no complete description has apparently been given. Carbohydrate free avidin preparations of high potency have been described (Woolley and Longsworth, 1942).

IV. ANALYTICAL PROCEDURES

The proof of purity and homogeneity in all these substances undoubtedly is a very difficult problem. The criteria applied in protein chemistry, namely constancy of analysis and rotation, electrophoresis, and ultracentrifugation, have been applied to these substances. To these might be added quantitative immunological reactions (for review see Kabat, 1943) especially for bacterial mucopolysaccharides, and quantitative enzymatic analysis (Meyer, Smyth, and Dawson, 1939; Meyer and Chaffee, 1940a). Constancy of the solubility (Soerenson, 1930) hardly seems to have been used. A similar principle has been employed in fractionating a serum mucoid fraction with alcohol in the presence of salt. By this method the ratio of hexosamine to nitrogen showed a shift, while an ovomucoid-a fraction by the same method gave a constant ratio, thus indicating homogeneity of the latter and complexity of the former. A similar procedure was used in the characterization of the mucopolysaccharides of skin (Meyer and Chaffee, 1941), where this method led to the recognition of mixtures of

two mucopolysaccharides, while a constant analysis of a number of mixed fractions suggested one complex polysaccharide acid.

The estimation of the carbohydrate components of mucopolysaccharides and mucoids depends to a very large degree on colorimetric methods, which undoubtedly have been relied upon too much. The isolation of component parts obviously is desirable.

The methods for the estimation of aminosugar are based on the condensation of acetylacetone with 2-aminosugars to form pyrrole derivatives, which in turn are condensed with dimethylaminobenzaldehyde to give colored products (Pauly and Ludwig, 1922). The method has been modified by a number of authors (for literature see Palmer, Smyth, and Meyer, 1937) and seems to give values with an error of about 5 per cent. The error is greater with sulfcmucopolysaccharides apparently due to destruction of hexosamine during hydrolysis, and may reach 20 per cent. In our opinion the methcd is specific and reliable within the limits mentioned. There is apparently no case known in which the color produced was proven to be due to the presence of a substance other than an aminosugar.

Hexosamines have been isolated as the hydrochlorides; as Schiff bases with anisaldehyde (Bergmann and Zervas, 1931), salicylaldehyde (Irving and Earl, 1922), 2, 4-dihydroxybenzaldehyde (Neuberger, 1938), 2-hydroxy-l-naphthaldehyde (Jolles and Morgan, 1940); as derivatives with phenyl isocyanate (Steudel, 1901) or naphthyl isocyanate (Neuberg and Hirschberg, 1910); as benzoyl (Muller, 1898) or carbobenzoxy (Chargaff and Bovarnick, 1937) compounds. The isolation as the crystalline hydrochloride has been used most extensively. It has the advantage of being successful both with glucosamine and chondrosamine, whereas the Schiff bases and the carbobenzoxy compound do not give good results with chondrosamine. Disadvantages in the hydrochlorides are first, the hydrolysis usually has to be severe, so as to destroy other sugars present, and second the material has to be free of salts, as otherwise the isolation proves extremely difficult. In the case of sulfuric acid esters, the barium or lead salt should be used.

The estimation of carbon dioxide on decarboxylation with strong acid or zinc chloride (Tollens-Lefèvre reaction) and the color reaction with naphthoresorcinol have been used most extensively for the determination of uronic acids. The estimation of furfural formed during decarboxylation seems to be insufficiently quantitative to be used as a micromethod. Methods based on the Tollens-Lefèvre reaction, in our experience, are far superior to the naphthoresorcinol method, especially in the presence of protein. The former has been used in this laboratory for the estimation of gluconic acid which yields carbon dioxide quantitatively and which seems to occur as a component of a mucopolysaccharide in submaxillary mucoid

(see below). Many modifications of the naphthoresorcinol reaction have appeared. From an extensive study of the reaction in this laboratory (Meyer, Bloch, and Chaffee, 1942) the following conclusions have been drawn. Pure uronic acids can be estimated quantitatively with naphtho-resorcinol if pure reagents are used, if the type of vessel, time of heating, and the acid concentration are kept constant and if benzol is substituted for ether in the extraction of the color, as pointed out by Neuberg and Saneyoshi (1911). The color is produced by an oxidation of the condensa-tion product of 1 mole of a furfural derivative and 2 moles of naphtho-resorcinol, producing a quinoid structure. This oxidation is catalyzed and the color intensified by adding, during the reaction, an oxidant like persul-fate instead of depending on the oxidation by air. In the presence of an excess of other sugars and of amino acids, the colorimetric determination of uronic acids has proven unsuccessful.

The isolation of the free uronic acids from mucopolysaccharides has apparently not been achieved. Glucuronic acid has recently been isolated from chondroitinsulfuric acid as the amide of 2, 3, 4-trimethyl-a-methyl glucuronide (Bray, Gregory, and Stacey, 1944) after exhaustive methyla-tion of the mucopolysaccharide. In other instances the presence and iden-tity of uronic acids (in the absence of hexoses) have been established by oxidation with nitric acid or bromine and isolation of potassium acid saccha-rate in the case of glucuronic or gluconic acid, and of mucic acid in the case of galacturonic acid. Glucosamine is oxidized with nitric acid to isosaccharic acid, which does not interfere, but can be isolated as the quinine or cinchonine salt from the oxidation mixture (Schulz and Becker, 1935). The oxidation with hypoiodite and subsequent condensation with o-phenylenediamine to a benzimidazole derivative (Moore and Link, 1940) may be a very useful method, although no crystalline products could be isolated in this laboratory with impure fractions of several mucopoly-saccharide acids in quantities of a few grams.

Various colorimetric methods for the estimation and characterization of hexoses in mucoids and glycoproteins have been described (Tilmans and Philippi, 1929; Soerenson and Haugard, 1933; Dische, 1930; Gurin and Hood, 1939). For purposes of identification of sugars in mixtures of sugars, such methods seem highly questionable, though they are of value for the approximate estimation of hexoses. For the identification of the sugars, recognized methods of isolation and characterization such as summarized by van der Haar (1925) are still imperative.

Among the best characterized mucopolysaccharides and mucoids, the basic units were found to consist of one hexosamine in combination with one or two molecules of uronic acid or other sugars. In one instance a tetrasaccharide seems to have been encountered, namely hexosamine to

galactose in ratio 1:3 in type XIV pneumococcus polysaccharide (Goebel, Beeson, and Hoagland, 1939). While still higher ratios may exist, it seems imperative to rigidly exclude mixtures of mucopolysaccharides as a reason for such high ratios. In the case of the mucopolysaccharide of ovomucoid (Stacey and Woolley, 1940; 1942) there has been postulated a hendeca-saccharide, having a central core of three mannose units to which are attached by glucosidic links seven N-acetylglucosamine and one galactose units. This conclusion was based on the isolation of the carbohydrates as methyl derivatives after exhaustive methylation. However, the ovomucoid used in this careful work must have been impure as judged by the high nitrogen value and the low rotation and reducing values as compared with figures reported by other authors. The values for hexosamine and "hexose" determined colorimetrically or by titration fit a 1 to 1 ratio better than a 7 to 4 ratio postulated by Stacey and Woolley. In spite of all the errors of the colorimetric and titrimetric methods, the isolation of the methyl derivatives of these components appears quantitatively even less reliable.

Very little is known about the molecular weights of mucopolysaccharides and mucoids. The great importance of the molecular weight of these compounds in biological reactions is clearly shown in a number of reports. Thus Morgan and King (1943) found the blood group A reactions of the gastric mucoid greatly influenced by heating in various solutions, which simultaneously led to a decrease in viscosity (see below). In this labora-tory the highly polymerized form of hyaluronic acid caused a great in-crease in erythrocyte sedimentation rate *in vitro* and *in vivo*, while the depolymerized form was practically without effect (Meyer, Hahnel, and Feiner, 1945). The failure of some commercial gastric mucin preparations to cause increased virulence of microörganisms, when they are injected as suspensions in this medium, may best be explained by depolymeriza-tion of the mucoid. Molecular weight of gastric mucoid preparations has been determined by osmotic pressure (Landsteiner and Harte, 1940) (see below). Recently Blix and Snellman (1944) found hyaluronic acid prepared from several sources to be polydisperse, a finding in agreement with the experience of this laboratory (Meyer and Palmer, 1936). The Swedish authors calculated from the double refraction of flow a particle length of hyaluronic acid from vitreous humor of 4800 Å, from synovial fluid about 7000 Å, and from umbilical cord still higher and outside the range of the apparatus used. By extrapolation of viscosity measurements they found for the latter material 10,000 to 12,000 Å. Chondroitin sulfate of cartilage was found to be less polydisperse with a particle length of about 4700 Å. Assuming 10 Å for the disaccharide unit of hyaluronic acid, they calculated

the molecular weight as 200,000 to 400,000, and for chondroitinsulfuric acid about 200,000. The actual particle size in the native forms is probably considerably larger than these figures.

V. Mucoids[3]

1. *Gastric Mucoid*

The mucoids of the first group, those containing glucosamine and galactose in the ratio of 1:1, probably are widely distributed in animals and in microörganisms. The mucopolysaccharide group of some of these mucoids is responsible for blood group A activity and is related to the Forssman antigen (Goebel, *et al.*, 1943). They occur in the gastric mucosa, in horse saliva, and in some ovarian cysts. It is unknown whether the mucoid of the oviduct of the frog (Schulz and Becker, 1935), *i. e.*, the glands that furnish the egg jelly, and that of salamander egg jelly (unpublished experiments) are related biologically to these substances.

In former papers (Meyer, 1938) the neutral substance obtained from hog gastric mucosa was listed as a mucopolysaccharide, although it was realized that most fractions contained only about 75 per cent of a polysaccharide, while the rest was made up of nitrogenous constituents of amino acid nature. This substance was shown by Landsteiner to be highly potent in serological tests for blood group A substance (for literature see Landsteiner, 1936b). This substance probably occurs in the mucosa and in the gastric secretion as a true mucoid. During its preparation the peptide moiety is partly split off leaving a more or less pure mucopolysaccharide which carries the serological activity. In the mucosa and the gastric secretion this substance occurs together with a sulfomucopolysaccharide acid, which like the neutral substance contains acetylated glucosamine, but instead of galactose has an uronic acid of unknown structure. The viscosity of gastric juice seems to be due to the neutral substance. The latter (Morgan and King, 1943) likewise probably is the factor in commercial "gastric mucin" which on injection into mice enhances the virulence of microörganisms (Nungester, Wolf, and Jourdonais, 1932).

For the isolation of the mucoid, different investigators extracted the crude material with hot carbonate (Meyer, Smyth, and Palmer, 1937), hot formamide (Landsteiner and Harte, 1940), aqueous carbon dioxide (Morgan and King, 1943), or 90 per cent phenol (Morgan and King, 1943). Protein was removed by proteolytic enzymes or by adsorption on Lloyd's reagent, zinc hydroxide, or charcoal, by shaking with chloroform-amyl alcohol, or by fractional precipitation with sodium sulfate. The final

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[3] A systematic discussion of mucopolysaccharides is omitted in accordance with the wishes of the Editors.

product was usually obtained by fractional precipitation with glacial acetic acid and alcohol. The material, especially that described by Morgan and King, forms viscous solutions. These investigators showed that heating in acid and especially in alkaline solutions leads to a considerable drop in viscosity and to loss of some of the biological functions. (However, the effect of alkali on the purified material is considerably greater than on the crude.) The chemical properties of samples prepared from commercial gastric mucin or from commercial pepsin were similar. The nitrogen values in the published figures range from 3.3 to 6.3 per cent, the hexosamine values lie between 31 and 39 per cent, and the specific rotation between −8 and +22°. The variation in the data indicates that the substances were either all impure or degraded to a varying degree. Phenol extracted fractions, further purified by fractionation by alcohol and containing between 5.9 and 6.2 per cent nitrogen, with $[a]_D$ +10 to +14°, were investigated by Kekwik (addendum to Morgan and King, 1943) in the Tiselius apparatus. In acetate and phosphate buffers at pH 8.0 and 4.0 a single component accounted for 90 per cent of the total refraction.

Galactose content by mucic acid determination was found to be 26.1 to 28.8 per cent (Meyer, Smyth, and Palmer, 1937) and 29 per cent (Landsteiner and Harte, 1940). By fermentation with a galactose yeast the value was 31 per cent (Landsteiner and Harte, 1940). The hexosamine was isolated (Meyer, Smyth, and Palmer, 1937; Landsteiner and Harte, 1940) and proved to be glucosamine.

An estimation of the molecular weight was attempted by Landsteiner and Harte. From the sedimentation rate in the ultracentrifuge a minimum of 40,000 was indicated. By measurements of the osmotic pressure, one preparation gave a molecular weight of 70,000, while others had a molecular weight of at least 120,000 to 200,000.

The biuret, xanthoproteic, and Sakaguchi reactions in most preparations were positive. Tests for tyrosine and tryptophan were negative.

The blood group A substance isolated from horse saliva by Landsteiner (1936a) was less pure than those described above. A similar material was isolated by King and Morgan (1944) from pseudomucinous ovarian cysts (nitrogen 6.0 per cent, acetyl 10 per cent, hexosamine 25 per cent, Van Slyke amino nitrogen 4.4 per cent, and a-amino acid nitrogen 2.5 per cent).

The majority of papers on the gastric mucoid dealt mainly with the properties of the substance as blood group A fractions. For this test either the inhibition of isoagglutination (the agglutination of human A erythrocytes by human anti-A serum) or the prevention of hemolysis of sheep erythrocytes by an anti-A rabbit serum (Landsteiner, 1936) is used. The A substance itself, in contrast to some similar bacterial antigens, is not fully antigenic. It can be made so, however, by combining it with a polypeptide

isolated from Shiga-Kruse bacillus (Morgan and Patridge, 1940, 1941). The inhibition of the isoagglutination was decreased in Morgan and King's experiments by lowering the viscosity of the fractions, while the property of inhibiting the hemolysis of sheep cells by anti-A immune serum was not lowered by the loss in viscosity or even was enhanced. The serological activity by the latter method of samples prepared by different laboratories was in the same order of magnitude (10^{-8} to 10^{-9}g., Morgan and King, up to 10^{-10}g. in some preparations made in this laboratory and tested by Dr. Landsteiner). The serological activity of the A substance is diminished by enzymatic decomposition by certain bacteria or bacterial extracts (Landsteiner and Chase, 1935; Schiff, 1939). In this laboratory the hydrolysis of the neutral polysaccharide by extracts of a strain of *Cl. welchii* was demonstrated by measuring reducing sugar (Meyer, *et al.*, 1940). In later experiments with Dr. Schiff it was shown that other strains of *Cl. welchii*, which he found ineffective in abolishing the serological activity, likewise failed to give an increase in reducing sugar. Inactivation by heat or oxidation led to a simultaneous disappearance of the two properties. These experiments seem to indicate that the blood group A activity is entirely dependent on the polysaccharide moiety regardless of the peptide groups attached to it.

2. *Gonadotropic Hormones*

The probable mucoid nature of the gonadotropic hormone of pregnancy urine was first suggested by Fischer and Ertel (1931). The presence of hexosamine in addition to reducing sugar, in a concentration of 7.7 per cent in highly purified preparations was demonstrated by Meyer (1937) The ratio of hexosamine to hexose was 1:1. The purification and chemical properties were then further studied by Gurin and his associates (1940). The gonadotropic hormone of pregnant mare serum, like the biologically distinct hormone of the urine in pregnant women also seems to be of mucoid nature (Hartman and Benz, 1938; Fleischer, Schwenk, and Meyer, 1938; Rimington and Rowlands, 1944). The gonadotropic hormones obtained from the pituitary gland probably are of mucoid nature too, at least the more soluble and biologically better defined follicle stimulating fraction (see Gurin, 1942, for some analyses).

The hormone of pregnancy urine was prepared by Gurin and his associates with an activity of about 6,000 to 8,000 I. U./mg. It contains 12 per cent nitrogen, 10.7 per cent hexose, 5.2 per cent hexosamine, and 3 per cent acetyl. The ratio of hexose to hexosamine thus was 2:1 as was the ratio of acetyl to hexosamine. This acetyl ratio in our experience is unique for this

hormone.[4] The hexose was thought, from colorimetric data, to be galactose. Electrophoretically the best fraction was apparently homogeneous with a mobility at pH 7.0 of -4.85×10^{-5} in phosphate of ionic strength 0.1. In the ultracentrifuge only one component was visible with a sedimentation constant (S_{20}) of 4.3×10^{-13}. The diffusion constant was (D_{20}) 4.4×10^{-7}, and the partial specific volume was 0.76 (Lundgren, *et al.*, 1942). A second fraction prepared as the iodo compound (Meyer, 1937) had a sedimentation constant of 5.4×10^{-13}. The molecular weight was calculated from the data as approximately 100,000. This molecular weight is surprisingly high for a substance excreted apparently at a constant rate through the kidney. The isoelectric point (microscopic electrophoresis of substance adsorbed on quartz) was given as at pH 3.2 to 3.3.

The most recent publication on the gonadotropins from pregnant mare serum was published by Rimington and Rowlands (1944). The serum was deproteinized by metaphosphoric acid, the hormone adsorbed on benzoic acid, followed by fractionation of aqueous extracts by alcohol at various pH levels. Fractions were obtained assaying 12,500 I. U./mg. The chemical data for these preparations include only the hexose value of between 14 and 16 per cent. For some less potent preparations a ratio of hexose to hexosamine of about 2 was found. It is interesting to note that from non-pregnant mare serum, the authors likewise obtained a small non-active fraction, which had a hexose content about equal to that of the active fractions. The carbohydrate in less pure pregnant mare serum preparations was stated to be either galactose or an equimolar mixture of glucose and mannose (Gurin, 1942). The ratio of hexose to hexosamine was approximately 2:1.

3. *Ovomucoid-a*

Most investigators have isolated ovomucoid-*a* from the supernatant fluids after heat coagulation of other proteins. From this fluid the mucoid was either precipitated by alcohol or by salting out with ammonium sulfate or sodium sulfate. An apparently identical fraction has been prepared by removing proteins by shaking with chloroform-amyl alcohol. Our laboratory has prepared ovomucoid from acetone precipitated eggwhite. The dried precipitate was extracted with a mixture of 50 per cent alcohol and 10 per cent acetic acid, and the filtered solution concentrated in vacuo to a small volume. The precipitate obtained by adding 5 volumes of alcohol

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[4] Hexosamine to acetyl ratios of 1:2 have been reported in other mucopolysaccharides (for example, see Blix, 1936). In these cases methyl alcoholic sodium hydroxide has been used in the saponification, which leads to the appearance of a second volatile acid. The saponification is best carried out with *p*-toluenesulfonic acid (Meyer and Palmer, 1936).

was dissolved in water. From this solution a further protein fraction could be removed either by shaking with a chloroform-amyl alcohol mixture or by adjusting the pH to about 4.0 followed by addition of sodium flavianate. From the supernatant solution the mucoid was precipitated by 3 volumes of alcohol.

The concentration of ovomucoid in eggwhite has been reported as about 13 to 15 per cent of dry weight by electrophoretic measurement (Longsworth, Cannan, and McInnes, 1940). By isolation we have obtained about 10 per cent (Meyer, 1938). Other authors have reported much lower values.

The mucoid fraction has been reported by Longsworth, *et al.* (1940) to be electrophoretically inhomogeneous. The isoelectric point was reported as 4.3. Hesselvik (1938), who heated eggwhite for 3 to 4 hours on a water bath, reported homogeneity and an isoelectric point at pH 4.5. Constant values for nitrogen and hexosamine were obtained by us on fractions obtained by increasing alcohol and salt concentrations.

The analytical values reported in the literature show considerable variation. The following table summarizes some of the more recent figures.

Nitrogen	Hexosamine	$[\alpha]_D$	Author
12.60%	9.70%	—	Young (1937)
—	13.5	—	Karlberg (1936)
11.0	12.5	—	Hesselvik (1938)
12.7	—	$-57°$	Stacey, Woolley (1940, 1942)
11.1 to 11.8	12 to 16	$-65°$ to $-70°$	Meyer (1938)
11.7	—	$-62.9°$ to $-64.6°$	Sevag (1934)

The amino acid composition of ovomucoid has received little attention, but values for cystine have been reported as 3.95 per cent (Young, 1937) and 4.10 per cent (Needham,[5] 1927).

Considerable work has been done on the carbohydrate radicle of ovomucoid. Fränkel and Jellinek (1927) were the first to show in eggwhite the presence of mannose, isolated as phenylhydrazone, beside glucosamine, isolated as the hydrochloride. It is unfortunate that these careful experiments were done only on whole coagulated eggwhite. The authors assumed the presence in eggwhite of a biose composed of glucosamine and mannose, linked through the amino group of the glucosamine. The evidence for a biose was based on the elementary analysis of the mucopolysaccharide. A few years later Levene and Mori (1929) extended the work of these authors. They likewise isolated glucosamine and mannose from polysaccharides which they obtained not only from whole eggwhite, but from crystalline

[5] The material studied by this author was undoubtedly very impure.

egg albumin and from ovomucoid as well.[6] The polysaccharides obtained by them from these sources agreed among each other fairly well in analysis and rotation. However, they concluded from elementary analysis that the unit of the polysaccharide was a glucosamine dimannoside. This conclusion apparently has been widely accepted. In the isolation of the mucopolysaccharide, Levene and Mori had hydrolyzed their material with 10 per cent barium hydroxide for much longer periods than Fränkel and Jellinek. The latter, however, obtained a fraction apparently very similar to their barium hydroxide digested product by enzymatic splitting of the eggwhite with trypsin, both being optically inactive and comparing well in analysis with each other. Levene and Mori undoubtedly had saponified the acetyl group of the glucosamine by the prolonged treatment with barium hydroxide, since in the final product total nitrogen and amino nitrogen gave identical values and acetyl was absent, while the preparations of Fränkel and Jellinek gave amino nitrogen only after acid hydrolysis.

The instability to alkali of the mucopolysaccharide of crystalline egg albumin is evident from the data of Neuberger (1938). Treatment with normal sodium hydroxide at 100° for 16 hours liberated 50 per cent of the total nitrogen as ammonia. The yield of mannose as phenylhydrazone after a subsequent acid hydrolysis was also greatly diminished.

By a colorimetric method or by determining the total reducing value after hydrolysis, Karlberg (1936) found in ovomucoid 13.5 per cent glucosamine and 10.2 per cent "mannose." Masamune and Hoshino (1936) likewise found hexosamine and "mannose" in equimolar ratio. By colorimetric methods (orcinol) or by determining the reducing value, our preparations of ovomucoid all gave a ratio for hexosamine and hexose as 1:1. In summary, one may conclude that the claim of a one to two ratio for hexosamine and mannose may be incorrect as it is based only on elementary analysis of degraded material. The work of Stacey and Woolley (1940; 1942), who found 7 moles of glucosamine for 4 moles of mannose plus 1 mole of galactose, has been discussed previously.

Apparently a fraction identical with ovomucoid from eggwhite exists in egg yolk (Fränkel and Jellinek, 1927).

4. *Seromucoid and Seroglycoid*

Zanetti (1903) and later Bywaters (1909) investigated the occurrence in serum of mucoids similar to those of ovomucoid. Rimington (1929) demonstrated the presence of mannose in serum proteins. The carbohydrate rich fractions of serum proteins were then intensely studied by

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[6] Levene had earlier concluded that eggwhite as well as serum contained a mucoitin-sulfuric acid (1925). In later papers he did not repeat this claim. Apparently there is neither in eggwhite nor in serum any demonstrable material of such nature.

Hewitt (1936 to 1939). Hewitt isolated one fraction from the mother liquor of the albumin portion which he called seroglycoid. The crude albumin was separated into a carbohydrate rich fraction, which he termed globoglycoid, and a crystalline albumin, which was practically devoid of carbohydrate. The latest study on seromucoid, seroglycoid, and globoglycoid was carried out by Rimington (1940) and Rimington and Van der Ende (1940). These last authors seem to have obtained good chemical and immunological evidence for the non-existence of globoglycoid. On repeated reprecipitation from ammonium sulfate solution, the globoglycoid fraction crystallized in fine needles. The crystals were free of carbohydrate and were indistinguishable from the albumin fraction in amino acid content, molecular weight, crystalline form, rotation, and immunological response on the uterine strip of guinea pigs.

The same authors, however, found seroglycoid and seromucoid fractions of horse serum, although both immunologically impure, distinctly different from each other. It is evident likewise from the chemical data of Rimington as well as from our own that the mucoid fractions of serum are not homogeneous. How many components it contains, their exact chemical nature, as well as their physiological significance is at present unknown.

The serum mucoids are usually obtained from the mother liquor of serum from which the bulk of the protein has been removed by heat coagulation at an acid pH. On fractionation of the serum with salts, most of the mucoid fractions seem to coprecipitate with the proteins, and only a small amount can be obtained on salt fractionation. On removing the acetone denatured proteins from slightly alkaline salt.solution with chloroform-amyl alcohol, the largest part of the mucoid fractions are also coprecipitated with the proteins.

Rimington prepared the mucoid fractions from defibrinated ox blood, which was diluted with an equal volume of 0.9 per cent sodium chloride. The cells were removed, the pH adjusted to 4.7, and the mixture heated to maximal coagulation by steam. The clear filtrate was concentrated to a small volume in vacuo and the crude mucoid precipitated with 10 volumes of alcohol. The precipitate was washed with alcohol and ether and dried. The aqueous extract of this material was dialyzed and from the clear fluid a bulky precipitate was obtained by 2.75 volumes of alcohol. The aqueous solution of this precipitate was then fractionated with alcohol. The main fraction with a ratio of carbohydrate to total nitrogen of 0.95 precipitated with 1 volume of alcohol. The end fraction, obtained with 4 volumes of alcohol, constituted only a small amount of material with a carbohydrate to total nitrogen ratio of 4.40. The main fraction was again fractionated with alcohol and showed a ratio of carbohydrate to total nitrogen of 0.79, corresponding to about 10 per cent glucosaminodihexose (see below for a

discussion of the carbohydrate moiety) and a total nitrogen of 13.60 per cent and sulfur of 1.59 per cent.

The hexoses were determined colorimetrically by the reaction with orcinol, as in the procedure of Tilmans and Philippi (1929), using a mixture of equal parts of mannose and galactose as a standard. Hexosamine, determined according to Elson and Morgan (1933) was very close to one-half the "hexose" value, the ratio of hexose and glucosamine in all preparations being close to 2:1. $[a]_D$ of the main fraction was about $-54°$, the cystine content 2.28 per cent, tyrosine and tryptophan 2.98 and 1.03 per cent, respectively. The most soluble fractions had a total carbohydrate content of about 45 per cent, with a nitrogen of 9.67 per cent and correspondingly lower total sulfur and amino acid contents.

The mucoid fractions were not precipitated by heating or by 2 per cent trichloroacetic acid, but were precipitated by saturation with ammonium sulfate. Rimington believes the main fraction, the constants of which are listed above, to be homogeneous.

The preparation of seroglycoid, as reported in various papers by Hewitt, was carried out either by ammonium sulfate fractionation or by alcohol precipitation after heat coagulation to remove globulin and albumin. From his work Hewitt concluded that seroglycoid was different from the seromucoid of previous publications. According to his author, seroglycoid has a carbohydrate content of between 10 and 11 per cent, while the seromucoid contained, according to Bywaters (1909) and Rimington (1933), about 24 per cent. It has been pointed out above that Rimington (1940) in his last publication gives the carbohydrate content of the main fraction of seromucoid as 10.7 per cent. Similar low values were obtained by Onoe (1936) and in this laboratory. The second argument for the difference between seroglycoid and seromucoid was the fact that the latter was prepared from the supernatant solutions after coagulation of the protein, while seroglycoid on boiling with protein was carried down to a large extent with the albumin. However, whether or not these mucoids coprecipitate with proteins is conditioned by the presence of salt and pH and holds true for both fractions equally well. It was, however, reported above that seroglycoid and seromucoid were distinctly different serologically. Whether this indicates essential differences remains doubtful, since the seromucoid used by Rimington and Van der Ende (1940) in these experiments was a rather crude fraction not corresponding in purity to the fractions described in the preceding paper (Rimington, 1940). In summary, it may be stated that seromucoid is a mixture. Whether seroglycoid is a single and distinct fraction has not been established.

In fever the total serum hexosamine is greatly increased (Nilsson, 1937; West, 1936). Whether the serum mucoid fraction is increased has appar-

ently not been determined. In some cases of carcinoma an increase of about 300 per cent over the normal was found in one serum mucoid fraction (Mayer, 1942). This fraction was thought to be responsible for a polaro-graphic carcinoma reaction.

Zanetti (1903) isolated glucosamine (as the tetrabenzoyl compound) from the hydrolyzate of a seromucoid fraction. The presence of mannose in seromucoid fractions, contrary to many statements in the literature, has apparently never been established by isolation. The presence of mannose in acid hydrolyzates of plasma treated with phosphotungastic acid was apparently established first by Dische (1929) who isolated mannose phenylhydrazone from the mixture. A second non-fermentable reducing substance was thought by this author to be glucosamine. Rimington in 1929 isolated glucosamine as the hydrochloride and mannose as phenyl-hydrazone from carbohydrate fractions obtained after barium hydroxide digestion of serum albumin, serum globulin, and mixed albumin-globulin. These protein preparations had been reprecipitated 8 times and, after denaturation with alcohol, washed thoroughly with distilled water to free them from easily soluble components. An identical component was isolated from an albumin-globulin fraction hydrolyzed for 4 weeks with trypsin without alkaline hydrolysis. The hydrolysis with barium hydroxide was carried out for only 3½ hours. In the isolation of the polysaccharide the author followed in general the procedure used by Fränkel and Jellinek (1927) for the isolation of the mucopolysaccharide of eggwhite. The analytical figures published, like those of the last named authors, agree well with those of a disaccharide. In 1931, apparently under the influence of the paper by Levene and Mori (1929), Rimington hydrolyzed the same proteins with 10 per cent barium hydroxide for periods up to 42 hours. The nitrogen values, as well as the yields, of the material decreased with the time of hydrolysis and apparently did not become constant. However, in most preparations the hydrolysis was stopped when the nitrogen values approached those required for a trisaccharide, the compound being assumed to be composed of one mole hexosamine and two moles of mannose. The orcinol reaction of Tilmans and Philippi corresponded to that required by a triose, while the indole reaction of Dische and Popper (1926) gave considerably lower values.

A later paper on serum mucoid by Rimington (1933) did not give detailed chemical data. The carbohydrate content determined by the orcinol method and expressed as glucosaminodimannose approached twenty-two per cent, but "some variation was noted."

In the last paper on seromucoid by Rimington (1940), the hexoses in the mucoid fractions are said to be composed of equimolar mixtures of mannose and galactose. This conclusion is apparently based on the exam-

ination with two different filters of the color produced by orcinol sulfuric acid, although the difference between mannose and galactose seems to have been slight. Since no significant deviation was found by using the two filters and equal mixtures of mannose and galactose, the author concludes that the two hexoses are present in equimolar ratio. The evidence for this statement appears insufficient.

5. *Ovomucoid-β*

As examples of insoluble mucoids, the so-called ovomucin of eggwhite and the chalazae are discussed briefly. On dilution of eggwhite with 2 to 3 volumes of water a mucilaginous precipitate is obtained, which can be redispersed with 5 to 10 per cent sodium chloride and reprecipitated by dilution with water. Even concentrated salt solutions or 50 per cent urea solutions do not result in a solution which can be filtered. The following data are given by Young (1937): nitrogen 11.8 to 13.1 per cent, total sulfur 1.59 and 1.80 per cent, cystine 3.25 to 5.61 per cent, hexosamine 10.4 to 12.1 per cent.

The chalazae of eggwhite washed repeatedly with 5 per cent sodium chloride and distilled water gave the following analysis: nitrogen 13.3 per cent, sulfur 1.08 per cent, cystine 4.10 per cent, hexosamine 11.4 per cent. It may be noted that the analysis of the ovomucoid is quite similar to that of the chalazae. Some of Young's experiments were repeated in this laboratory and similar results were obtained (Meyer, 1938).

6. *Submaxillary Mucoid*

The carbohydrate-protein compounds obtained from submaxillary gland have been studied by Blix and his collaborators and by this laboratory. Blix (1936) used extraction at 0° C. by water followed by precipitation by dilute hydrochloric acid according to Hammarsten (1882). On the basis of a "direct positive color test" with *p*-dimethylaminobenzaldehyde and the orcinol reaction, Blix distinguished two substances in such extracts, one a neutral mucoid in which a ratio of one mole hexosamine to 2 moles of hexose was established by color reactions. This fraction was not further investigated. In the "crude mucin" fraction it was present in a concentration of about 5 per cent. From the other fraction a small quantity of a crystalline nitrogenous acid was isolated by a process not well defined. The crude dehydrated extracts were refluxed with 95 per cent alcohol. The residue was boiled under nitrogen with water for 2 hours, the extracts were evaporated in vacuo, and extracted twice with methanol. To the concentrated methanol solution ether was added, the resulting precipitate was discarded and petroleum ether added to the supernatant solution. On standing in the cold the acid deposited in crystal clusters along with oily material.

The acid was recrystallized from methanol-petroleum ether solution. The yield was 0.05 to 0.075 per cent of "mucin." By color reactions Blix found the "mucin" to contain 20 to 25 per cent of this acid.

The product was strongly acid in nature, gave positive direct Ehrlich reaction with p-dimethylaminobenzaldehyde, negative Molisch and orcinol reactions, and strongly reduced alkaline copper solution on warming.

The analysis for carbon, hydrogen, and nitrogen fitted best the formula $C_{14}H_{23}$ or $_{25}NO_{11}$ (Blix erroneously gives H_{24}), although other formulae could be fitted to the analysis. Micro acetyl determination gave values between 22.6 and 23.4 per cent indicating two acetyl groups, one of which Blix believed to be due to degradation of the molecule (see footnote 4). Titration indicated an equivalent weight of 381 which is in good agreement with the theoretical value of 381–383 for the C_{14} formula.

In experiments in this laboratory a disappearance of carbohydrate was noted when fresh submaxillary glands were used for extraction, especially when the temperature became elevated. The cold minced glands were therefore dehydrated with acetone and ether and the extraction carried out at room temperature with 50 per cent alkaline urea at a pH of about 9. From the diluted extracts an acidic mucoid was obtained after acidification with acetic acid, while a neutral mucoid, not further investigated, remained in the supernatant fluid. The acid fraction was redissolved and reprecipitated and inert protein removed by stirring with chloroform-amyl alcohol solution and by adsorption on Lloyd's reagent. A further purification was achieved by precipitating an aqueous solution with 10 to 12 volumes of glacial acetic acid. After being washed with alcohol and ether the resulting material was not acidic in contrast to other acid polysaccharides, such as hyaluronic acid, which under the same conditions are converted from their sodium or calcium salts to the free acid. A free acid is obtained, however, from the basic lead salt after removing lead with sulfuric acid and precipitating with alcohol. Fractions thus obtained contain 8 to 9 per cent of nitrogen, 18 to 22 per cent of hexosamine, and an amount of carbon dioxide (obtained on decarboxylation with zinc chloride according to Freudenberg, et al., 1941) equivalent to the hexosamine value.

As in Blix's compound the naphthoresorcinol reaction for uronic acids is negative, the Molisch and orcinol reactions are very weakly positive, and the substance gives the direct Ehrlich's reaction as described by Blix. After hydrolysis by normal hydrochloric acid in a sealed tube the reducing values corresponded closely to the hexosamine content. It can be concluded that a uronic acid or a ketose and aldose are absent or present only in minimal concentrations. After hydrolysis with 6 N hydrochloric acid hexosamine was isolated and characterized by analysis and rotation as glucosamine hydrochloride. On oxidation with nitric acid according to Van der

Haar (1925) saccharic acid was isolated as acid potassium saccharate, which was converted to the silver salt and analyzed. This finding strongly indicates that the carbohydrate portion contains gluconic acid, since glucuronic acid is excluded by the negative naphthoresorcinol reaction and by the absence of a reducing group. Gluconic acid on decarboxylation gives one equivalent of carbon dioxide under the same conditions as glucuronic acid.[7] Gluconic acid or its lactone in our experience gives a weak Molisch reaction. In our mucoid the orcinol reaction corresponded to the carbon dioxide value.

The material as isolated contains a peptide chain. The biuret reaction is positive. It contains neither sulfur nor phosphorus, and tests for tyrosine, tryptophan and histidine are negative. The Sakaguchi reaction for arginine is positive. No carbohydrate is lost on prolonged dialysis. In the Tiselius apparatus one sample migrated in a single boundary with a mobility of -6.95×10^{-5} at pH 7.8 in barbiturate buffer. It must be concluded from these experiments that the substance is a mucoid containing an acidic group. On incubation of the mucoid with filtered saliva in the presence of toluene, an increase in reducing power is obtained at different rates with different samples of saliva. Some extracts prepared from autolyzates of pneumococci contain a heat labile enzyme which in 24 hours will liberate reducing groups equivalent to the concentration of hexosamine in the mucoid. Amylase, lysozyme, and hyaluronidase do not attack the mucoid. This laboratory is testing other bacteria, especially those involved in upper respiratory infections, for the presence of this enzyme. The acid carbohydrate isolated by Blix is probably identical with the reducing carbohydrate liberated by this enzymatic hydrolysis. Blix's analytical figures agree well with those calculated for an acetyl glucosamine-gluconic acid. Glucosamine and gluconic acid may be linked to each other by an ether linkage, while the "disaccharide" units are linked together by the glucosidic linkage through the amino sugar. This conclusion is based on the presence of only one glucosidic group in the disaccharide, the acetylation of the glucosamine, the presence of a free carboxyl group, and of a long chain type carbohydrate as evidenced by viscosity and enzymatic hydrolysis.

In sublingual gland extracts, mucoids with properties identical with those described by Blix were found by Tanabe (1939).

VI. GLYCOPROTEINS

The distinction between mucoids and glycoproteins, based on a hexosamine content of the former of 4 per cent or over, is arbitrary. In many instances a substance cannot be placed properly at the present time.

[7] For these tests crystalline glucono-lactone was prepared from twice recrystallized calcium gluconate.

Furthermore homogeneity of fractions in this group has been rigidly proved even less often than in the mucoids. For example, complex formation is known to occur among the blood proteins and the question whether carbohydrate is an essential part of a molecule or whether it occurs as a complex between different proteins in some instances is not yet settled. This problem is still more complicated for the tissue proteins, where our knowledge is very meager. However, in at least two instances, in a serum albumin fraction and in egg albumin, the data strongly support the theory that carbohydrate is an essential part of these protein molecules.

1. Serum Albumin

The serum albumin fraction is not homogeneous, as shown by electrophoresis or by salt fractionation. Kekwick (1938) partly in confirmation of work by Soerenson (1930) obtained from horse serum two crystalline albumin fractions A and B. A was more soluble than B, both were recrystallized from sodium sulfate solution. In the two fractions the hexose content, determined with orcinol, was 1.95 per cent for fraction A and 0.083 per cent for fraction B. No hexosamine values were given. Electrophoretically both fractions were homogeneous; serologically there was some difference between A and B, as reported in an addendum by Gell and Yuill.

Blix, Tiselius, and Svensson (1941) found in the albumin fraction of two human sera, obtained by two electrophoretic separations, 1.1 and 1.2 per cent hexose, while the hexosamine values were 0.45 and 0.56 per cent, respectively. (The authors state that the hexose values according to the method of Soerenson and Haugaard cannot be considered very accurate.) In one serum albumin fraction from a patient with lobar pneumonia the hexose was 5.8 per cent, the hexosamine 3.6 per cent.

Carbohydrate-free preparations of serum albumin have been reported by Soerenson (1930), Hewitt (1936), and by McMeekin (1939). Electrophoretically even the carbohydrate-free and crystalline fraction is inhomogeneous (Luetscher, 1939).

2. Serum Globulin

The serum globulin fractions all seem to contain carbohydrate. The homogeneity of these fractions is still more open to question as their properties depend on the method of preparation. Complex formation in these proteins seems to be the rule (Jameson and Alvarez-Tostado, 1943; Hewitt, 1938). Values varying from 1.4 to 5.6 per cent have been given for the carbohydrate content of different globulin fractions from different animals. Tiselius (1937a, b) reports hexose values of 0.4 per cent for α-globulin, 2.2 per cent for β-globulin, and 0.7 per cent for γ-globulin of horse serum.

Blix, Tiselius, and Svensson (1941) gave much higher hexose values for 2 normal sera and one serum from a pneumonia patient as follows:

	α-Globulin	β-Globulin	γ-Globulin
Normal I	—	8.3%	3.6%
Normal II	6.0%	6.2	3.0
Pneumonia	9.9	1.5	3.7

A very high carbohydrate value of 10.3 per cent (orcinol method) for one euglobulin fraction, which the authors found to be one component of complement, has been reported (Pillemer, et al., 1941).

Serum globulins have occasionally been observed to crystallize from certain pathological sera. One such globulin, not identical with Bence-Jones protein, was reported recently. It gave a strong Molisch reaction (Holmberg and Grönwall, 1942).

Prothrombin and thrombin prepared by Seegers (1940) had a carbohydrate content (Soerenson and Haugaard) of 3.8 to 6.2 per cent. The carbohydrate seems to be associated with the activity of these preparations.

3. Egg Albumin

Most authors have agreed on the presence of carbohydrate in crystalline egg albumin. However, some have ascribed it to contamination by coprecipitated ovomucoid, while others considered it an essential component of the molecule. Levene and Mori (1929) favored the first assumption. M. Soerenson (1934) by the orcinol method found 1.7 per cent hexose in crystalline egg albumin which she believed to be mannose. Neuberger (1938) carefully reëxamined the question of carbohydrate in egg albumin. He found by the orcinol method a hexose content of 2.8 per cent in the first crystallization, which fell to 1.8 per cent in the third and remained constant over seven recrystallizations. The carbohydrate, even after denaturation of the protein by heat or acid, did not pass through membranes of known porosity which retained the protein, and the ratios of hexoses to nitrogen remained constant in ultra filtrates obtained with membranes of lower porosities.

The isolation by acetylation and deacetylation (Neuberger, 1938) of a carbohydrate group after tryptic digestion of crystalline egg albumin has already been mentioned. The carbohydrate isolated had a molecular weight of 1200 and contained 2 moles of hexosamine, 4 moles of mannose, and an unidentified nitrogenous constituent. The polysaccharide was regarded as forming a single prosthetic group with one point of attachment to the molecule of egg albumin. Neuberger has estimated that egg albumin of a molecular weight of about 40,000 contains one such polysaccharide unit of molecular weight of 1200.

REFERENCES

Bergmann, M., and Zervas, L. (1931). *Ber.* **64**, 975.

Blix, G. (1936). *Z. physiol. Chem.* **240**, 43.

Blix, G., and Snellman, O. (1944). *Nature* **153**, 587.

Blix, G., Tiselius, A., and Svensson, H. (1941). *J. Biol. Chem.* **137**, 485.

Booth, V., and Green, D. E. (1938). *Biochem. J.* **32**, 855.

Bray, H. G., Gregory. J. E., and Stacey, M. (1944). *Biochem. J.* **38**, 142.

Bywaters, H. W. (1909). *Biochem. Z.* **15**, 322.

Chambers, L. A., and Flossdorf, E. W. (1936). *Proc Soc. Exptl. Biol. Med.* **34**, 631.

Chargaff, E., and Bovarnick, M. (1937). *J. Biol. Chem.* **118**, 421.

Charles, A. F., and Scott, D. A. (1936). *Biochem. J.* **30**, 1927.

Charles, A. F., and Todd, A. R. (1940). *Biochem. J.* **34**, 112.

Dische, Z. (1929). *Biochem. Z.* **201**, 74.

Dische, Z. (1930). *Mikrochemie* **8**, 4.

Dische, Z., and Popper, H. (1926). *Biochem. Z.* **175**, 371.

Elson, L. A., and Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.

Fischer, F. G., and Ertel, L. (1931). *Z. physiol. Chem.* **202**, 83.

Fleischer, G., Schwenk, E., and Meyer, K. (1938). *Nature* **142**, 835.

Fränkel, S., and Jellinek, C. (1927). *Biochem. Z.* **185**, 392.

Freudenberg, K., Gudjons, H., and Dumpert, G. (1941). *Ber.* **74B**, 245.

Goebel, W. F., Beeson, P. B., and Hoagland, C. L. (1939). *J. Biol. Chem.* **129**, 455.

Goebel, W. F., Shedlovsky, T. H., Lavin, G. I., and Adams, M. H. (1943). *J. Biol. Chem.* **148**, 1.

Gurin, S. (1942). *Proc. Soc. Exptl. Biol. Med.* **49**, 48.

Gurin, S. (1945, in press). Symposium on Hormones, Am. Assoc. Advance Sci.

Gurin, S., Bachman, C., and Wilson, D. W. (1940). *J. Biol. Chem.* **133**, 467, 477.

Gurin, S., and Hood, D. B. (1939). *J. Biol. Chem.* **131**, 211.

Hammarsten, O. (1882). *Z. physiol. Chem.* **12**, 163.

Hartman, M., and Benz, F. (1938). *Nature* **142**, 115.

Hesselvik, L. (1938). *Z. physiol. Chem.* **254**, 144.

Hewitt, L. F. (1936). *Biochem. J.* **30**, 2229.

Hewitt, L. F. (1937). *Biochem. J.* **31**, 360, 1047, 1534.

Hewitt, L. F. (1938). *Biochem. J.* **32**, 26.

Hewitt, L. F. (1939). *Biochem. J.* **33**, 1496.

Holmberg, C. G., and Grönwall, A. (1942). *Z. physiol. Chem.* **273**, 199.

Irving, J. C., and Earl, J. C., (1922). *J. Chem. Soc.* **121**, 2376.

Ivánovics, G. (1940). *Z. Immunol.* **97**, 402.

Jameson, E. and Alvarez-Tostado, C. (1943). *J. Am. Chem. Soc.* **65**, 459.

Jolles, Z. E., and Morgan, W. T. J. (1940). *Biochem. J.* **34**, 1183.

Jorpes, E., and Bergstroem, S. (1937). *J. Biol. Chem.* **118**, 447.

Kabat, E. A. (1943). *J. Immunol.* **47**, 513.

Karlberg, O. (1936). *Z. physiol. Chem.* **240**, 55.

Kekwick, R. A. (1938). *Biochem. J.* **32**, 552.

King, H. K., and Morgan, W. T. J. (1944). *Biochem. J.* **38**, X.

Landsteiner, K. (1936a). *J. Exptl. Med.* **63**, 185.

Landsteiner, K. (1936b). The Specificity of Serological Reactions. Charles C. Thomas.

Landsteiner, K., and Chase, M. W. (1935). *Proc. Soc. Exptl. Biol. Med.* **32**, 713, 1208.

Landsteiner, K., and Harte, R. A. (1940). *J. Exptl. Med.* **71**, 551.

Levene, P. A. (1925). Hexosamines and Mucoproteins. Longmans, Green, and Co.

Levene, P. A., and Mori, T. (1929). *J. Biol. Chem.* **84**, 49.

Longsworth, L. G., Cannan, R. H., and McInnes, D. A. (1940). *J. Am. Chem. Soc.* **62**, 2580.

Luetscher, J. A. (1939). *J. Am. Chem. Soc.* **61**, 2888.

Lundgren, H. P., Gurin, S., Bachman, C., and Wilson, D. W. (1942). *J. Biol. Chem.* **142**, 367.

Masamune, H., and Hoshino, S. (1936). *J. Biochem. (Japan)* **24**, 299.

Mayer, K. (1942). *Z. physiol. Chem.* **275**, 16.

McMeekin, T. L. (1939). *J. Am. Chem. Soc.* **61**, 2884.

Meyer, K. (1937). *in* Kurzrock, R. The Endocrines in Obstetrics and Gynecology, p. 115. Williams and Wilkins Co., Baltimore.

Meyer, K. (1938). *Cold Spring Harbor Symposia Quant. Biol.* **6**, 91.

Meyer, K., Bloch, H.·S., and Chaffee, E. (1942). *Federation Proc.* **1**, 125.

Meyer, K., and Chaffee, E. (1940a). *J. Biol. Chem.* **133**, 83.

Meyer, K., and Chaffee, E. (1940b). *Am. J. Ophthalmol.* **23**, 1320.

Meyer, K., and Chaffee, E. (1941). *J. Biol. Chem.* **138**, 491.

Meyer, K., Hahnel, E., and Feiner, R. (1945, in press). *Proc. Soc. Exptl. Biol. Med.*

Meyer, K., Hobby, G. L., Chaffee, E., and Dawson, M. H. (1940). *J. Exptl. Med.* **71**, 137.

Meyer, K., and Palmer, J. W. (1936). *J. Biol. Chem.* **114**, 689.

Meyer, K., Palmer, J. W., and Smyth, E. M. (1937). *J. Biol. Chem.* **119**, 501.

Meyer, K., and Smyth, E. M. (1937). *J. Biol. Chem.* **119**, 507.

Meyer, K., Smyth, E. M., and Dawson, M. H. (1939). *J. Biol. Chem.* **128**, 319.

Meyer, K., Smyth, E. M., and Palmer, J. W. (1937). *J. Biol. Chem.* **119**, 73.

Moore, S., and Link, K. P. (1940). *J. Biol. Chem.* **133**, 293.

Morgan, W. T. J. (1938). *Helv. Chim. Acta.* **21**, 469.

Morgan, W. T. J., and King, H. K. (1943). *Biochem. J.* **37**, 640.

Morgan, W. T. J., and Patridge, S. M. (1940). *Biochem. J.* **34**, 169.

Morgan, W. T. J., and Patridge, S. M. (1941). *Biochem. J.* **35**, 1140.

Mörner, K. A. H. (1895). *Skand. Arch. Physiol.* **6**, 332.

Müller, F. (1898). *Ber. Ges. Beförderung Natur.* p. 118.

Needham, J. (1927). *Biochem. J.* **21**, 733.

Neuberg, C., and Hirschberg, E. (1910). *Biochem. Z.* **27**, 339.

Neuberg, C., and Saneyoshi, S. (1911). *Biochem. Z.* **36**, 56.

Neuberger, A. (1938). *Biochem. J.* **32**, 1435.

Nilsson, I. (1937). *Biochem. Z.* **291**, 254.

Nungester, W. J., Wolf, A. A., and Jourdonais, L. F. (1932). *Proc. Soc. Exptl. Biol. Med.* **20**, 120.

Onoe, T. (1936). *J. Biochem. (Japan)* **24**, 1.

Palmer, J. W., Smyth, E. M., and Meyer, K. (1937). *J. Biol. Chem.* **119**, 491.

Pauly, H. and Ludwig, E. (1922). *Z. physiol. Chem.* **121**, 170.

Pennington, D., Snell, E. E., and Eakin, R. E. (1942). *J. Am. Chem. Soc.* **64**, 469.

Pillemer, L., Ecker, E. E., Oncley, J. L., and Cohn, E. J. (1941). *J. Exptl. Med.* **74**, 297.

Rimington, C. (1929). *Biochem. J.* **23**, 430.

Rimington, C. (1931). *Biochem. J.* **25**, 1062.

Rimington, C. (1933). *Ergb. Physiol.* **35**, 712.

Rimington, C. (1940). *Biochem. J.* **34**, 931.

Rimington, C. and Rowlands, I. W. (1944). *Biochem. J.* **38**, 54.

Rimington, C. and Van der Ende, M. (1940). *Biochem. J.* **34**, 941.

Schiff, F. (1939). *J. Infectious Diseases* **65**, 127.

Schulz, F. N. and Becker, M. (1935). *Biochem. Z.* **280**, 217.

Seegers, W. H. (1940). *J. Biol. Chem.* **136**, 103.
Sevag, M. G. (1934). *Biochem. Z.* **273**, 419.
Soerenson, M. (1934). *Biochem. Z.* **269**, 271.
Soerenson, M. and Haugaard, G. (1933). *Biochem. Z.* **360**, 247.
Soerenson, S. P. L. (1930). *Compt. rend. trav. Lab. Carlsberg* **18**, 1.
Stacey, M. and Woolley, J. M. (1940). *J. Chem. Soc.*, p. 184.
Stacey, M., and Woolley, J. M. (1942). *J. Chem. Soc.*, p. 550.
Steudel, H. (1901). *Z. physiol. Chem.* **34**, 353.
Tanabe, Y., (1939). *J. Biochem. (Japan)* **30**, 181.
Tilmans, J. and Philippi, K. (1929). *Biochem. Z.* **215**, 36.
Tiselius, A. (1937a). *Biochem. J.* **31**, 313, 1464.
Tiselius, A. (1937b). *Trans. Faraday Soc.* **33**, 524.
Van der Haar, A. W. (1925). Anleitung zum Nachweis, zur Trennung und Bestimmung
    der Monosaccharide und Aldehydsäuren. Berlin.
West, R. (1936). *Trans. Assoc. Am. Physicians* **51**, 230.
Woolley, D. W. and Longsworth, L. G. (1942). *J. Biol. Chem.* **142**, 285.
Young, E. G. (1937). *J. Biol. Chem.* **120**, 1.
Zanetti, C. (1903). *Gazz. chim. ital.* **33** I, 160.
Zittle, C. A. and Harris, T. N. (1942). *J. Biol. Chem.* **142**, 823.

The Reactions of Formaldehyde with Amino Acids and Proteins

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CONTENTS

I. INTRODUCTION

Formaldehyde is a reagent familiar to all protein chemists through its employment in the formol titration of amino acids and peptides, following the classical studies of Sörensen (1907). It is also of immense importance to the immunologist, because of its use in converting the toxins of diphtheria and tetanus into the relatively inert toxoids which are far less dangerous than the original bacterial poisons, but still possess the power of evoking specific antitoxins on injection into animals or man. Moreover, formaldehyde has been widely used as a tanning agent for collagen and other fibrous proteins. Like other such agents, it renders these proteins relatively inert to digestion with trypsin, greatly decreases their tendency to swell in water, or in acid or alkaline solutions. In the specific case of collagen, it markedly increases the temperature required to produce thermal shortening.

All these properties of formaldehyde are of great interest, both theoretical and practical. Its reactions with proteins, however, are inevitably numerous and complex. Formaldehyde can combine with any one of a number of different kinds of functional groups found in proteins. Moreover, when steric relations are favorable, it can react with two such groups, forming a methylene bridge between them. It is reactions of the latter type which are most likely to modify the mechanical properties of the protein. Thus, the interpretation of observed changes in the properties of the proteins is rendered extremely difficult by the multiplicity of possible reactions which must be considered. The remarkable variety of these reactions, indeed, has not always been fully realized by those who have employed formaldehyde as a reagent. Some of the reactions are rapid, some are slow; some are readily reversible, some practically irreversible; some proceed readily even at room temperature, others only at higher temperatures. Their nature is, of course, far more readily discerned when only one or two functional groups are involved, as in the amino acids and dipeptides, rather than in the more complex peptides and the proteins. We have, therefore, devoted the major part of this review to a discussion of the

simpler compounds, and have given only a relatively brief discussion of a small number of recent studies on proteins, with emphasis on work which can be at least tentatively interpreted in terms of definite reactions with functional groups in the protein. The discussion of proteins makes no attempt to cover the vast technical literature relating to formaldehyde, but simply to present the results of a few significant researches which may serve to stimulate the further investigations which are needed in this field.

II. General Properties of Formaldehyde[1]

1. Anhydrous Formaldehyde

Pure monomeric formaldehyde may be obtained by dry distillation of paraformaldehyde in a stream of inert gas, drying the vapors thoroughly and condensing at a low temperature. Liquid formaldehyde so obtained is fairly stable in the absence of moisture and boils at $-21°$ C. If traces of moisture are present, the monomeric form polymerizes, often suddenly and violently. Several polymers, more or less well defined, are known. The open chain hydrated polyoxymethylenes, $H-(-O-CH_2-)_n-OH$, range from the water-soluble di- and trioxymethylenes to the highly insoluble paraformaldehyde with $n = 100$ or more. For detailed discussions of the polymers of formaldehyde, with extensive references, see Staudinger (1933, pp. 224-287 incl.); and Walker (1944), Chapter 7.

The cyclic trimer of formaldehyde, *sym*-trioxane, is a clear, crystalline solid with a low melting point (m.p. 60–61° C.) and none of the characteristic reactivity of formaldehyde. The odor resembles that of chloroform or *p*-dichlorobenzene. It can be distilled unchanged, but at high temperatures in the presence of acid catalysts it is broken down into formaldehyde.

2. Structure of Formaldehyde in Aqueous Solution: Methylene Glycol

Technical formalin contains about 37 per cent formaldehyde and varying amounts of methanol in water. By fractional distillation, discarding the low-boiling fractions, it is possible to obtain a relatively methanol-free product boiling at 99–100° C.

Solutions of pure formaldehyde in water may be readily obtained by passing a stream of formaldehyde gas, mixed with nitrogen, into water. Solutions containing as much as 30 per cent formaldehyde are relatively stable for short periods of time, although the methanol present in commercial formalin appears to stabilize the formaldehyde and inhibit the forma-

[1] A comprehensive monograph on formaldehyde has recently been published by J. F. Walker (1944), and the reader is referred to it for thorough discussion of many points which can only be mentioned briefly, if at all, in the present review.

tion of higher polymers. For accurate studies, involving determination of equilibrium and velocity constants, solutions of purified formaldehyde are, of course, decidedly preferable to those containing methanol.

Freezing-point measurements of aqueous solutions of formaldehyde (Auerbach and Barschall, 1905) indicate that at concentrations up to one molar or more, the formaldehyde is present chiefly in monomeric form. However, the ultra-violet spectra of such solutions (Bielecki and Henri, 1912) show no evidence of the characteristic absorption band near 2800 Å, which is typical of compounds containing the carbonyl group. Likewise, the Raman spectra of aqueous solutions of formaldehyde (Hibben, 1931; Krishnamurti, 1931; Kohlrausch and Köppl, 1934) show no evidence of the typical carbonyl vibration frequency near 1700 cm.$^{-1}$. These facts, combined with the low volatility of formaldehyde in aqueous solution, indicate that it exists chiefly as the hydrate, methylene glycol $CH_2(OH)_2$.[2] At higher concentrations, the molal freezing point depression of formaldehyde in water is considerably less than that calculated for methylene glycol, suggesting the presence of formaldehyde polymers in equilibrium with the monomeric form. Auerbach and Barschall (1905) calculated an equilibrium constant for the monomer and an assumed open chain-trimeric form, assuming the reaction to be

$$HO\text{--}CH_2\text{--}O\text{--}CH_2\text{--}O\text{--}CH_2\text{--}OH + 2\ H_2O \rightleftharpoons 3\ HO\text{--}CH_2\text{--}OH \qquad (1)$$
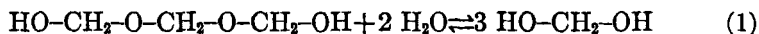
They found that a value of $K = 0.0264$ gave a good fit to the observed data. On this basis, the concentration of monomeric formaldehyde approaches a maximum of about 11 per cent when the total concentration of formaldehyde is 20 per cent. The monomer concentration would not increase above this value with increase of total formaldehyde up to 40 per cent. It seems certain, however, that Equation 1 must be an inadequate representation of the equilibrium actually existing in formaldehyde solutions. There is every reason to expect the presence of the dimer and (in smaller concentrations) of a series of polymers larger than the trimer.

On diluting a concentrated solution of formaldehyde, a measurable amount of time is required for the depolymerization to take place. Wadano, Trogus, and Hess (1934) have studied the kinetics of the depolymerization reaction. They found the rate to vary markedly with pH, reaching a

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[2] Confirmatory evidence may be obtained from the apparent molal volume of formaldehyde in such solutions. From the density data of Auerbach and Barschall (1905), this volume is calculated as approximately 21 cc. per mol. Employing the atomic volumes deduced by Traube (1899) (see also Cohn and Edsall, 1943, p. 157) and a co-volume of 13 cc, the value for $H_2C = O$ should be near 34.5 cc. The calculated value for $CH_2(OH)_2$ is 38.0 cc. However, one mol of water, with a volume of 18 cc. is consumed in the formation of a mol of methylene glycol; hence, the expected value for the latter compound is 20 cc./mol, in very good agreement with the apparent molal volume actually observed.

minimum in the pH range 2–4 and increasing rapidly as the pH deviates from this minimum in either direction. At pH values of 7 and above, the equilibrium state is reached very quickly. There is no convincing evidence that the final equilibrium state is dependent on pH, and no such dependence would be expected if the stable forms of the monomer and polymers are uncharged molecules; see the discussion by Levy and Silberman (1937).

Formaldehyde in aqueous solution is a very weak acid, the pK value according to Wadano (1934) being 12.79, and according to Levy (1934) being 12.87. Even this very weak acidity, however, leads to the neutralization of significant amounts of alkali at pH values of 9 or even below, in moderately concentrated formaldehyde solutions — a fact which has important implications, as Levy has pointed out, for the accuracy of the formol titration.

3. REACTIONS OF FORMALDEHYDE WITH FUNCTIONAL GROUPS FOUND IN AMINO ACIDS AND PEPTIDES

a. *Addition and Condensation Reactions*

The most frequently encountered reaction of formaldehyde is its addition to a compound containing an active hydrogen atom with the formation of a hydroxymethyl compound:

$$R-H+CH_2O \rightleftharpoons R-CH_2(OH) \tag{2}$$

If the formaldehyde is present as methylene glycol, this may be formulated as a condensation reaction,

$$R-H+CH_2(OH)_2 \rightleftharpoons R-CH_2(OH)+H_2O \tag{2a}$$
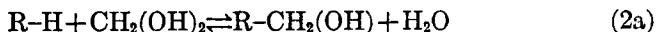
The hydroxyl group thus formed is usually reactive and may condense with another atomic grouping containing an active hydrogen atom to form a methylene bridge:

$$R-CH_2(OH)+H-R' \rightleftharpoons R-CH_2-R'+H_2O \tag{3}$$

This condensation may take place intramolecularly with the formation of cyclic structures, intermolecularly with the formation of molecular aggregates, or with more formaldehyde molecules to form polyoxymethylene chains or bridges. In general, one may expect the formation of cyclic structures in polyfunctional amino acids if steric conditions are favorable, and there is now abundant evidence, presented later in this discussion, that such structures are actually formed.

Numerous groups found in amino acids, peptides, and proteins are capable of undergoing addition and condensation reactions with formaldehyde.

(1) *The Amino Group*. The reactions of this group with formaldehyde have certainly received more intensive study than those of any other group.

Sprung (1940) has reviewed the reactions of aldehydes with amines. The oldest formulation of the reaction with primary amines

$$R-NH_2+CH_2O \rightleftharpoons R-N=CH_2+H_2O \tag{4}$$

with the formation of monomeric Schiff bases appears to be of little importance in the case of aliphatic compounds. Compounds having the chemical composition of Schiff bases are more likely cyclic trimers:

$$
\begin{array}{c}
\text{R} \\
| \\
\text{N} \\
\diagup\ \ \ \diagdown \\
\text{H}_2\text{C}\ \ \ \ \ \ \text{CH}_2 \\
|\ \ \ \ \ \ \ \ \ \ | \\
\text{R-N}\ \ \ \ \ \text{N-R} \\
\diagdown\ \ \ \ \diagup \\
\text{C} \\
\text{H}_2
\end{array}
\tag{4a}
$$
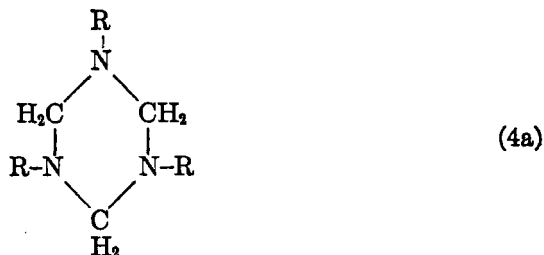
There is some evidence that dioxytrimethylene rings may be formed under special circumstances (Bergmann, Jacobsohn, and Schotte, 1923).

Physical-chemical methods of analysis make it clear that each amino group is capable of forming unstable hydroxymethyl derivatives with either one or two moles of formaldehyde:

$$R-NH_2+CH_2O \rightleftharpoons R-NH-CH_2 \cdot OH \tag{5}$$
$$R-NH_2+2\ CH_2O \rightleftharpoons R-N(CH_2 \cdot OH)_2 \tag{5a}$$
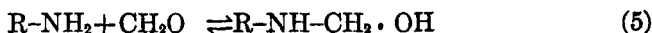
(2) *The Imino Group.* The imino group can react with one molecule of formaldehyde to form a hydroxymethyl compound

$$R_2=NH+CH_2O \rightleftharpoons R_2=N-CH_2 \cdot OH \tag{6}$$

and two imino groups may react to form a methylene compound

$$2\ R_4=NH+CH_2O \rightleftharpoons (R_4=N)_2CH_2+H_2O \tag{7}$$
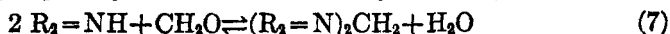
(3) *The Amide Group.* The classical researches of Einhorn (1905, 1908) have shown that acid amides can react with formaldehyde to form hydroxymethyl compounds

$$R-CO \cdot NH_2+CH_2O \rightleftharpoons R-CO \cdot NH-CH_2 \cdot OH \tag{8}$$
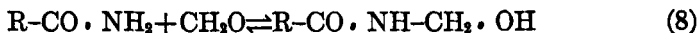
in which the hydroxyl group is unusually reactive and readily condenses with another amide molecule to give methylene diamides:

$$2\ R-CO \cdot NH_2+CH_2O \rightleftharpoons (R-CO \cdot NH)_2CH_2+H_2O \tag{9}$$

These compounds are usually prepared at elevated temperatures. The hydroxymethyl compounds are somewhat unstable at room temperature and markedly so at higher temperatures, while the methylene diamides are quite stable.

(4) *The Peptide Linkage.* Diketopiperazine reacts with one or two

molecules of formaldehyde to form hydroxymethyl groups. Presumably the peptide linkage is capable of reacting in the same way.

$$-CO-NH-+CH_2O \rightleftharpoons -CO-N(CH_2 \cdot OH)- \tag{10}$$

(5) *The Guanidino Group.* The Sakaguchi test for the monosubstituted guanidino group is drastically altered when arginine has been allowed to react with formaldehyde for 20 or 30 minutes. The exact nature of the underlying reaction is not yet clear (see the discussion of arginine in Part V).

(6) *The Hydroxyl Group.* Formaldehyde reacts with alcoholic hydroxyl groups to form acetals and hemiacetals:

$$R-OH+CH_2O \rightleftharpoons R-O-CH_2 \cdot OH \tag{11}$$
$$2\ R-OH+CH_2O \rightleftharpoons (R-O)_2CH_2 \tag{12}$$

Hemiacetals are rather unstable while acetals are stable in neutral or alkaline media, unstable in acid.

(7) *The Carboxyl Group.* Under condensing conditions formaldehyde reacts with organic acids to form esters of methylene glycol:

$$2\ R-COOH+CH_2O \rightleftharpoons (R-COO)_2CH_2+H_2O \tag{13}$$

In aqueous solutions, however, conditions scarcely favor this reaction and, in general, it appears to be unimportant for amino acids and proteins except when they are in the dry or nearly dry state.

(8) *The Sulfhydryl Group.* Thio analogs of acetals and hemiacetals are readily formed, and are considerably more stable than the oxygen derivatives:

$$R-SH+CH_2O \rightleftharpoons R-S-CH_2 \cdot OH \tag{14}$$
$$2\ R-SH+CH_2O \rightleftharpoons (R-S)_2CH_2+H_2O \tag{15}$$
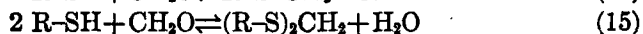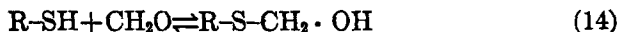
(9) *Aromatic Rings.* Under favorable conditions the active hydrogen atoms of an aromatic ring are capable of reacting with formaldehyde to form hydroxymethyl groups:

$$\equiv C-H+CH_2O \longrightarrow \equiv C-CH_2 \cdot OH \tag{16}$$
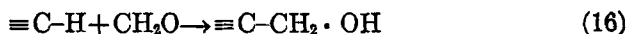
The C–C bond formed is very stable and so the reaction, though it often proceeds slowly, is practically irreversible. The hydroxyl group formed is comparable in reactivity to that of benzyl alcohol, and Reaction 16 is frequently followed by condensation with another reactive group, according to Equation 3, with the formation of a methylene bridge. Alternatively condensation may occur between the aromatic $\equiv$CH and a neighboring hydroxymethyl group attached to another functional group in the same or another molecule. Such reactions underlie the formation of phenolformaldehyde polymers. In the amino acids they lead to formation of additional rings in tryptophan, tyrosine, phenylalanine, and histidine, as discussed in Part V below.

b. *Reduction, Alkylation, and Other Reactions*

Aldehydes in general, formaldehyde in particular, are good reducing agents and under appropriate conditions alkylating agents. Clarke, Gillespie, and Weisshaus (1933) have shown that amino acids may be methylated by boiling with formaldehyde in the presence of very concentrated formic acid. The process eventually leads to considerable deamination with the simultaneous formation of methylamines. *N*-Dimethyl glycine may be prepared in this way, but many other amino acids are rapidly deaminated.

It is possible that formaldehyde may reduce disulfide linkages and then react with the newly formed sulfhydryl groups. Convincing evidence on this point is lacking (see the discussion of keratin in Part VI).

III. Methods Applicable to the Study of the Reactions of Formaldehyde with Amino Acids and Proteins[3]

We shall, at this point, give only a brief summary of the methods employed for studying the reactions of formaldehyde with amino acids and proteins, since the methods can in general be more conveniently presented in connection with the discussion of the various reactions that have been studied. The isolation, crystallization, and analysis of well defined compounds has been repeatedly attempted. Particularly in the case of the polyfunctional amino acids, which can react with formaldehyde to give ring structures containing a methylene bridge, important evidence has been obtained by the actual isolation of the resulting compounds (see Part V). However, the reactions of formaldehyde with the amino group are so rapid and so readily reversible that little evidence of the nature of the equilibria existing in solution has been obtained by isolation procedures. In the study of compounds which release formaldehyde on hydrolysis, the formaldehyde can be distilled off and the distillate analyzed for formaldehyde. The difficulties involved in this procedure, and the precautions which should be taken, are further discussed below. Also, the amount of unbound and reversibly bound formaldehyde in a system may be determined by adding dimedon (dimethyldihydroresorcinol) to the mixture, and filtering off and weighing the insoluble methylene addition product. This method has been employed by Wadsworth and Pangborn (1936) in their study of the combination of various amino acids with formaldehyde. It was also employed by Nicolet and Shinn (1941) and by Martin and Synge (1941) for the isolation of the formaldehyde produced by periodate oxida-

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[3] A general survey of methods for the detection and analysis of formaldehyde has been given by Walker (1944), Chapters 16 and 17.

tion of serine and hydroxylysine in protein hydrolyzates.[4] The compounds form slowly, and several days must generally be allowed for complete precipitation. Clearly, analytical methods of this sort cannot be applied to obtain information about the equilibrium state of systems in reversible equilibrium. For information about systems of the latter type, one must turn to the methods of physical chemistry.

Two methods of the latter sort have proved particularly powerful in the study of the equilibria between formaldehyde and the free amino group of amines, amino acids, peptides, and proteins:

(1) *Potentiometric Analysis.* In this method, the pH of a buffer system, made up of the acidic and basic forms of the substance under investigation, is studied as a function of the concentration of formaldehyde added. From observed changes in pH, and from the fundamental equations of acid-base equilibrium, important inferences may be drawn concerning the affinity of formaldehyde for the combining groups in the molecule being studied, and concerning the number of formaldehyde molecules which can react with such groups. The method is discussed in detail in Section IV below.

(2) *Polarimetric Analysis.* The optical rotation of optically active amino acids and their derivatives is markedly altered when formaldehyde combines with a group adjoining the center of asymmetry. If a second molecule of formaldehyde combines with the reacting group, a further change in optical rotation occurs which can, in general, be clearly distinguished from that due to the addition of one molecule of formaldehyde. Here again the detailed discussion of the application of the method is presented in Section IV.

In recent years, intensive study has been devoted to the mechanism of the action of formaldehyde as a tanning agent on collagen, casein, and other fibrous or potentially fibrous proteins. A detailed discussion of some of the principal methods employed in such studies, and the results obtained, is given in Section VI.

IV. The Reactions of Formaldehyde
with the Amino Group in Simple Amino Acids
1. AMMONIA AND AMINES

Ammonia, the simplest amine, is somewhat unusual in its reaction with formaldehyde, yielding the very compact and symmetrical hexamethylenetetramine:

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[4] As Neuberger (1944) has recently pointed out, formaldehyde formed by periodate oxidation from serine may react irreversibly with histidine or other aromatic amino acids present in the hydrolyzate, the analytical determination of serine being thereby falsified. An extensive discussion of periodate oxidation methods, in the analysis of hydroxyamino acids, is given by Martin and Synge in this volume.

$$6 \ CH_2O + 4 \ NH_3 \rightleftharpoons (CH_2)_6N_4 \tag{17}$$

Each of the four nitrogens is linked to three CH_2 groups, and each CH_2 group links two nitrogens. The four nitrogens are arranged at the corners of a regular tetrahedron; each face of the tetrahedron contains three of the nitrogens and three CH_2 groups arranged in a puckered hexagonal ring like that shown in Formula 4a. The structure is best visualized with the aid of a space model; it has been definitely established by X-ray diffraction studies (Dickinson and Raymond, 1923; Wyckoff and Corey, 1934). The reaction which gives rise to the formation of hexamethylenetetramine proceeds nearly to completion at room temperature in neutral or alkaline solution, and a large amount of heat is evolved in the process. Hexamethylenetetramine is a moderately weak monoacid base ($pK_a = 6$) and in the cationic form reacts with an equimolar amount of F:[8]

$$C_6H_{13}N_4^+ + CH_2O \rightleftharpoons C_7H_{15}ON_4^+ \tag{18}$$

with an appreciable increase in basic strength (French, unpublished). On heating in acid solution, hexamethylenetetramine can be decomposed into F and ammonium salts and the F can then be removed by distillation.

Methylamine reacts with an equimolar amount of formaldehyde to form a cyclic trimer, trimethyl trimethylenetriamine (see for instance Duden and Scharff, 1895; Einhorn and Prettner, 1904; other references are given by Sprung, 1940).

$$3CH_3NH_2 + 3CH_2O \rightleftharpoons \quad\begin{matrix} CH_3 \\ | \\ N \\ \diagup \ \diagdown \\ H_2C \quad\quad CH_2 \\ | \quad\quad\quad | \\ H_3C-N \quad\quad N-CH_3 \\ \diagdown \ \diagup \\ C \\ H_2 \end{matrix}\quad + 3H_2O \tag{19}$$
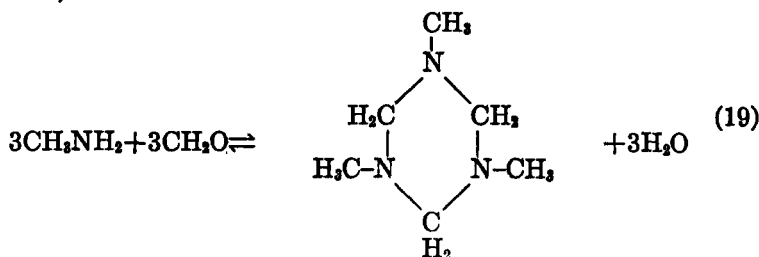
Likewise, ethylamine and F give triethyl trimethylenetriamine, as Dybing (1936) has shown by freezing point and pH measurements. He deduces an association constant, for the ethylamine reaction corresponding to Equation 19 for methylamine, of the order of 17×10^{13}.

The higher aliphatic amines appear to undergo analogous reactions. At very low concentrations of amine, however, and at high concentrations of formaldehyde, they may also react to form mono and di (hydroxymethyl) amines. The trimethylenetriamines of n-butyl and higher normal amines are relatively insoluble in water. However, they dissolve in concentrated F solutions, perhaps due to the formation of N-dihydroxymethyl amines.

[8] We use the symbol F, when convenient, as an abbreviation for formaldehyde.

The formation of the cyclic trimers from their components involves apparently a very low energy of activation; the reactions are rapid, and run practically to completion within comparatively few minutes.

2. GLYCINE

The reactions of glycine with formaldehyde have been studied more extensively than those of any other simple aliphatic amino acid. To a large extent, glycine may be considered typical of the monoamino monocarboxylic acids. However, it shows notable differences in certain respects from most of the other amino acids of this type, and in some respects its behavior appears to be more complex than that of its homologues with longer carbon chains. The isolation of numerous compounds has been reported as a result of the action of formaldehyde on glycine (RNH_2) or its esters or salts. In most cases, the reactions involve the *uncharged* amino group. The following is a partial list of the reported reactions, in which R denotes $HOOC \cdot CH_2 -$ or $^-OOC \cdot CH_2 -$:

$$RNH_2 + CH_2O \rightleftharpoons \quad RN = CH_2 \tag{20}$$
$$\text{Methylene glycine}$$
(Ba salt reported by Franzen and Fellmer, 1917, and Bergmann, *et al.*, 1923)

$$RNH_2 + 3CH_2O \rightleftharpoons \quad R-N \underset{CH_2-O}{\overset{CH_2-O}{\diagup \diagdown}} CH_2 \tag{21}$$
(Bergmann, *et al.*, 1923)
Triformal glycine ester and amide

$$RNH_2 + 2CH_2O \rightleftharpoons \quad R-NH-CH_2 \cdot OH \tag{22}$$
N-Hydroxymethyl glycine   (Krause, 1919)

$$RNH_2 + 4CH_2O \rightleftharpoons \quad R-N \overset{CH_2}{\underset{CH_2}{\diagup \diagdown}} CH\ OH \quad + \quad HCOOH + H_2O \tag{23}$$
(Krause, 1919)
N-Hydroxytrimethylene glycine

$$RNH_2 + CH_2O + 2H \rightleftharpoons \quad RNHCH_3 + H_2O \tag{24}$$
Sarcosine
(Löb, 1913)

$$RNH_2 + 2CH_2O + 2HCOOH \rightarrow \quad RN(CH_3)_2 \quad + \quad 2CO_2 + 2H_2O \tag{25}$$
N-Dimethyl glycine   (Clarke, *et al.*, 1933)

$$2RNH_2 + CH_2O \overset{HCl}{\underset{boil}{\longrightarrow}} \quad R-NH-CH_2-NH-R \tag{26}$$
Methylene diglycine   (Löb, 1913)

In addition, physical chemical methods have indicated the following reactions:

$$RNH_2 + 2CH_2O \rightleftharpoons \quad R-N(CH_2 \cdot OH)_2 \hspace{4cm} (27)$$

$$N\text{-Di(hydroxymethyl) glycine} \hspace{1.5cm} \text{(Levy, 1933; Levy}$$
$$\text{and Silberman, 1937)}$$

$$3RNH_2 + 3CH_2O \rightleftharpoons R-N \begin{matrix} \diagup CH_2-N \diagup^{R} \\ \diagdown CH_2 \\ \diagdown CH_2-N \diagdown_{R} \end{matrix} \hspace{3cm} (28)$$

$$\text{(French, 1945)}$$

Tricarboxymethyl
trimethylenetriamine
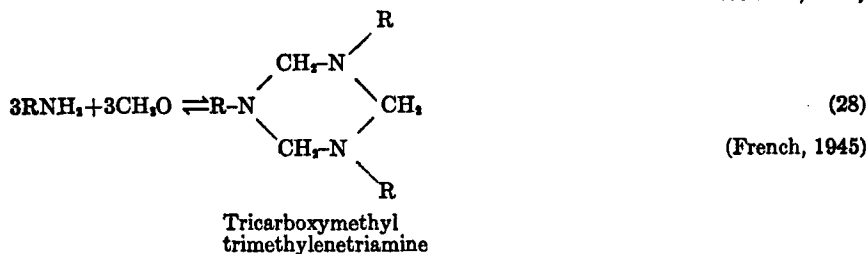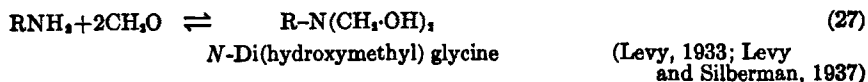
This is by no means an exhaustive list of the reactions which have been postulated to occur between glycine and formaldehyde, but it does give some indication of the varied possibilities that must be considered. In Reactions 21, 22, 24 and 25, there appears to be satisfactory evidence of the nature of the compounds formed. Reaction 23 is dubious. The assumed occurrence of Reaction 22 is in harmony with the potentiometric measurements of Levy (1933) and of Levy and Silberman (1937). The products of Reactions 24 and 25 have been compared with authentic specimens of sarcosine and N-dimethyl glycine. The isolation of the barium salt of methylene glycine (Reaction 20) by Bergmann, Jacobsohn and Schotte (1923) (see also Bergmann and Ensslin, 1925) appears to be substantiated by the analytical data.[6] However, it should be noted that this salt crystallized from solution with 4 or 5 molecules of water of crystallization, which could only· be removed by heat and intensive drying. Thus, while the methylene derivative could apparently be obtained by thorough dehydration, it appears very unlikely in the light of all the other evidence available that it exists as such in aqueous solution. In all probability, the substances present in the solution are the N-hydroxymethyl and N-di(hydroxymethyl) derivatives of glycine, as indicated by Reactions 22 and 27, and the trimer shown in 28. Sprung (1940) has pointed out that the monomeric Schiff bases of formaldehyde and aliphatic amines are practically unknown in aqueous solutions.[7]

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[6] It may be, however, that the anion of this salt is a trimer, as shown in Reaction 28, which would give identical analytical data. Crystallographic studies might serve to decide this point.

[7] Well defined Schiff bases, formed by the reaction of acetaldehyde and higher aliphatic aldehydes with methyl amine, have been prepared and their Raman spectra studied by Kahovec (1939). He also studied the Raman spectra of a series of N-alkyl trimethylene triamines (Formula 19).

The formation of "methylene diglycine" postulated by Löb (Reaction 26) appears extremely improbable, especially in the highly acid media employed by Löb in his preparation. The compound obtained is probably sarcosine, since Löb himself and later Clarke showed that sarcosine can be prepared under almost the same conditions as the hypothetical methylene diglycine. Löb's analytical data for "methylene diglycine" are in better agreement with sarcosine.

3. REVERSIBLE EQUILIBRIA INVOLVING ONLY THE AMINO OR IMINO GROUP

a. Potentiometric Analysis

The utility of the formol titration (Sörensen, 1907)[8] depended on the fact that formaldehyde appeared to decrease greatly the basicity of the amino group, while leaving practically unaltered the degree of alkalinity required to cause an indicator such as phenolphthalein to change color. Birch and Harris (1930) titrated a number of amines, fatty acids, and amino acids potentiometrically, in water and in formaldehyde solution (generally 1–2 per cent). Those portions of the curves associated with ionization of carboxyl groups were virtually unchanged by addition of formaldehyde (F), but those associated with amino groups were shifted markedly to the acid side. In the case of glycine, determinations at several different concentrations of F showed a progressive decrease in the apparent pK_2 value as F increased.

The first systematic treatment of the effect of F on the apparent dissociation constants was given by Levy (1933, 1934, 1935), and extended and summarized by Levy and Silberman (1937). Levy's conclusions, for substances containing only a single amino or imino group, may be summarized as follows: (1) only the uncharged amino or imino group reacts with formaldehyde, (2) the formaldehyde addition products are such weak bases that their basicity may be neglected in considering the equilibria involved, (3) amino groups can combine, rapidly and reversibly, with either one or two molecules of formaldehyde; imino groups with only one. The reactions of the amino group are probably those shown in Equations 22 and 27.

Thus, in a solution containing isoelectric amino acid and amino acid anion, the following equilibria determine the state of the system ($A^{\pm}$ denotes the dipolar ion of the amino acid, A^- the anion). The association constants of the reactions with F are denoted by L_1 and L_2.

$$L_1 = \frac{(AF^-)}{(A^-)F} \qquad (29) \qquad\qquad L_2 = \frac{(AF_2^-)}{(A^-)F^2} \qquad (30)$$

[8] As Sörensen pointed out, the fundamental observations on which the formol titration is based were made by Schiff (1900, 1901, and other papers).

For an imino acid, $L_2 = 0$. If the amino acid is titrated in water, pH is determined by the equation

$$pH = pK_2 + \log \frac{(A^-)_{H_2O}}{(A^\pm)_{H_2O}} \qquad (31)$$

For an extensive table of such pK values, see, for instance, Cohn and Edsall (1943, p. 84).

Let C_A denote the sum of the concentrations of all the different anions derived from the amino acid.

$$C_A = (A^-)_{H_2O} = (A^-) + (AF^-) + (AF_2^-) = (A^-)(1 + L_1F + L_2F^2) \qquad (32)^9$$

It is assumed that in formaldehyde solution the activity coefficients of the component species, and the liquid junction potentials, are the same as in water, and that $(A^\pm)$ is equal to $(A^\pm)_{H_2O}$. The decrease in pH, as formaldehyde is added, is thus determined only by the decrease of (A^-), due to its partial conversion into (AF^-) and (AF_2^-).

Then, in the solution containing formaldehyde at concentration F:

$$pH = pK_2 + \log \frac{C_A}{(A^\pm)} - \log (1 + L_1F + L_2F^2) = pG_f + \log \frac{C_A}{(A^\pm)} \qquad (33)$$

Thus, if the solution is so made up that $pH = pK_2$ in water, then in formaldehyde solution $pH = pG_f$.

$$pK_2 - pG_f = \log (1 + L_1F + L_2F^2) \qquad (34)$$

At high F values, the first two terms in the parenthesis become negligible, and as an approximation:

$$pK_2 - pG_f = \log L_2 + 2 \log F \qquad (35)$$

On plotting $(pK_2 - pG_f)$ against $\log F$, a curve is obtained which at high F values approaches a straight line of slope -2. The intercept of this line at $\log F = 0$ is $\log L_2$. Similarly for imino compounds, the limiting slope at high F is -1 and the intercept $\log L_1$.

Fig. 1 shows clearly the distinction between primary, secondary, and tertiary $(L_1 = 0)$ amino acids when plotted according to Levy's method. This type of plot is very useful for exploratory work, as it permits the investigator to cover quickly a wide range of formaldehyde concentrations and to infer by inspection (a) whether acidic, basic, or both forms are reacting; (b) the number of moles of F reacting, from the limiting slope at

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[9] It is assumed in this treatment that C_A is a constant, not affected by (F). This is strictly true only if the concentrations of H^+ and OH^- ions, and of the anions (CHO^-) derived from the acidic dissociation of F, are regarded as negligible in the equation for electroneutrality of the system. In the more alkaline solutions considered, (OH^-) and especially (CHO^-) if (F) is large, are not negligible. The corrections so introduced have important implications for the formol titration (p. 301), but we shall omit them in this discussion for the sake of simplicity.
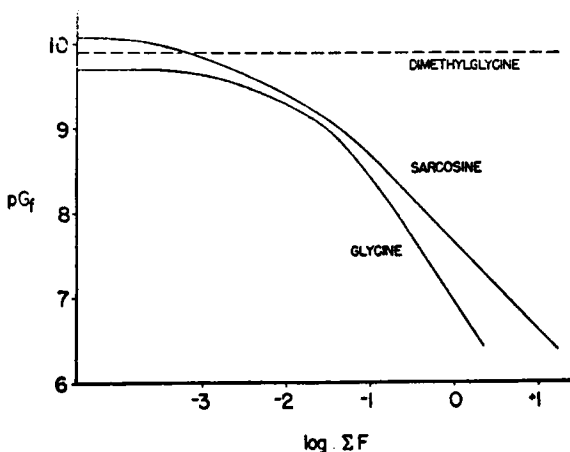
Fig. 1. Plots based on Levy's equations 34 and 35, showing the distinctive differences in behavior of primary, secondary, and tertiary amino acids in formaldehyde solution.
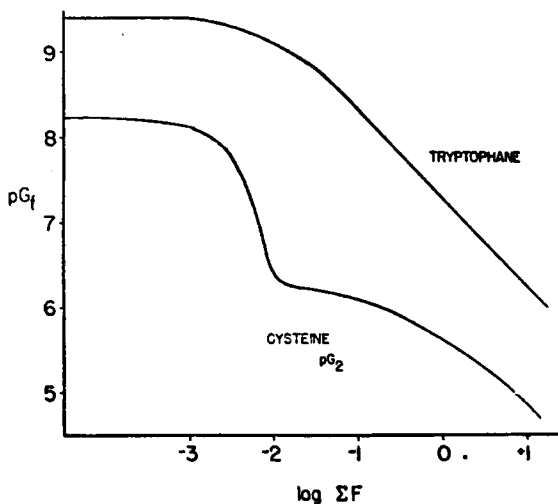
Fig. 2. The Levy plot for cysteine and tryptophan in formaldehyde solutions. Note that tryptophan behaves as a secondary amino acid (see equations 46 and 47).

high F concentrations; (c) the approximate values of the association constants. If an unsuspected reaction is taking place it is often revealed by the Levy plot. For example, cysteine (Fig. 2) shows a rapid drop in pG_f on addition of an equivalent amount of formaldehyde, followed by a plateau and a gradual falling off to a slope of -1. The interpretation of these phenomena is discussed in Part V below.
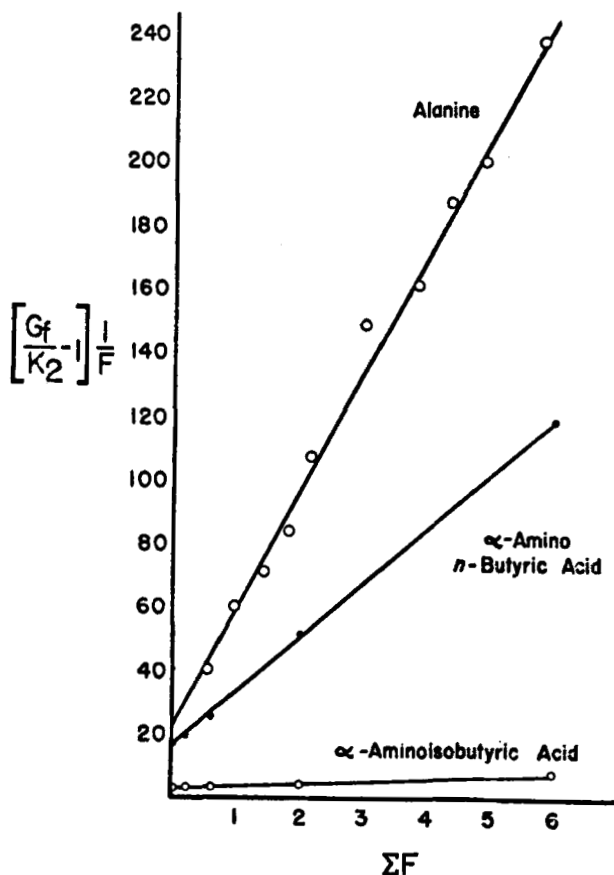
Fig. 3. The evaluation of the association constants between amino acids and formaldehyde by equation 37. Data for alanine from Frieden (1942); data for the aminobutyric acids from French (1945a).

The second step in Levy's treatment is the graphical determination of the association constants L_1 and L_2. From Equation 34,

$$G_f/K_2 = 1 + L_1F + L_2F^2 \qquad (36)$$

and therefore

$$\frac{G_f/K_2 - 1}{F} = L_1 + L_2F \qquad (37)$$

By plotting the function $(G_f/K_2-1)/F$ against F one should obtain a straight line of slope L_2 and intercept L_1. Values of L_1 and L_2 so obtained, for a number of amino and imino acids, are given in Table I on p. 293. Fig. 3 shows the plot of Equation 37 for alanine, a-aminobutyric acid, and a-aminoisobutyric acid.

TABLE I

Association Constants of Amino and Imino Acids with Formaldehyde

Substance	Association Constants			Method	Temp. C.	Reference
	L₁	L₂	L₂'=L₂/L₁			
Ethylamine[1]	1700	2000	1.2	GP	—	F(1945a)
Glycine[2]	60	290	4.8	HP	30°	LS(1937)
Glycine[2]	160	500	3.1	HP	25°	BL(1936)
α-Alanine	23	36.8	1.6	GP	—	FR(1942)
α-Alanine	22	75	3.4	HP	30°	LS(1937)
α-Alanine	24	66	2.7	HP	25°	BL(1936)
α-Aminobutyric acid	21	28	1.2	HP	25°	BL(1936)
α-Aminobutyric acid	15	17	1.1	GP	—	F(1945a)
α-Aminoisobutyric acid	3	0.5	0.16	GP	—	F(1945a)
α-Aminovaleric acid	18	18	1.0	GP	—	F(1945a)
α-Aminocaproic acid	17	18	1.0	GP	—	F(1945a)
α-Amino-α-methyl butyric acid	0.85	0.15	0.2	GP	—	F(1945a)
Valine	22	12	0.55	HP	30°	LS(1937)
Valine	23	10	0.43	HP	25°	BL(1936)
Leucine	16	35	2.2	HP	30°	LS(1937)
Leucine	17	32	1.9	HP	25°	BL(1936)
Leucine	19.9	31	1.56	POL	—	FDC(1943a)
Leucine	50	86	1.7	GP	3.1°	FR(1942)
Leucine	20	35	1.65	GP	24.5°	FR(1942)
Leucine	11.3	10.2	0.90	GP	44.9°	FR(1942)
β Alanine	230	780	3.4	GP	—	F(1945a)
ε-Aminocaproic acid	600	1500	2.5	GP	—	F(1945a)
Lysine (pK₂)[3]	89	251	2.8	HP	30°	LS(1937)
Lysine (pK₃)[3]	240	309	1.3	HP	30°	LS(1937)
Glycylglycine	230	880	3.8	GP	—	F(1945a)
Leucylglycine	22	35	1.6	HP	25°	BL(1936)
Leucylglycylglycine	25	38	1.5	HP	—	BL(1936)
α-Aminophenylacetic acid	13	77	6	HP	—	LS(1937)
Phenylalanine[4]	16	23	1.5	HP	—	LS(1937)
Phenylalanine[4]	22	25	1.1	HP	—	BL(1936)
Phenylalanine[4]	10.5	15.8	1.5	GP	—	FR(1942)
Tyrosine[4]	10	5	0.5	HP	—	LS(1937)
Proline	112	0	0	HP	30°	LS(1937)
Proline	126	0	0	HP	25°	BL(1936)
Proline	105	0	0	POL	—	FDC(1942)
Proline	67	0	0	HP	—	Tomiyama
Hydroxyproline	79	0	0	HP	30°	LS(1937)
Tryptophan[5]	83	0	0	HP	30°	LS(1937)
Sarcosine	320	0	0	HP	25°	BL(1936)
Sarcosine (N-Methyl glycine)	151	0	0	HP	30°	LS(1937)

[1] These "constants" depend on the amine concentration, since the complex forms a cyclic trimer (see Equation 19 and Dybing's (1936) paper).
[2] The apparent association constants determined vary with the glycine concentration, presumably due to polymerization of the glycine formaldehyde complex. See text.
[3] For a further discussion of lysine, see Part V of the text.
[4] For a further discussion of phenylalanine and tyrosine, see Part V of the text.
[5] Tryptophan behaves like an imino acid because of its rapid conversion to a carboline derivative through ring closure by the action of one molecule of formaldehyde. The observed association constant is a hybrid constant as defined in Equation 47 of the text.

TABLE I (Continued)

Substance	Association Constants			Method	Temp.	Reference
	L_1	L_2	$L_2'=L_2/L_1$			
N-Methyl leucine	4.77	0	0	GP	—	FDC(1943a)
N-Methyl leucine	2.27	0	0	POL	—	FDC(1943a)
N-Dimethyl glycine	0	0	0	HP	—	BL(1936)
Aspartic acid	26	37	1.42	HP	30°	LS(1937)
Aspartic acid	—	—	2.03	POL	—	CL(1943)
Glutamic acid	22	24	1.09	HP	30°	LS(1937)
Glutamic acid	38	43.7	1.15	POL	—	FDC(1943a)
Glutamic acid	—	—	0.923	POL	—	CL(1943)
Asparagine[6]	13	54	4.15	HP	30°	LS(1937)
6-oxytetrahydro-pyrimidyl- 4-carboxylic acid[7]	7.04	0	0	POL	—	CL(1942)

[6] This estimate was derived from the measurements of Levy and Silberman (1937, page 728ff), from the extrapolated initial points from the reaction of formaldehyde with asparagine.

[7] This compound has commonly been designated as methylene-asparagine. Concerning the evidence for its structure see page 307.

Methods: The symbols employed are as follows:
      HP denotes potentiometric measurements on the hydrogen electrode
      GP denotes potentiometric measurements on the glass electrode
      POL denotes polarimetric measurements

References: LS = Levy and Silberman
      BL = Balson and Lawson
    FDC = Frieden, Dunn and Coryell
      FR = Frieden
      CL = Carpenter and Lovelace
       F = French (the approximate temperature of French's measurements is 25° C.).

Balson and Lawson (1936), who carried out experiments similar to Levy's, concluded that amino acid anions could combine with a third molecule of formaldehyde. Evidence for the third association constant, however, depended on measurements at very high formaldehyde concentrations, where uncertainties due to activity coefficients and liquid junction potentials are considerable. Levy and Silberman (1937) and French (1945a) could find no evidence for a third association constant in their data. The phenomena observed by Balson and Lawson could equally well be explained on the assumption that their formaldehyde contained a trace of formic acid, although they took precautions to minimize this.[10] The assumption that a third mol of F can combine with the amino group is not inherently unreasonable — compare, for example, the triformal derivatives of glycine ester and glycine amide: Reaction 21 — but there appears to be insufficient evidence to establish its occurrence in solution.

The titrations of Dunn and Loshakoff (1936b) and Dunn and Weiner

[10] It is extremely difficult to prepare and maintain F acid free. At high F or low amino acid concentrations, it is imperative to correct for the amount of acid in the formaldehyde.

(1937), carried out with the glass electrode, show satisfactory agreement with Levy's measurements, in which the hydrogen electrode was used.[11]

b. Polarimetric Analysis

The following discussion of the polarimetric method is taken directly from the work of Frieden, Dunn, and Coryell (1942):

"The polarimetric method used in studying the formaldehyde-amino acid equilibria . . . is based on the principle that the rotations of the complexes resulting from the combination of formaldehyde with optically active amino acid anions should differ from each other and from the rotation of the amino acid. By inspection of the curve relating rotation and formaldehyde concentration it should be possible to determine the number of complexes. From a detailed study of the changes in rotation occurring in amino acid solutions varying in formaldehyde concentration, it should be possible to determine the relative concentrations of the complexes and the equilibrium constants of the reactions."

The molecular rotations of an amino acid anion, (A^-), and of its association compounds with formaldehyde, AF^- and AF_2^-, are respectively denoted by a_1, a_2, and a_3. The observed molecular rotation, $[M]$, is then the sum of the rotations of the components present, or

$$[M] = \frac{a_1(A^-) + a_2(AF^-) + a_3(AF_2^-)}{(A^-) + (AF^-) + (AF_2^-)} = \frac{a_1 + a_2 L_1 F + a_3 L_2 F^2}{1 + L_1 F + L_2 F^2} \qquad (38)$$

(See Frieden, Dunn and Coryell, 1943a).

For imino acids, $L_2 = 0$ and the solution of 38 is relatively simple. In Fig. 4, the molecular rotation of a solution of anionic proline is plotted as a function of the concentration of added formaldehyde. $[M]$ in water is $-112°$. As formaldehyde increases, $[M]$ progressively decreases and approaches asymptotically a limiting value of approximately $-168°$, which is the molecular rotation of the proline formaldehyde complex, AF^-. The form of the curve is that of a segment of a rectangular hyperbola, as

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[11] Svehla (1923) attempted to determine association constants by freezing point measurements, and Baur (1941) employed both freezing point and conductivity measurements for the same purpose, using solutions of F and isoelectric amino acids. However, their calculated values can not be relied on, because the nature of the underlying reactions was inadequately formulated in these papers. Qualitatively, the results appear to agree well with those discussed in the text here.

Baur (1941) made rough estimates of reaction velocity for the combination of F with glycine and alanine, by following the change of conductivity with time. Rather surprisingly, alanine appeared to react faster than glycine, combination being essentially complete in about 30 seconds at 25°, whereas, in the case of glycine the reaction was not fully complete for about two minutes. This may mean that glycine reacts in two stages (see Reactions 22 and 28) while alanine undergoes only Reaction 22 and does not react further.
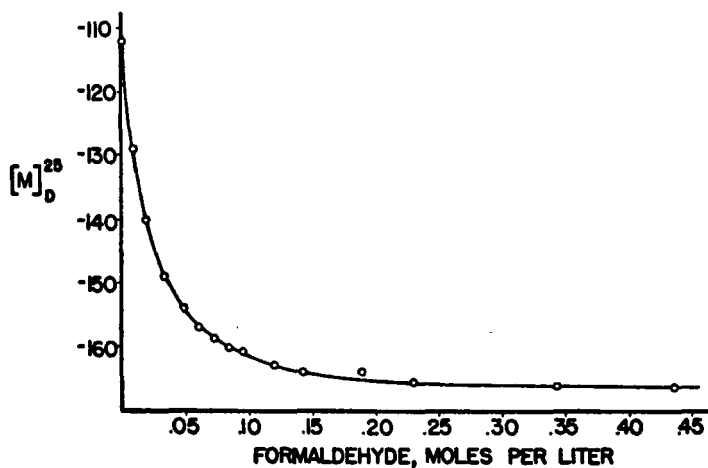
Fig. 4. Optical rotation of proline anion in solution as a function of formaldehyde concentration. Data from Frieden, Dunn, and Coryell (1942).
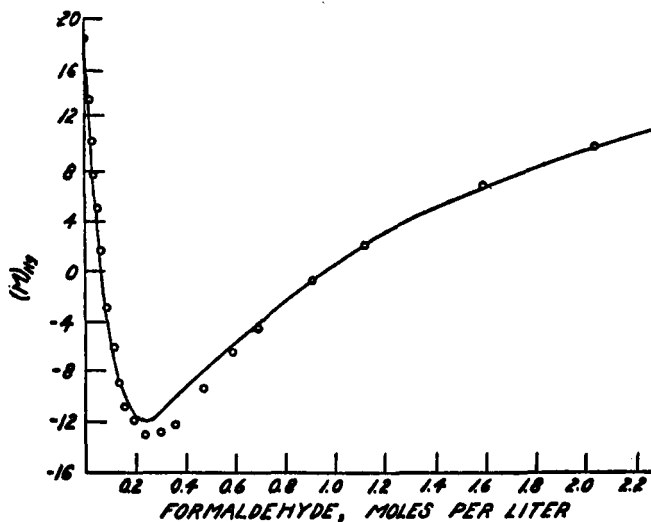
Fig. 5. The molecular rotation of dianionic $1(+) -$ glutamic acid as a function of total formaldehyde concentration. The line is calculated from the association constants given in Table I, assuming $a_2 = -23.4°$ and $a_3 = +25.0°$. From Frieden, Dunn, and Coryell (1943a).

would be expected from the law of mass action for a single molecule of formaldehyde combining with one of proline anion.

In Fig. 5, a curve for an amino acid, anionic $l(+)$-glutamic acid, is shown. Here it is immediately apparent that the curve falls into two sec-

tions. The first descending portion, at low formaldehyde values, is determined by the formation of the complex AF . As the concentration of formaldehyde increases further, [M] rises again as AF_2^- is formed, the latter having clearly a larger positive molecular rotation than the simple anion A^-. There is, of course, some overlapping between the two successive formaldehyde addition compounds; a considerable amount of AF_2^- has begun to form long before A^- is completely converted into AF^-. In order to derive the association constants from data like those shown in Fig. 5, Equation 38 must be solved. This involves four unknowns: a_2, a_3, L_1, L_2. Moreover, in solutions containing little formaldehyde, F must be regarded as a fifth unknown, since this symbol represents the *free*, not the total formaldehyde in the solution. The equation can be solved only by successive approximations. At high F concentrations, it may be assumed that A^- is absent from the solution.

$$[M]_{\text{high F}} = \frac{a_2 + a_3 L_2' F}{1 + L_2' F} \qquad (39)$$

Here $L_2' = L_2/L_1$ is the equilibrium constant of the reaction

$$AF^- + F \rightleftharpoons AF_2^- \qquad (40)$$

Under these conditions, it is fairly simple to evaluate a_2, a_3, and L_2. Having thus obtained a_2, and since a_1 is known from measurements at $F = 0$, a similar calculation can then be carried out at low F concentrations, assuming that under these conditions AF_2^- is present in negligible amounts. Equation 38 then becomes:

$$[M]_{\text{low F}} = \frac{a_1 + a_2 L_1 F}{1 + L_1 F} \qquad (41)$$

A full discussion of the derivation of the constants from the equations will not be given here; the method is presented in detail by Frieden, Dunn, and Coryell (1943a).

Equation 41 is obviously applicable also to imino acids at all F concentrations, since 38 automatically reduces to 41 if $L_2 = 0$. Here the association constant may be derived most conveniently by a plot of log $(AF^-)/(A^-)$ against log F, since from Equation 29

$$\log L_1 = \log \frac{(AF^-)}{(A^-)} - \log F \qquad (42)$$

When a_1 and a_2 have been evaluated, the observed value of [M] allows the calculation of log $(AF^-)/(A^-)$, and F may be calculated by difference as the total added formaldehyde minus (AF^-). Here again, however, successive approximations are required in evaluating these quantities. A tentative value of L_1 must be assumed in order to estimate a_1 and a_2, and

two or three approximations may be required before the best value of L_1 is obtained.

The values of the association constants obtained by the polarimetric method on all the amino acids, except the dibasic acids which are discussed in Part V below, are given in Table I, together with those derived from potentiometric measurements. The results of the measurements by the two different methods are generally in fairly good agreement. The proline values of Levy and of Frieden, Dunn, and Coryell, for instance, agree within about 6 per cent. On the other hand, the values for N-methyl leucine, by the two methods, differ by a factor of more than 2 to 1. Frieden, Dunn, and Coryell (1943a), who carried out both sets of measurements on this compound, are unable to find a reason for the discrepancy. Each set of measurements appears entirely consistent within itself. However, equally marked discrepancies appear between different potentiometric determinations: for example, L_1 for sarcosine, as reported by Balson and Lawson (1936) is more than twice as great as that reported by Levy and Silberman (1937). A factor of 2 in these values corresponds to a difference of approximately 0.3 in the pH measurements at high formaldehyde concentration. Actually, the measurements of Balson and Lawson were made at a temperature 5° lower than those of Levy and Silberman, but there is no indication, in the other compounds studied by these authors, of any systematic differences in the L values due to temperature.[12] Some of the discrepancies in the reported values may be due to an inadequacy of the underlying assumptions concerning the reaction mechanism. This is particularly true in the case of glycine, which appears to form a cyclic trimer with F, similar to those formed by the aliphatic amines (pp. 282 and 286).

4. INFLUENCE OF STRUCTURE ON OBSERVED ASSOCIATION CONSTANTS

Allowing for the uncertainties just indicated, some definite deductions concerning the effect of structure on L_1 and L_2 may be drawn from the data in Table I. The data for ethylamine and for glycine must be treated with reserve, since the first formaldehyde addition products of both tend to condense, forming cyclic trimers. In any case, it is clear that the association reactions are markedly inhibited by substituting a $-COO^-$ for the CH_3 group in ethylamine. On further replacing one hydrogen on the α-carbon

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[12] However, the data of Frieden (1942) for L_1 and L_2 of leucine at three different temperatures, given in Table I, show clearly a *decrease* of association with rising temperature. From these data, Frieden calculates ΔH values as follows:

$$A^- + F \rightleftharpoons AF^- \; ; \; \Delta H_1 = -6200 \text{ cal/mol}$$
$$AF^- + F \rightleftharpoons AF_2^- \; ; \; \Delta H_2 = -9000 \text{ cal/mol}$$
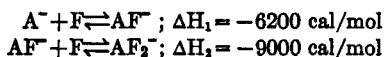
Also, with respect to the glycine$-$F complex, Hobohm (1944) states that rise of temperature causes the complex to dissociate; but he gives no quantitative data.

by a methyl group, to give alanine, association is still further inhibited, L_1 falling from 60–80 to 22, and L_2 from 300–550 to about 70. On inserting a second methyl group on the a-carbon (a-aminoisobutyric acid), L_1 falls to 3, and L_2 to 6.4. An equally striking decline in L_1 and L_2, on a-methylation, is shown by the comparison of a-amino-n-valeric acid and a-amino-a-methyl butyric acid; for the latter compound $L_1 = 0.85$, $L_2 = 0.15$. The effect of changing from a-methyl to a-ethyl (alanine to a-amino butyric acid) is comparatively small, and the higher members of the a-amino-n-alkyl carboxylic acid series have nearly identical values, $L_1 \cong L_2 \cong 18$. The effect of branching at the far end of the alkyl chain, as in leucine or valine, is small.

All these observations indicate that steric factors, depending primarily on the size and perhaps on the charge of the groups attached to the a-carbon atom, have a profound influence in inhibiting the tendency of an amino group to combine with formaldehyde. If the steric inhibiting factors are weak or absent, as in the n-alkylamines and to a lesser extent in glycine, condensation of the amino group with F, and formation of a cyclic trimer, proceed rapidly.

Similar effects of steric hindrance are revealed in the imino acids: compare sarcosine ($L_1 = 150 - 320$) with N-methyl leucine ($L_1 = 2.3 - 4.8$).[13] For proline L_1 is not much smaller than for sarcosine, indicating that the two additional CH_2 groups of the pyrrolidine ring interfere very little with the approach of a formaldehyde molecule to the $=NH$ group.

The effect of N-methylation does not appear systematic from the available data. L_1 for sarcosine is greater than for glycine; but for N-methyl leucine, it is only one-fourth to one-eighth as large as for leucine.

Increasing the distance between the amino and carboxyl groups increases the tendency of the amino group to combine with F. For β-alanine, L_1 and L_2 are much greater than for glycine; for ϵ-aminocaproic acid, they are much greater still. It is not unlikely that these substances, like glycine, form cyclic trimethylenetriamine derivatives.

In peptides similar relations are found. $L_1 = 230$ and $L_2 = 880$ for glycylglycine are similar to the values for β-alanine, and much larger than for glycine. For leucylglycine and leucylglycylglycine, $L_1 = 22-25$, $L_2 = 35-38$, revealing again the steric effect of the bulky leucyl group in inhibiting combination with F. Evidence on this point is also given by the studies of Dunn and Loshakoff (1936b) whose data for some amino acids and peptides in 9 per cent HCHO are given in Table II.

It is clear that ($pK_2 - pG_f$) is about one unit greater here for amino groups without alkyl substitution on the adjoining a-carbon than for those

[13] For substances with association constants as low as those of a-aminoisobutyric acid or N-methyl leucine, it would be impossible to get accurate values in the formol titration, because of the very small downward shift of pG_f with increasing F.

TABLE II

Dissociation Constants of Amino Acids and Peptides in Water and Formaldehyde

Substance	pK$_2$ (Water)	(9% HCHO) pG$_f$	pK$_2$ −pG$_f$
Glycine	9.60	5.92	3.66
dl-Norleucine	9.76	7.10	2.66
Glycylglycine	8.13	4.27	3.86
Diglycylglycine	7.91	4.24	3.67
Glycyl-dl-leucine	8.29	4.40	3.89
dl-Alanylglycine	8.18	5.52	2.66
dl-Leucylglycine	(8.18)	5.55	2.63

pK$_2$ values from Cohn and Edsall (1943, p. 84); pG$_f$ values from Dunn and Loshakoff (1936b). For leucylglycine, pK$_2$ in water is not available; it was assumed to be the same as for alanylglycine.

with attached alkyl groups. Particularly striking is the difference between the isomers glycylleucine and leucylglycine, for which pK$_2$ probably differs by only about 0.1 pH unit in water, while the pG$_f$ values in 9 per cent F differ by 1.15. Here again it is the distance between the large alkyl group and the amino group that determines the tendency to associate with formaldehyde. This shows strikingly the difference between formaldehyde association constants, which are profoundly affected by the steric effects of non-polar groups, and acid dissociation constants, which are primarily affected by charged and polar groups, and only in minor degree by variation in the size of neighboring alkyl groups.

5. LIMITATIONS TO THE SIMPLE FORMULATION OF THE REACTIONS OF AMINO GROUPS WITH FORMALDEHYDE

Practically the entire discussion given in the preceding section is based on the two association equilibria expressed by Equations 29 and 30, which presumably correspond to the chemical Reactions 22 and 27. If Equations 29 and 30 represent completely the nature of the reactions involved, it is to be expected that (a) the titration curve in the presence of a constant amount of F should be an undistorted Henderson-Hasselbalch curve which is shifted to a region of lower pH, and (b) that the amount of shift should be independent of the amino acid concentration. With glycine neither condition is fulfilled. The titration curve is appreciably broader than a typical Henderson-Hasselbalch curve and the extent of shift depends considerably on the glycine concentration. Irregularities of this sort had been observed by Levy (1933) who concluded that "the formaldehyde complex is polymerized to a marked extent and the values of the constants are dependent to some extent on the actual glycine concentration" (p. 776). Polymerization alone, however, should tend to make the titration curve

steeper than a simple Henderson-Hasselbalch curve, whereas the curves actually observed are broader.

Recent experiments (French, 1945b) have indicated that the observed phenomena can be explained if it is assumed that the hydroxymethyl complex first formed condenses with itself to form a cyclic trimer, tricarboxymethyl trimethylenetriamine (Reaction 28). In this compound, one of the nitrogen atoms of the ring can accept a proton ($pK_a = 8.0$). The reaction is quite analogous to that of the aliphatic amines with F. Addition of an excess of F may lead to the formation of the di(hydroxymethyl) compound. As expected, this shows no tendency to polymerize.

The above reactions appear to account quantitatively for the titration behavior of glycine throughout a wide range of F concentration (0.0001–10 M) with glycine from 0.001 to 0.1 M. Freezing point measurements of sodium glycinate at varying F concentrations are also in good agreement with this theory.

In addition to the reactions discussed above, which are all quite rapid, slow reactions involving a gradual loss of amino nitrogen appear to take place in alkaline solutions of even the simple amino acids. Such a slow loss of amino nitrogen has been observed by Holden and Freeman (1931) and by Wadsworth and Pangborn (1936) when the amino acids are incubated for several weeks in mildly alkaline solution. Thus, at a pH near 8, Wadsworth and Pangborn found that in 28 days at 39° C. the free amino nitrogen of glycine had decreased by about 25 per cent below its initial value. It is possible that the reaction involved is due to contaminating microörganisms. (Toluene was added as a preservative in these experiments, but rigid sterility was not maintained.) The observed changes are, in any case, exceedingly slow and certainly have no influence on the results obtained in the formol titration, which is ordinarily carried out very promptly after the addition of F to the amino acid solution. The rapid loss of amino nitrogen, due to the reaction of F with tryptophan, histidine, tyrosine, cysteine, and other bi-functional amino acids, represents an entirely distinct type of reaction of which the simple amino acids are incapable. The mechanisms involved are discussed further in Part V.

6. CHOICE OF CONDITIONS FOR THE FORMOL TITRATION

The general principles of the formol titration should be immediately apparent from the foregoing discussion. The essential object involved is to add sufficient F so that the pG_f values of the amino acids become so low that they are completely titrated before the indicator used changes color. Levy (1934) has shown that phenolphthalein changes color at the same pH in F solution and in water, up to F concentrations of 3.5 M, and that the color is apparently unaltered by the change of solvent. If F itself did not

react with the added alkali, the optimum conditions for titration would lie at the highest obtainable F concentration. However, F is an acid of pK near 12.9 (Levy, 1934). Its presence in the solution at high concentration, therefore, has an effect which, from the point of view of the titration, is equivalent to a large increase in K_w. Thus there is an optimum region of F concentration for the formol titration, in which the pG_f values are sufficiently depressed to make the titration satisfactory, but in which the concentration of anions at the end point, due to the dissociation of F itself, is small enough to produce little error. A detailed analysis of the problem has been given by Levy (1934) who concludes that the maximum accuracy is obtained when the F concentration is 6–9 per cent at the end volume. He recommends that neutral formalin, adjusted to pH 7, should be used, that no correction for a blank should be made, and that the amino acids be titrated at as high a concentration as possible. The error of the titration is smallest when the solution at the end point contains equivalent quantities of the amino acid and the added alkali. If $F = 2.3 M$, this occurs when pH is equal to $9.6 + \frac{1}{2}$ the log of the molar amino acid concentration. For a $0.1 M$ amino acid at the end volume, the proper end point is somewhere near pH 9.1.[14] In the presence of large amounts of proline, as in gelatin hydrolyzates, the end point of the formaldehyde titration should be somewhat more alkaline than otherwise (Levy, 1934, p. 164).

The stoichiometric relations involved in the formol titration have been discussed by Van Slyke and Kirk (1933). If the titration is begun at the isoelectric point of the amino acid and carried to an end point near pH 9 in formaldehyde, the titration gives a measure of the free carboxyl groups. If the titration is started at pH 7 in water and carried to the same end point, it gives a measure of the free amino groups present. The latter method is generally the condition of choice; obviously the first method can only be applied to a single amino acid, or to a mixture of amino acids which are all of the same charge type.

Highly accurate formol titrations have been carried out by Dunn and Loshakoff (1936a) who determined pH, during the progress of the titration, on the glass electrode. Choosing the conditions of the titration to be nearly optimum according to the directions given by Levy and summarized above, they determined the electromotive force (E) as a function of the volume (V) of added alkali. The function $\Delta E / \Delta V$ passes through a sharp maximum near the stoichiometric end point, which can thus be located with very high precision. In this method, the absolute pH values are not of importance, but only their variation with V. This method is admirably suited to

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[14] The conditions for the titration of the dibasic amino acids require special consideration (Levy, 1935). In particular, significant errors are to be expected for histidine, if it is titrated under the same conditions as the other amino acids.

titrations of individual amino acids for determination of analytical purity; and Dunn and Loshakoff report that the error of the method for this purpose can be reduced to 0.1 per cent or less. With some modifications, methods based upon the same principle should be applicable to more complex mixtures of amino acids, but the accuracy of the titration in a mixture must necessarily be somewhat lower.

Borsook and Dubnoff (1939) described an ultramicro modification of the glass electrode formol titration of Dunn and Loshakoff. It should be noted that the conditions chosen by them for the titration deviate markedly in several respects from those recommended by Levy; presumably these conditions were chosen deliberately for special purposes, and could be suitably modified by other workers without alteration of the ultramicro technique. Sisco, Cunningham, and Kirk (1941) have described a micro titration by quantitative drop analysis, which permits the determination of 4 to 10 μg. of amino N with an accuracy comparable to that of the macro method as ordinarily carried out. Here again (as the authors have indicated) accuracy could be improved by using a higher F concentration, with F adjusted to pH 7, and a final pH of approximately 9, following Levy's recommendation.

Martin and Synge discuss the formol titration as an analytical tool elsewhere in this volume.

V. POLYFUNCTIONAL AMINO ACIDS AND PEPTIDES

When functional groups other than the amino group are present in an amino acid, the possible complexities of the formaldehyde reaction are increased. Reactions of the types already outlined may occur at two or more points in the molecule, and any one of the initial products may react with another free functional group, with elimination of water and methylene bridge formation. However, the relative rates of the various possible reactions may be so different that a satisfactory description of the reaction mechanism, and of the products obtained, may be achieved. In the cases of cysteine and asparagine, the products obtained are well characterized, and there is considerable knowledge of the steps in the reaction. In other cases — tryptophan, tyrosine, phenylalanine, histidine — crystalline reaction products have been obtained and identified, although the mechanism of the reaction is still almost unknown. The guanidino group of arginine, and the peptide linkage, are known to react with formaldehyde, but neither the exact mechanism of the reaction, nor the exact nature of the derivatives formed, is yet known for arginine or the peptides.

1. CYSTEINE

The reaction of F with cysteine has been examined by several workers with substantially identical results. The most complete report is given by
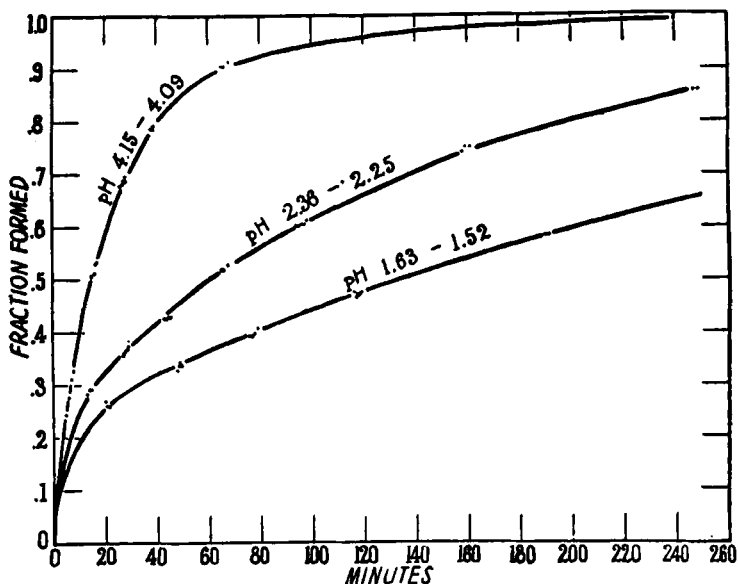
Fig. 6. Rate of thiazolidine carboxylic acid formation in buffered solutions at 25°. Initial concentrations of cysteine and formaldehyde both 0.068 M at all pH values. From Ratner and Clarke (1937).

Ratner and Clarke (1937); somewhat earlier Schubert (1935, 1936) published similar accounts. Ratner and Clarke show that the reaction of one mole of formaldehyde with cysteine gives a crystalline amphoteric derivative, $C_4H_7O_2NS$, which is remarkably stable toward acid and alkali. The nitrogen exists as a secondary amino group; the sulfur is present as a thioether, for the nitroprusside reaction is negative. Oxidation of the acetyl derivative by hydrogen peroxide leads to a sulfoxide or a sulfone. All the properties of the compound are readily explained by the assumption that it is thiazolidine-4-carboxylic acid (Reaction 43).

$$
\begin{array}{c}
CH_2 \cdot SH \\
| \\
CH \cdot NH_3^+ \\
| \\
COO^-
\end{array}
\quad + H_2CO \quad \rightleftarrows \quad
\begin{array}{c}
H_2C-S \\
| \qquad \diagdown \\
| \qquad\quad CH_2 \\
| \qquad \diagup \\
HC-NH_2^+ \\
| \\
COO^-
\end{array}
\quad + H_2O \qquad (43)
$$
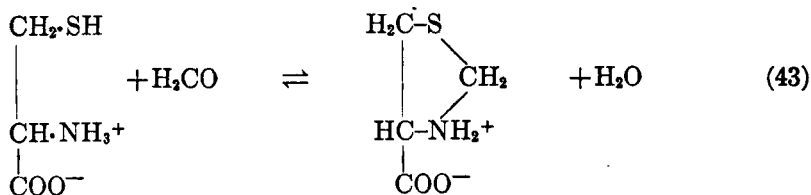
The reaction is readily followed by observing the very large change in optical rotation which it involves. The course of the reaction, at several different pH values, is shown in Fig. 6. The rate increases rapidly with increasing pH, and above pH 5 becomes too fast to follow by the technique employed. S-Ethylcysteine, at pH 5.1, does not react rapidly with formal-

dehyde, as shown by change of rotation; while N-acetylcysteine does react very quickly. All these facts suggest that the initial step in the reaction with cysteine involves combination of formaldehyde at the sulfur atom, probably to form a derivative of the type $RSCH_2OH$. This then condenses with the uncharged amino group, with formation of a methylene bridge. This second reaction is the rate determining step and is therefore very sensitive to pH, since the concentration of uncharged $-NH_2$ groups of cysteine is small, and increases rapidly with pH in the range studied.[15]

Reaction 43 is reversible, but the equilibrium lies very far to the right, On boiling a solution of thiazolidine carboxylic acid in N hydrochloric acid, formaldehyde can be distilled from it, but the rate of liberation is very slow, about 15 per cent of the possible yield being obtained by nine hours' distillation. Oxidation with H_2O_2 or iodine produces cystine and formaldehyde almost quantitatively; with bromine, cysteic acid is formed.

Thiazolidine carboxylic acid is an ampholyte, pK_1 (COOH) $= 1.51$, pK_2 ($=NH_2^+$) $= 6.21$, isoelectric pH $= 3.86$. Thus, although a hydroxy-methyl amino group ($R-NH-CH_2OH$) is too weak a base to be measured, the basicity of the nitrogen is readily measurable (although weak) after further condensation, with methylene bridge formation.[16] Proline differs from thiazolidine carboxylic acid only by the substitution of a $-CH_2-$ group for the sulfur atom; but pK_2 of proline is 10.6. Thus the presence of the sulfur decreases the pK value of the $=NH_2^+$ group by 4.4 pH units — a remarkably large shift. The sulfur also decreases the affinity of the neighboring imino group for formaldehyde. For proline (Table I), L_1 is approximately 110; for thiazolidine carboxylic acid (French, unpublished) it is near 2.2.

Djenkolic Acid, $CH_2[S \cdot CH_2 \cdot CH(NH_3^+) COO^-]_2$. This amino acid was isolated from the djenkol bean by Van Veen and Hyman (1935), who correctly deduced its structure. It was synthesized by du Vigneaud and Patterson (1936) by the action of methylene chloride on l-cysteine in liquid ammonia. The synthetic product gave $[a]_D^{26} = -44.5°$ (2 per cent solution in 1 per cent HCl) and the isoelectric amino acid has a solubility of 1.02

---

[15] In more alkaline solutions (pH >8.5) the amino groups are largely in the uncharged form, and the $-SH$ groups have begun to ionize. It is probable that the $-SH$, like the $-NH_2$ group, reacts with F only when uncharged. In that case, the order of the steps in the reaction in highly alkaline solution may be the reverse of that in acid solution; but the great speed of the reaction under these conditions has hitherto prevented further study.

[16] The effect of formaldehyde on the titration behavior of cysteine was presented in a graph by Birch and Harris (1930) who wrongly interpreted the decrease in pK_2 and increase in pK_3 as indicating ꞏhat the groups responsible for these constants are the amino and sulfhydryl groups respectively. (Concerning the assignment of these pK values, see Ryklan and Schmidt (1944)).

g./l. in water at 30° C. It decomposes in the range 300–350° C. Djenkolic acid has not as yet been identified as a hydrolysis product of any protein and would indeed be decomposed by hydrolysis with strong acids (compare Lillevik and Sandstrom, 1941). It may arise in nature through the interaction of cysteine (or cysteine peptides) and formaldehyde. Likewise, djenkolic acid residues might arise in formaldehyde treatment of a protein such as keratin, through rupture of disulfide linkages and subsequent methylene bridge formation between the sulfur atoms. This would maintain the cross linkage between adjacent peptide chains, with an $-S-CH_2-S-$ bond replacing the original S–S. No positive proof of the formation of such linkages, however, has yet been given (Middlebrook and Phillips, 1942).

2. SERINE AND THREONINE

By analogy with cysteine, these hydroxyamino acids might be expected to react with F, with methylene bridge formation between the hydroxyl and amino groups, giving oxazolidine-4-carboxylic acid and its methyl derivative, respectively. Oxazolidines have been prepared (Knorr and Matthes, 1901) by interaction of formaldehyde and acetaldehyde with 2-aminoethanol or its N-methyl and N-isobutyl derivatives. The reactants were heated in ethereal solution over potash; the products were fairly volatile, alkaline oils, which could be purified by distillation at atmospheric pressure. These oxazolidines are very unstable, being largely and rapidly decomposed into aldehyde and ethanolamine by acids, alkalies, or dilution with water. These facts suggest that serine and threonine, in aqueous formaldehyde solution, probably undergo reactions analogous to 43, but that the products are very unstable and the equilibrium lies much further to the left than in the case of cysteine.[17] The pH measurements of Dunn and Weiner (1937) on serine with the glass electrode show a very large decrease in pG_f on the addition of small amounts of F, and a relatively small further decrease at higher F concentrations — decidedly smaller than that of most α-amino acids. Qualitatively these findings appear to be in harmony with the picture suggested above, but further study of the hydroxyamino acids is clearly needed.

3. ASPARAGINE

Schiff (1900) isolated a well defined crystalline substance, $C_5H_7O_3N_2$, from the interaction of equimolar amounts of asparagine and F. This compound combined with a second molecule of F, giving also a crystalline product. The second molecule of F, however, was very loosely bound, and on standing in a desiccator over sulfuric acid the second compound wa

[17] Bergmann and Zervas (1926) have prepared salts of O-hydroxybenzylidene serine from serine and salicylaldehyde. These structures probably contain an oxazolidine ring

reconverted to the first. Schiff assumed the first compound to be methylene asparagine, in which the amino group has been converted to an $-N = CH_2$ group (compare Equation 20). He noted, however (Schiff, 1901) that several other amino acids failed to give well defined crystalline derivatives analogous to "methylene asparagine." In particular it was impossible to isolate a similar derivative of aspartic acid, and Schiff concluded that the neighboring amide group of asparagine exercised a determining influence on the reactivity of the amino group.

Cherbuliez and Stavritch (1922) showed that "methylene asparagine" was converted by oxidation with hypobromite to 6-hydroxy-5-bromo-pyrimidine-4-carboxylic acid, from which they prepared a series of other pyrimidine derivatives. In the light of these findings, and of their own electromotive force measurements, Levy and Silberman (1937) advocated a cyclic formula for the asparagine-F condensation compound $C_5H_7O_3N_2$. They determined pH at varying F concentration in an asparagine solution initially adjusted to $pH = pK_2 = 8.8$. The potentials varied with time, the pH values decreasing and gradually approaching a limiting value which was reached and maintained within two or three days. The major part of the reaction (in molar F) was complete in half an hour at 30° C. The initial pH (or pG_f) values, extrapolated to zero time, when plotted against log F gave a characteristic curve for an *amino* acid, with a limiting slope of -2 at high F values. The final pH values, after the steady state had been attained, gave a typical curve for an *imino* acid, with a slope of -1 at high F. These findings, combined with those of Cherbuliez and Stavritch, are most satisfactorily explained by the assumption that the amino group of asparagine (A) comes into practically instantaneous equilibrium with F, forming mono-and dihydroxymethyl derivatives, like those of other amino acids. Following this, however, is a slow reaction, involving methylene bridge formation between the amino and the amide nitrogen, the resulting product being 6-hydroxy-tetrahydropyrimidyl-4-carboxylic acid (B). This is the true formula of "methylene asparagine."

$$
\begin{array}{ccc}
\begin{array}{l} CO-NH_2 \\ H_2C \\ CH-NH_3^+ \\ | \\ COO^- \\ (A) \end{array} & +HCHO \rightleftharpoons & \begin{array}{l} CO-NH \\ H_2C CH_2 \\ CH-NH_2 \\ | + \\ COO^- \\ (B) \end{array} +H_2O \quad (44)
\end{array}
$$
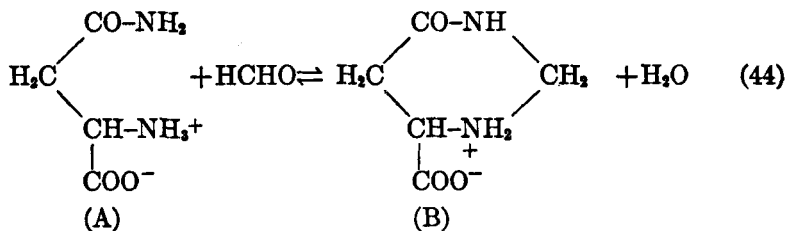
It is an imino acid which, when the imino group is uncharged, can combine with one more molecule of F, giving the unstable "dimethylene" derivative of Schiff. Levy and Silberman calculated the velocity constant for Reac-

tion 44 in two ways: (1) From the change of pH with time, knowing the initial and final pG_f values at any given F value. (2) From the rate of disappearance of amino nitrogen. The two methods gave results in satisfactory agreement, the velocity constant K (Equation 45) being $0.029\pm.003$ (A_0 = initial asparagine concentration) at 30° C.

$$K = \frac{1}{Ft} \log \frac{A_0}{A_0 - B} = 0.029 \text{ (t in minutes, logs to base 10)} \qquad (45)$$

The F concentration was at least ten times as great as the asparagine concentration in all experiments. Notable is the fact that the rate is independent of pH, and therefore the rate determining step of the reaction presumably takes place at the amide group; a fact quite incompatible with Schiff's formulation of the reaction. The pK value of the imino group in compound B is of the order of 6.0. As with cysteine, the conversion of an open chain amino to a cyclic imino compound decreases markedly the basic strength of the nitrogenous group.

The optical rotation measurements of Carpenter and Lovelace (1942, 1943) on asparagine are readily interpreted in terms of Reaction 44, although these authors themseves interpreted them differently. We believe their interpretations to be incompatible with the findings of Levy and Silberman, of which Carpenter and Lovelace were evidently unaware.

The possible biological importance of the reaction between asparagine and aldehydes, as a source of pyrimidines and their derivatives in plant metabolism, has been pointed out by Cherbuliez and Stravritch.

Schiff also studied α-methyl asparagine and glutamine, reporting that they combined with F to form derivatives similar to that of asparagine. Aspartic and glutamic acids have been studied by several authors. No well defined derivatives have been isolated, but the optical rotation measurements of Carpenter and Lovelace (1943) and of Frieden, Dunn, and Coryell (1943b) indicate association of F with the amino group similar to that of simple amino acids (see L_1 and L_2 values in Table I). Carpenter and Lovelace noted, however, that the solutions required a very long time to reach equilibrium — five to seven days for disodium glutamate, and 45 days for disodium aspartate solutions! Perhaps this indicates formation of a methylene bridge between the amino and carboxyl groups, the resulting compound being presumably very unstable. Frieden, Dunn, and Coryell make no mention of a variation with time in their studies.

4. DIKETOPIPERAZINE AND THE PEPTIDES

Diketopiperazine readily forms a dihydroxymethyl derivative on heating with F in neutral or slightly acid solution. It is a well defined, crystalline compound melting at 179–180° C., more soluble in water than is diketo-

piperazine itself. It was first prepared independently by Cherbuliez and Feer (1922) and by Bergmann, Jacobsohn, and Schotte (1923). The former authors prepared a large number of derivatives from it.

$$
\begin{array}{c}
CH_2 \cdot OH \\
| \\
N \\
OC \quad\quad CH_2 \\
| \quad\quad\quad | \\
H_2C \quad\quad CO \\
N \\
| \\
CH_2 \cdot OH
\end{array}
$$
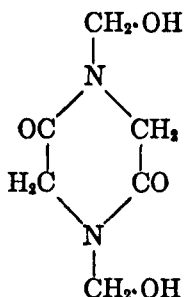
Thus the peptide group, in a diketopiperazine, shows a reactivity with F comparable with that of a primary amide group. This is perhaps surprising, since Einhorn (1905, 1908) found that N-alkyl amides of fatty acids were very unreactive with F; however, he reported that an N-hydroxymethyl derivative was readily formed by the action of F on *sym*-dimethylurea. Studies on proteins, reported in Part VI, suggest that most of the peptide groups in proteins react with F either very slowly or not at all. Further studies on synthetic peptides are clearly desirable; our present information is very limited. Wadsworth and Pangborn (1936) found a progressive decrease in free and "reversibly bound" F in solutions of glycylalanine and alanylglycine at pH 8 and 39° C. The reaction was slow and was not entirely complete, even after four weeks; but the bound formaldehyde was very firmly held, not being removed by exposure to dimedon at pH 4.4–5.0 for three days at 39°. Since in the dipeptides a free amino group is present as well as the peptide group the reaction may be analogous to that of asparagine, involving ring closure and methylene bridge formation to give an imidazolidone derivative (Reaction 45).

$$
\begin{array}{c}
NH_3^+ \\
/ \\
R{-}CH \\
| \quad\quad +H_2CO \rightarrow \\
OC \\
\backslash \\
N \cdot H \\
| \\
R'
\end{array}
\qquad
\begin{array}{c}
NH_2^+ \\
/ \quad\quad \backslash \\
R{-}HC \quad\quad CH_2 \quad +H_2O \\
| \\
OC \\
\backslash \quad / \\
N \\
| \\
R'
\end{array}
\qquad (45)
$$
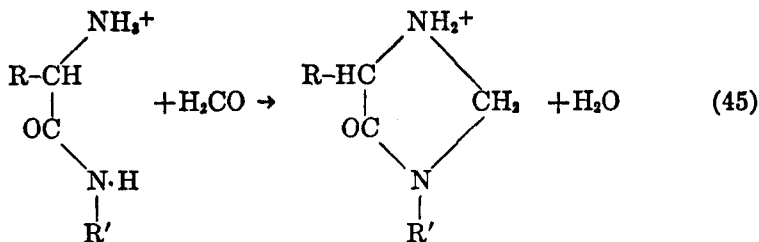
A compound which may be of this type was prepared by Franzen and Fellmer (1917) from glycylglycine. The reactions of peptides with F

clearly call for further study, which should yield information of great value for elucidating the reactions of proteins with F.

5. TRYPTOPHAN

Annie Homer (1912), in Hopkins' laboratory, obtained the crystalline mono- and dihydrates of a substance $C_{12}H_{12}O_2N_2$, from the interaction of tryptophan and F, but did not establish its structure. Wellisch (1913) attempted to investigate the reaction further, but did not obtain a homogeneous product. Jacobs and Craig (1936) prepared what was evidently the same compound described by Homer, by heating the reactants in moderately acid solution, and identified it as 3, 4, 5, 6-tetrahydro-4-carboline-5-carboxylic acid (Reaction 46).

$$(46)$$
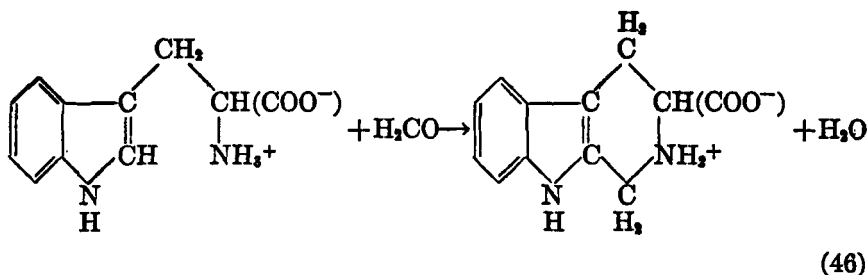
It is an ampholyte, and is clearly a dipolar ion, melting with decomposition at 306°. It is rather insoluble in water near the isoelectric point, but dissolves in moderately acid or alkaline solutions. It is formed readily and rapidly in neutral or slightly alkaline solutions, at ordinary temperatures (Wadsworth and Pangborn, 1936; Ross and Stanley, 1938). Jacobs and Craig showed that a number of other aldehydes readily undergo reactions with tryptophan exactly analogous to 46, with the formation of carboline derivatives. The reaction is of interest as indicating a possible mechanism for the formation of certain ergot and harmala alkaloids from tryptophan (concerning these alkaloids, see for instance Small, 1943, p. 1228). The reactions between tryptophan and aldehydes, considered as a source of humin formation in protein hydrolyzates, have been investigated in a series of researches in Gortner's laboratory (see Zeleny and Gortner, 1931; Lillevik and Sandstrom, 1941; further references are given in these papers).

The reaction with F has been examined by Levy and Silberman from the point of view of the formol titration. They found that tryptophan behaves as an imino acid, the reaction product combining reversibly with only one mol of F. The association constant ($L_1 = 83$) given by Levy for the reaction must be considered as a composite of the ratio of the basic dissociation constants of tryptophan and tetrahydrocarboline carboxylic acid and the F association constant of the latter:

$$L_1 \text{ ("tryptophan")} = L_1 \text{ (carboline)} \frac{K_2 \text{ (carboline)}}{K_2 \text{ (tryptophan)}} \qquad (47)$$

Measurements by one of us (French, unpublished) indicated that the ratio of the basic dissociation constants is not large, perhaps two or three (a few tenths in logarithmic units).

In order to obtain more quantitative information about this interesting system, the authors attempted a polarimetric study, after the method of Frieden, Dunn, and Coryell. Although large effects were observed, the course of the reaction appeared to be so complex as to admit no satisfactory solution. The rotations were not constant even after several days, and the necessary alkalinity led to a high development of color in the solutions.

Tryptamine (Hahn, Bärwald, Schales, and Werner, 1935) reacts in a manner similar to that of tryptophan. Glycyl tryptophan also reacts, as judged by the formation of an insoluble "methylene" derivative (Birch and Harris, 1930) and by the effect on the reaction of the peptide with the Folin reagent (Ross and Stanley, 1938).

6. PHENYLALANINE AND TYROSINE

The reaction of these amino acids with F is somewhat similar to that of tryptophan, and leads to the formation of tetrahydroisoquinoline derivatives (Reactions 48 and 49). β-Phenylethylamine gives tetrahydroisoquinoline itself on treatment with F,

$$(48)$$

$$(49)$$
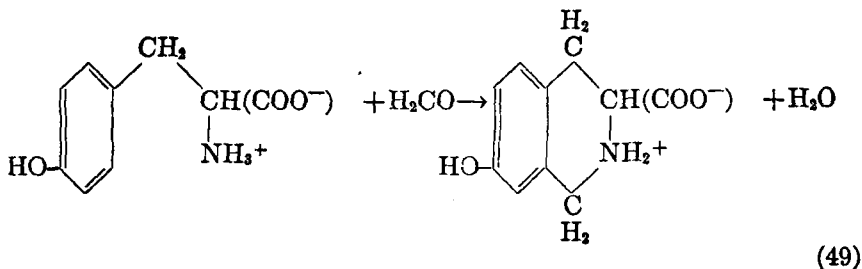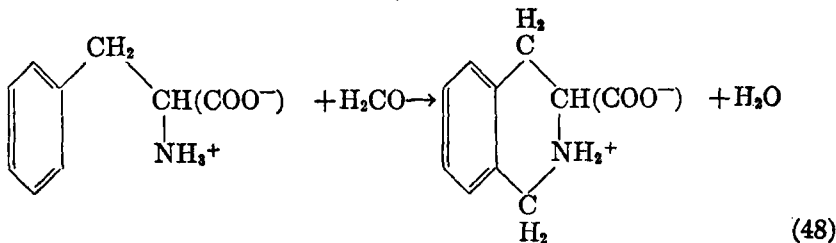
while the amino acids react even more readily to give the carboxylic acid derivatives shown in Equations 48 and 49. These products were first

obtained by Pictet and Spengler (1911), by heating the reactants for several hours in acid solution. Both are dipolar ions, as shown by their very high melting points (with decarboxylation) and their extreme insolubility in non-polar solvents. The phenylalanine derivative is fairly soluble in hot water; but the tyrosine derivative, near its isoelectric point, is extremely insoluble in almost every solvent. As Pictet and Spengler pointed out, the products of the reaction — in which F can be replaced by many other aldehydes — are very closely related to the important class of alkaloids which contain the isoquinoline ring structure (see Small, 1943, p. 1209), and reactions such as 48 and 49 may play a significant role in the biosynthesis of alkaloids.

In these studies, *dl* amino acids were employed; Wellisch (1913) carried out Reaction 49 with *l*-tyrosine, the crystalline reaction product being very similar to the *dl* compound. The high acidity and high temperature employed in these preparations are perhaps unnecessary, especially in the case of tyrosine. Holden and Freeman (1931) showed a rapid disappearance of amino N in moderately alkaline tyrosine solutions at room temperature; the observed effect was probably due to Reaction 49, although the product was not isolated.

Levy and Silberman (1937) reported F association constants of $L_1 = 10$; $L_2 = 5$ for tyrosine. It is likely that these rather low values do not represent fairly the reactivity of tyrosine with F. The pK values of the phenolic hydroxyl group and the α-amino group in tyrosine lie so close together that pK_2' cannot be taken as the pK of the amino group; therefore, the drop in pH of a tyrosine solution, initially adjusted to $pH = pK_2'$, on the addition of F might be expected to be considerably retarded due to the buffering effect of the phenolic group. The true association constants are probably appreciably higher than those reported. Attempts to determine the association constants by the polarimetric technique, in which the buffering capacity of the phenolic group should introduce no error, were unsuccessful as in the case of tryptophan.

The reaction with phenylalanine is not complicated by the presence of the phenolic group. Levy determined the association constants: $L_1 = 16$, $L_2 = 23$. Balson and Lawson gave similar values $L_1 = 22$; $L_2 = 25$.

7. HISTIDINE

Histidine undergoes a reaction with F exactly analogous to that of tryptophan, the product being 1, 2, 3, 4-tetrahydropyrido-3, 4-imidazole-6-carboxylic acid. This was isolated by Wellisch (1913) in the form of the dihydrochloride, after boiling a strongly acid solution of histidine in for-

$$(50)$$
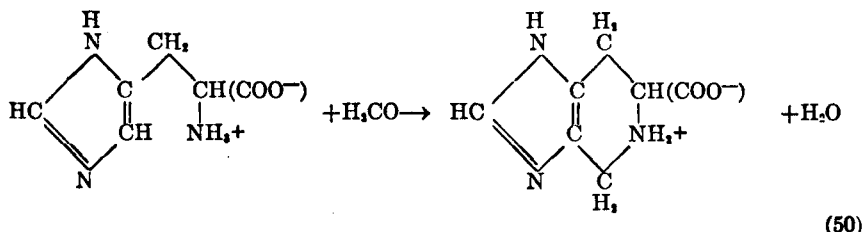
maldehyde for several hours (Equation 50). This treatment was undoubtedly more drastic than necessary. Holden and Freeman (1931) observed a very rapid disappearance of amino N on incubating histidine with F at pH 8 and 37° C. A crystalline product, much less soluble than histidine itself, separated from the solution. Holden and Freeman were evidently unaware of the work of Wellisch, but there seems little doubt that Reaction 50 describes the process they observed. Similar observations were made by Wadsworth and Pangborn (1936), although they did not obtain a crystalline product. Smith, Handler, and Mrgudich (1940), also in ignorance of Wellisch's work, treated histidine (free base) with an excess of formaldehyde, precipitated the complex with alcohol and dried for an extended period of time. The X-ray powder diagram of the complex, at first amorphous, gradually became crystalline and decidedly different from that of histidine. Probably this product also is that shown in Equation 50, although the analytical figures of Smith, et al. do not check very well with those expected.

Decisive evidence for the occurrence of Reaction 50, and for the exact structure of the reaction product, has recently been furnished by Neuberger (1944). He has shown clearly that the methylene group in the final product of the reaction of F with histidine is linked to a carbon (not nitrogen) atom of the imidazole nucleus. He determined the dissociation constants of tetrahydropyrido-3,4-imidazole-6-carboxylic acid as pK_2' (imidazole) $=4.73$; pK_3' (imino) $=8.58$. The isoelectric point $pI = (pK_2'+pK_3')/2$ is at pH 6.65, as compared with 7.59 for histidine (Cohn and Edsall, 1943, p. 85). Both pK_2' and pK_3' are decreased by addition of F, the latter being the more strongly affected. This indicates further reaction with F of the product of Reaction 50, and indeed a monohydroxymethyl derivative of this product is readily obtained; on heating or acidifying this derivative, one mol of F is again readily given off. Neuberger has studied the kinetics of Reaction 50, and deduces that it occurs in two steps, the first (involving disappearance of amino N) being nearly complete in a few minutes, while the second requires many hours to run to completion. The reaction is very slow below pH 4, very fast above pH 5, the rate being virtually independent of pH between 7.4 and 11.2.

Neuberger has suggested several possibilities for the mechanism of the reaction. Perhaps the most likely is an initial formation of a new ring by means of a methylene group joining the amino group to one of the *nitrogen* atoms of the imidazole ring. On this hypothesis the slow secondary rearrangement involves an intramolecular shift of the attachment of the methylene group from nitrogen to carbon in the imidazole ring. This hypothesis would explain why both the initial and the final reaction products contain no amino nitrogen. Final proof of this or any other reaction mechanism remains for future investigation.

The potentiometric analysis of the reaction is complicated by the fact that the imidazole group has its greatest buffering action in the pH range in which the effect of F on the amino group is normally examined. In order to determine the association constants it was found necessary to obtain data in the acid range (pH < 6). Levy (1935) concluded that the major changes produced in the titration behavior of histidine on the addition of F could be interpreted on the basis of a reaction with the amino group, together with internal shift of a hydrogen ion. Thus the histidine dipolar ion can react with F, a proton being transferred from the $-NH_3^+$ to the imidazole group in the process; and a similar reaction occurs with the monocation, the COO^- group acting as proton acceptor. The imidazole group is considered to be a simple buffer which does not react with F. The behavior of histamine was found to be quite analogous to that of histidine. There was no mention of a slow reaction taking place.[18]

Levy also examined in the same way the effect of F on 4-methyl-imidazole and imidazole lactic acid. The effect was fairly small as compared to that on an amino group; however, the shape of the curve is quite characteristic of the reaction of F with both the basic and cationic forms, the reaction with the basic forms predominating at low F concentration. The effects are probably too large to be ascribed to changes in the solvent, since the bulk of the effect is manifest in solutions containing only 1 per cent F.

The polarimetric studies of Frieden, Dunn, and Coryell (1943c) led them to general conclusions not dissimilar to those of Levy. They concluded, however, that the anion, the dipolar ion, and the monocation of histidine can all react with either one or two mols of F, several of the reactions involving intramolecular proton shifts. Like Levy, they recorded no drift of their readings with time, such as might be expected from Reaction 50. However, the change in the molecular rotation on the addition of the first mol of F was very great—from $-16.3°$ for anionic *l*-histidine to about $-320°$ for anionic histidine plus an equimolar amount of F. This is much greater than the change generally observed for the simple combination of F with an

[18] Concerning the effect of F on the depressor activity of histamine, see Best and McHenry (1930, p. 357ff).

amino group, and is comparable to the very large negative shifts of rotation which occur in the reactions of cysteine (Ratner and Clarke, 1937) and asparagine (Carpenter and Lovelace, 1942) with F. Since both the latter reactions involve ring closure near the asymmetric carbon atom, the data here suggest a similar reaction. It is well known that the absolute value of the molecular rotation in ring compounds is generally much higher than for closely related open chain compounds (Kauzmann, Walter, and Eyring, 1940, pp. 376–81; Kauzmann and Eyring, 1941). The extremely large values for the association constants (Table III), especially for anionic histidine, also suggest that a distinctive type of reaction is taking place. The association constants reported by Levy (1935) and by Frieden, Dunn, and Coryell (1943c) are given in Table III.

TABLE III

Association Constants for the Reactions between Histidine and Formaldehyde

Authors	L_1^+	$L_1^\pm$	$L_2^\pm$	L_1^-	L_2^-
Levy	0.59	430	—	32,000	—
Frieden, Dunn, and Coryell	0.74	660	1680	42,000	145,000

The subscript of the symbol L (1 or 2) indicates whether the reaction involves 1 or 2 mols of F (see Equations 29 and 30). The superscript ($+$, $\pm$ or $-$) indicates whether the histidine cation, dipolar ion, or anion is involved. In Levy's notation, the five constants given here, reading from left to right, are denoted as L_{11}, L_{12}, L_{22}, L_{13}, and L_{23}, respectively.

8. ARGININE

Both pH and optical rotation measurements indicate that arginine, in the presence of F, undergoes a rapid reaction which attains equilibrium almost at once, followed by a slow reaction which is not complete for 24 hours or more. A steady state, however, appears to be finally achieved. The pH measurements of Levy (1935) on arginine in this steady state indicated that both the dipolar ion and the monocation of arginine react with *two* mols of F, the association constants being $L_2^\pm = 2.57 \times 10^5$, and $L_2^+ = 0.71$.

Frieden, Dunn, and Coryell (1943d) concluded from their polarimetric measurements that the instantaneously established equilibrium involved reaction between one mol of arginine and one of F; the final, slowly attained equilibrium state appeared to involve 2 moles of F per mol of arginine. They therefore formulated the slow reaction as

$$AF^\pm + F \rightleftharpoons AF_2^\pm \qquad (51)$$

Measurements of the change of optical rotation with time, and also of the change of pH with time in a solution initially at pH 7.85, indicated a velocity constant for this reaction close to 10^{-2} (t in minutes, logs to base e).

A slow alteration in the response of arginine, in the presence of F, to the Sakaguchi test, was also observed. The usual deep carmine color was obtained with the Sakaguchi reagent immediately after mixing F and arginine; but if the reagent was not added until a half hour later, a dark green color appeared, which gradually changed to a reddish brown. This is strong evidence that the slow reaction involves the guanidino group. It should also be noted, however, that the change in optical rotation accompanying this reaction is extraordinarily great. In the presence of a large excess of F, the initial molecular rotation is near $-100°$, the final value greater than $+700°$. Such an extremely large change would be surprising if none of the groups immediately adjoining the asymmetric carbon were involved. The total picture suggests methylene bridge formation, involving both amino and the guanidino nitrogen — compare the preceding discussion of histidine — but conclusive evidence is lacking. Wadsworth and Pangborn (1936) reported that all the F in an arginine –F mixture could be precipitated as the dimedon derivative, on standing at pH 4.4–5.0 with dimedon for three days, even when arginine and F had first been allowed to react as long as 11 days at pH 8 and 39° C. This suggests that Reaction 51 is reversible, at least with respect to F, although it has not been proved that unaltered arginine can be recovered from such systems. Further studies of the arginine-formaldehyde system should prove of great value.

9. LYSINE

Each of the two amino groups of lysine should presumably be capable of reacting, in the uncharged form, with either one or two molecules of F. Levy (1935) reported for the lysine dipolar ion the association constants $L_1^{\pm} = 89$, $L_2^{\pm} = 250$; for the anion he reported $L_1^{-} = 240$, $L_2^{-} = 310$. Frieden, Dunn, and Coryell (1943d) however, could not confirm these values by the optical rotation method, although their analysis was incomplete. They evaluated $L_2^{\pm}/L_1^{\pm}$ as 8.72, as against 2.7 from Levy's results; and L_1^{-} as 35.0, much lower than Levy's figure. They concluded that the ε-amino group has a much stronger tendency to associate with F than the α-amino group — a result to be expected in view of the steric factors affecting the reaction (p. 299). The possibility of methylene bridge formation between the two amino groups should be considered, although there is no positive evidence for it from the available data. Titherly and Branch (1913) believed that hexahydro pyrimidine was formed from propylene diamine by a similar reaction with F. Their observations indicated a very mobile equilibrium between hexahydro pyrimidine and the open chain hydroxymethyl derivatives of propylene diamine. Further knowledge of the conditions of formation, and the stability, of such cyclic methylene diamine

derivatives may be important for understanding of the reactions of proteins with F.

VI. PROTEINS

1. INFLUENCE OF FORMALDEHYDE ON TITRATION CURVES

The formol titration of proteins does not differ in principle from that of the amino acids. The effect of F in modifying the titration curves has been studied most systematically by Kekwick and Cannan (1936) (see also Cannan, 1942, Fig. 1 and p. 402). An alkaline segment of the curve is displaced by F (1 to 8 per cent) from a pK value near 10 to one near 7. This very large displacement corresponds to what would be expected for the free ε-amino groups of lysine. The best procedure for titrating these groups appears to involve: (1) an initial adjustment of the aqueous protein solution to a pH of approximately 8.5; (2) addition of F, which, of course, causes a marked decrease in pH; (3) titration to a final pH of 8.5 in the F solution. The alkali consumed should be equivalent to the ε-amino groups. If terminal free α-amino groups are present, they should contribute very little to the titration, provided their pK values are similar to those found in peptides (pK values of 8.1 or less). If the initial adjustment of pH in water is made to 6.5 instead of 8.5, the α-amino groups are presumably included; there is then also a contribution from the imidazole groups of histidine. The ε-amino groups, as determined by formol titration, frequently exceed the lysine determined by analysis of the protein hydrolyzate; often this is due to inadequacies in the analytical procedure (Cannan, 1942). Lichtenstein (1940) has shown that deamination of gelatin causes virtually complete disappearance of that portion of the titration curve which is affected by F, other portions being virtually unaffected. The effect of F on the titration curves of numerous other proteins has been studied: among those investigated most systematically, we may cite egg albumin (Cannan, Kibrick, and Palmer, 1941), β-lactoglobulin (Cannan, Palmer, and Kibrick, 1942) and myosin (Dubuisson, 1941).

2. THE DETERMINATION OF BOUND FORMALDEHYDE IN PROTEINS

In the study of such processes as the tanning of collagen or casein by F, it is necessary to expose the protein to F at a given concentration and temperature, for a known time, and study the effects on the protein. At the end of the time of treatment, the sample is removed from the solution and thoroughly washed to remove F which is not firmly bound. Some of the limitations of such a procedure must be immediately apparent. Any F which is held only in a loose reversible combination will be rapidly removed by the washing procedure. This fact has generally been clearly recognized. However, F which can readily be removed in this way is not likely to play

an important part in the reactions underlying the tanning process. More important is the fact revealed by the very careful studies of Nitschmann and Hadorn (1943a) on casein, that a considerable amount of F, which still remains adherent to the solid protein even after several hours of quite thorough washing, can be removed if the washing is prolonged for a period of many days. On one sample of casein, for example, which had been soaked for 64 hours at room temperature in 38 per cent F, they were unable to demonstrate any further F in the flowing wash water after five hours of washing, even by the very delicate test of Rimini and Schryver.[19] However, if the wash water were allowed to stand at this stage for 10 minutes in contact with the casein, a positive test appeared. The test did not become finally negative, in this case, until after 24 days of continuous washing. At the end of this time, analysis of the casein, by the method discussed in the following paragraph, showed an F content of 1.78 per cent. This amount of F, therefore, is very firmly held, and cannot be removed even by such prolonged washing at room temperature. In another experiment, a sample of casein which had been treated 24 hours with 38 per cent F was divided into two portions, one being continuously washed for 1½ hours and the other for 12 days. The formaldehyde content of the former was 2.59 per cent; of the latter, 1.91 per cent, so that the amount removed by the more prolonged washing was more than 35 per cent of the residual amount which remained firmly bound. It seems clear that the F which can be removed, but only by very prolonged washing at room temperature, must exist in the form of a chemical complex which does not readily dissociate. At the same time, it must be less firmly bound than the residual F which cannot be removed even by weeks of washing at room temperature. It is impossible, at present, to differentiate the chemical groups involved in these two forms of binding. This work of Nitschmann and Hadorn, however, reveals clearly the difficulties which arise in defining the concept of "bound formaldehyde" in proteins. Such a term, in order to have a definite meaning, must be very precisely defined in terms of the operations used in measuring it.

Various different techniques have been employed in the determination of bound F in proteins. Most of the linkages between F and the groups existing in the protein molecule can be broken, with the release of free formaldehyde, by prolonged hydrolysis in hot acid solution.[20] On thorough

[19] This test is carried out by adding phenylhydrazine hydrochloride and potassium ferricyanide to the solution to be tested, and then adding a little concentrated hydrochloric acid. In the presence of formaldehyde, a strong red color appears; one part in a million of formaldehyde can be detected by this method.

[20] This is probably not true, however, of F which has been incorporated into tryptophan, tyrosine, phenylalanine, or histidine residues by the reactions described in Section V.

distillation, the formaldehyde passes over into the distillate, and can be determined there, for instance, by collecting it in a solution of $NaHSO_3$ and subsequent titration with iodine. Highberger and Retzsch (1938) determined bound formaldehyde in collagen by digesting the sample to be analyzed in 2 N H_2SO_4 and distilling over nearly all of the digestion fluid into a receiving flask containing $NaHSO_3$. A sufficient length of time was permitted to allow complete formation of the bisulfite addition product in the receiving flask. Iodine solution was then added in amount just sufficient to oxidize the free bisulfite, and the solution was made alkaline after the addition of a little ethanol to inhibit oxidation of sulfite by oxygen. In the alkaline solution, at pH between 9 and 10, the formaldehyde addition compound is decomposed. The liberated sulfite is then rapidly titrated with iodine and the formaldehyde content calculated from the amount of sulfite thus liberated.

Nitschmann and Hadorn (1941) confirmed the accuracy of this method of determining F in collagen digests, but showed that it gave low results for formaldehyde-treated casein, and also for vitellin and probably for certain other proteins. For casein, they discovered that accurate results could be obtained if solutions less strongly acid than those recommended by Highberger and Retzsch were employed for the digestion. Approximately 0.1 molar phosphoric acid was found to give quantitative yields. Later (Nitschmann, Hadorn, and Lauener, 1943), it was found that low results were sometimes obtained even by this technique unless the digestion fluid was twice distilled. After about 85 per cent of the original volume of liquid had been distilled, more water was added and a second distillation carried out, to a very small final volume. The second distillation leads to an added recovery, generally of the order of 1 or 2 per cent of the total amount of bound formaldehyde determined.

Theis and Jacoby (1942, 1943) and Theis (1944, 1945) have chosen not to remove free and reversibly bound F by washing, but instead press out the collagen (or other fibrous protein) several times between sheets of blotting paper at a pressure of 10,000 lbs. per sq. in. They present evidence that free water and free electrolytes are removed by this treatment, and therefore infer that free F is also removed from a protein preparation previously exposed to F solution. However, this pressure method certainly must leave in the protein considerable amounts of F which are removed even by brief washing. The values of bound F, determined by the method of Theis, are therefore not directly comparable with those determined after the protein has been thoroughly washed.

3. COLLAGEN[21]

The use of formaldehyde in the tanning of leather has led to a long series of empirical studies. The action of F renders the product inert to trypsin and other proteolytic enzymes, greatly decreases the amount of swelling in water, acid, or alkali, and increases the temperature at which collagen undergoes thermal shortening[22] from 65–70° C. to about 90 °C. Moreover, the thermal shortening of F-treated fibers, unlike that of normal fibers, is reversible. Such a treated fiber, heated briefly to 90–100° C. in water, contracts to about one-third of its original length. On cooling it extends and uncoils very rapidly to about two-thirds of its original length, and over a period of several hours, lengthens further, returning to the same dimensions as before heating (Ewald, 1919; Küntzel and Prakke, 1933).

Interpretation of the reactions underlying these phenomena involves a knowledge of the amount of F actually bound by collagen under various conditions, a determination of the chemical groups in collagen responsible for the binding, and also of the nature of the linkages formed. Recent years have seen significant beginning of knowledge on these points. Highberger and Retzsch (1939) equilibrated collagen with formaldehyde solutions of various concentrations, adjusted to various pH values, generally for periods of 24 hours.

After equilibration, the collagen was washed for several hours to remove unbound or loosely bound F, and then digested with $2 N H_2SO_4$ and analyzed for bound F as described in Section 2. Similar studies were made on partially deaminated collagen. The amounts of bound F, as a function of pH, after equilibration with 1 per cent F, are shown in Fig. 7. The uptake of F, up to pH values of 7 to 8, is greatly decreased by deamination; but the rate of increase of bound F with increasing pH above 8 is not much affected. Similar observations were made independently by Bowes and Pleass (1939). The plausible inference was drawn that the binding of F below pH 8 is due chiefly to combination with the ε-amino groups of lysine. at higher pH values to combination with the guanidino groups of arginine, The latter concept was strengthened by the finding that treatment with hypochlorite — which chemically alters the guanidino groups — led to a decrease in bound F in the alkaline portion of the curve, above pH 8 (Highberger and O'Flaherty, 1939). These findings were confirmed and extended by Gustavson (1943), who has given a particularly critical analy-

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[21] For general discussion of the structure of the collagen fiber, see Küntzel and Prakke (1933); Küntzel (1941); Schmitt (1944).

[22] The exact value obtained for this temperature depends on the particular type of collagenous fiber under study and also on the manner and duration of its exposure to high temperature.
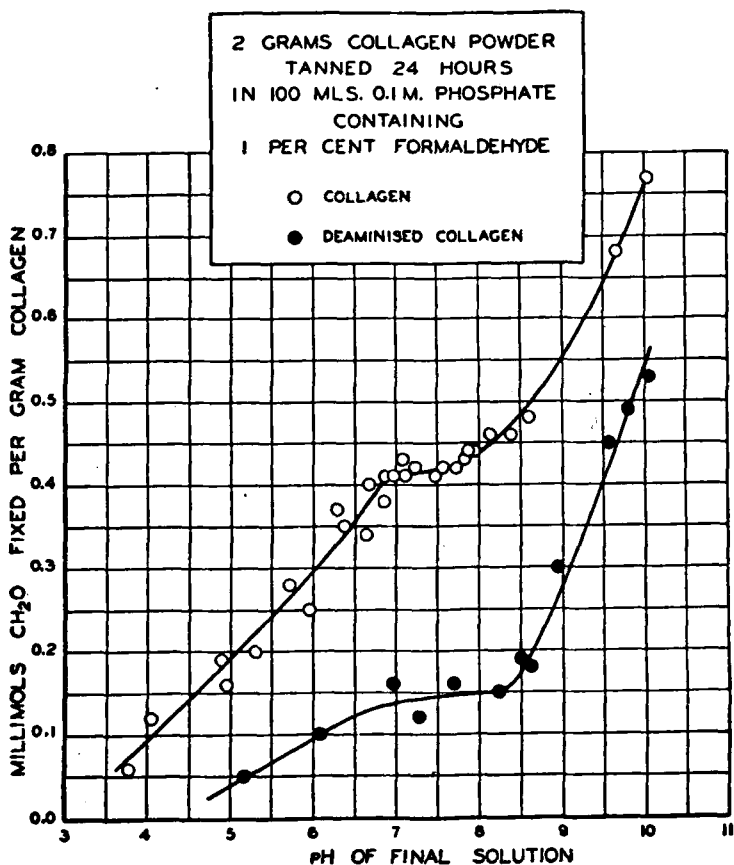
Fig. 7. Formaldehyde bound by collagen as a function of pH. Data from Highberger and Retzsch (1939).

sis of the factors involved.[23] Table IV, taken from his paper, shows the effect of treatment with F at several pH values on native, and on partly and completely deaminated collagen, as indicated by the elevation of the temperature of thermal shortening (T.), the loss of digestibility by trypsin, and the decreased swelling in solutions far from the isoelectric point. It will be noticed that all these alterations are achieved almost as completely by treatment with F at pH 8 as at either of the higher pH values, although the amount of bound F increases very greatly as the pH rises. The difference in bound F between native and completely deaminated collagen, at pH 11 and 13, is 0.44–0.48 $mM/g.$, which is very nearly equivalent to the lysine content of native collagen. The completely deaminated material

[23] Gustavson's paper is also useful for its very full bibliography of work in this field.

TABLE IV

Binding of Formaldehyde by Collagen and Deaminated Collagen

	Untreated			pH =8			pH =11			pH =13		
	Collagen	Completely deaminated	Partially deaminated	Collagen	Completely deaminated	Partially deaminated	Collagen	Completely deaminated	Partially deaminated	Collagen	Completely deaminated	Partially deaminated
Bound CH_2O millimols/g. collagen	0	0	0	0.31	0.02	0.12	0.72	0.28	0.38	0.94	0.46	0.58
T_s in °C.	69	70	69	89	70	74	90	70	74	91	68	73
Tryptic digestion in per cent of original collagen	12	60	46	0	54	38	0	52	40	1	63	58
Swelling: g.H_2O/g. collagen	2.3	2.4	2.3	1.7	2.5	2.5	1.8	4.2	3.9	1.7	5.6	5.4

From Gustavson (1943).

can still bind large amounts of F at high pH, but this binding is associated with none of the alterations characteristic of the tanning of native collagen. Gustavson infers from the data that the tanning reaction is determined by combination of F with the amino groups of lysine; and that, although reactions with guanidino groups and peptide linkages also occur, the latter contribute nothing to the strength of the internal "lattice structure" of the fiber. This structural alteration is therefore assumed to be due to methylene bridge formation[24] involving the ϵ-amino groups of lysine. Gustavson assumes that a $-CH_2-$ group derived from formaldehyde links two lysine ϵ-amino groups. On this hypothesis, however, the ratio of bound F to bound amino groups should be 1 : 2, while Gustavson's data (and also those of Highberger, and of Bowes and Pleass) indicate that it is more nearly 1 : 1. This suggests that each $-CH_2-$ bridge links a lysine amino group to another type of functional group. The most likely group to be involved here is the peptide linkage, for such linkages are far more numurous than any other functional group in the protein molecule; and the type of reaction involved is exactly analogous to that which has been shown to occur in asparagine (p. 307) and to be probable in simple peptides (compare also the work of Nitschmann and Hadorn on casein, discussed below).

It is possible to produce the typical alterations associated with the tanning process even at very low pH (1 to 2), but high F concentrations must be employed, and the reaction runs very slowly. Thus Gustavson (1943) reports that at pH 8 the reaction is essentially complete in 8 hours even for thick samples and in one hour for thin ones; while more than a week, and high F concentrations, may be required to produce an equivalent effect at pH 1-2. This is what might be expected if an essential step in the process is the combination between an F molecule and an uncharged amino group.

Theis (1945, and earlier references there cited) has studied the F binding of collagen after heat denaturation, and finds it to be much increased over that of the native collagen. He also finds much less change in F binding capacity after deamination than do Highberger and Retzsch, or Gustavson. The differences reported proceed largely from differences in method of removing bound F (see pp. 317-319). The method of Theis certainly allows much F to remain with the protein, which would be removed by the thorough washing procedures of the other authors.

Bear (1944) has shown that the long X-ray spacing parallel to the collagen fiber axis — approximately 640 Å in dried, and 675-680 Å in wet

[24] The explanation of the alteration in mechanical properties of proteins by F through methylene bridge formation between adjoining functional groups was perhaps first suggested by K. H. Meyer (1929).

native collagen — is reduced to about 615 Å in a dried sample which had previously been treated with 4 per cent F (10 per cent formalin) for 24 hours, and thoroughly washed. Another F-treated fiber (rat tail collagen) was shortened to 30 per cent of its initial length on brief treatment with water at 100° C.; after reversible spontaneous extension to 87 per cent of its initial length, the long spacing was 550 Å. In contrast to the change in the long spacing, the characteristic short spacings of collagen — 11.5 Å, equatorial; 2.86 Å, meridional — were virtually unaffected by treatment with F.

4. CASEIN

The use of formaldehyde in the tanning of casein has also been of major technological importance. As with collagen, treatment with F makes the material tougher, more resistant to swelling and to enzymatic action. We shall consider here only the recent careful studies of Nitschmann and Hadorn. They confined their studies to a narrow pH zone, approximately 5.5 ± 0.5. Nitschmann, Hadorn, and Lauener (1943) determined the amount of bound F in casein as a function of time and F concentration. A discussion of the analytical methods employed, and of their very pains-taking study of the effects of prolonged washing in removing part of what has commonly been called "bound" F, has already been given on p. 317. Nitschmann and Hadorn (1943b) studied the swelling of F-treated casein. When the amount of F bound is near 1.5 per cent, the casein becomes far more resistant to swelling, not only in acid and alkaline solutions, but in concentrated solutions of urea or thiocyanates; only drastic hydrolysis breaks down the network structure produced by F. It is only up to about 1.5 per cent bound F that the mechanical properties depend upon the amount bound; much more than this can be bound, but the additional F contributes nothing to the tanning process. In a further study, Nitschmann and Hadorn (1944) give evidence that, as in collagen, the lysine amino groups play a major part in the process. The comparison of casein and deaminated casein is shown in Table V. If the tanning process is carried out for only 24 hours (10 per cent F) the decrease in bound F on deamina-tion is close to, but somewhat less than, the decrease in maximum acid binding or amino N. If the tanning is much prolonged (30 days), deamin-ated casein binds nearly as much F as native casein. Nitschmann and Hadorn believe this to be due to a slow reaction with F of the –OH groups which have replaced the –NH₂ groups on deamination; but the evidence for this particular conclusion is not compelling. The decrease in free amino N of native casein, on tanning in the cold with F under a variety of condi-tions, is near 0.4 millimol/g., or about two-thirds of the total van Slyke

amino N, a figure very close to the difference in bound F between native and deaminated casein after 24 hours tanning (Table V).

TABLE V

Binding of Formaldehyde by Casein and Deaminated Casein

Mixture	Casein (I)	Deamino-casein (II)	Difference (I–II)
Acid binding capacity in millimols/g.	0 763	0 263	0 500
van Slyke amino N in millimols/g.	0 597	0.102	0 495
CH₂O bound in per cent	2 18	0.96	1 22
CH₂O bound in millimols/g.	0.726	0.32	0.406
(Tanning 48 hours with 10 per cent CH₂O, washed 24 hours)			
CH₂O bound in per cent	3.42	3.10	0 32
CH₂O bound in millimols/g.	1.14	1.033	0.166
(Tanning 30 days with 10 per cent CH₂O, washed 24 hours)			

From Nitschmann and Hadorn (1944).

Strong evidence for the existence of methylene bridge formation is given by the change of weight of casein after tanning by exposure to gaseous F. The casein samples were first dried, then exposed to F vapor over a 38 per cent F solution in a desiccator for 28 days at room temperature. After this, they were exposed to the air for 14 days to remove all unbound and loosely bound F, and finally dried again, weighed, and analyzed for bound F. The results are shown in Table VI. If F is bound as a hydroxymethyl compound, the change in weight should be equivalent to the F bound; if –CH₂– bridges are formed, the loss of water in the reaction should make the weight increase correspondingly less. The results show clearly that some

TABLE VI

Loss of Water and Methylene Bridge Formation in the Reaction of Formaldehyde with Casein

	Experiment I	Experiment II
Weight of casein before tanning	0 3262 g.	0.3307 g.
Weight of casein after tanning	0 3352 g.	0.3395 g.
Increase in weight	0.0090 g.	0 0088 g.
Increase in per cent (final wt. = 100)	2 69%	2 59%
Bound CH₂O in g.	0.01186 g.	0.01152 g.
In per cent	3.54%	3 39%
CH₂O bound as methylol	2.11%	2 06%
CH₂O bound as methylene	1.43%	1.33%
CH₂O bound as methylene in millimols/g.	0.476	0.443

From Nitschmann and Hadorn (1944).

water is lost in the reaction between casein and F. The number of methylene groups formed calculated on this basis is 0.44–0.48 mM/g. casein, a figure in close correspondence with the previously estimated number of lysine groups reacting with F. Nitschmann and Hadorn (1944) deduce, from all these results, that the essential reaction involved in the tanning of casein at pH 5–6 is methylene bridge formation between the ε-amino groups of lysine and the nitrogen of adjacent peptide linkages. Cross linkage between two lysine residues is excluded, since the ratio of bound lysine to bound F is very nearly 1 : 1, not 2 : 1 as the latter hypothesis would require. Moreover, methylene bonds between an amino group and an amide or peptide group appear to be considerably more stable than those between two amino groups (compare the discussion of asparagine and the peptides, and of lysine, in Part V).

This work of Nitschmann and Hadorn stands out as a carefully planned and critical study of the action of F on a protein. However, the recent work of Fraenkel-Conrat, Cooper and Olcott (1945) indicates that the terminal amide groups of glutamine or asparagine residues bind F more readily than the peptide groups. In a series of proteins and related compounds, they found that the F bound in four days, at pH 3.5–4 and 70°, was always somewhat less than the sum of the free basic groups plus the amide groups. Partial deamidation of casein, gliadin and zein was accompanied by a diminution of the F binding capacity, which closely paralleled the number of amide groups hydrolyzed. Polyglutamic acid (from B. Subtilis) bound very little F, while the polyglutamine prepared from it, by amidation of the free carboxyls, bound between ten and twenty times as much. (Compare also Wormell and Kaye, 1945.)

All this is strong evidence of the importance of the terminal amide groups. It does not, of course, cast doubt upon the work of Nitschmann and Hadorn indicating methylene bridge formation; but it does suggest that such bridges in casein may (as in asparagine) link chiefly amino and amide, rather than amino and peptide groups.

5. KERATIN

Except in one respect, the behavior of keratin is fairly similar to that of collagen and casein (Bowes and Pleass, 1939; Hegman, 1942). Between pH 6 and 8, the results can be interpreted as indicating the reaction of 1 mol of F with each ε-amino group of lysine; and above pH 8 at low F concentration, reaction with arginine appears to account for the data. At higher F concentrations, the amount bound in alkaline solution is definitely greater than can be explained in terms of the lysine and arginine content and appears to be explicable only on the basis of reaction with the disulfide linkages of cystine. Bowes and Pleass and also Hegman assume that F

acts as a reducing agent in alkaline solutions, transforming the S–S groups of cystine to –SH groups. Middlebrook and Phillips (1942), on the other hand, assume preliminary hydrolysis of the S–S linkage, giving –SH and –SOH groups in equivalent amounts. Presumably, however, this must be followed by a reduction of the –SOH groups to –SH, in order to explain the observed reactions. When –SH groups have once been formed, they might conceivably react with F in any one of three ways: (1) to form thiazolidine carboxylic acid residues with an adjoining peptide nitrogen of the same peptide chain, (2) to form analogous cross linkages with the peptide nitrogen of an adjoining peptide chain, (3) to form djenkolic acid residues by methylene bridge formation between adjacent –SH groups of what had been a cystine residue before reduction. Middlebrook and Phillips favor the first of these possibilities,[25] but the basis for their argument rests on the failure of treatment with F to inhibit the subsequent supercontraction of the treated wool. The exact interpretation of the supercontraction mechanism is still open to some doubt (compare also Stoves, 1944).

The conditions employed by Middlebrook and Phillips were significantly different from those of the other investigators. They found that at pH 5.6 wool reacts with F at 70°, but not at room temperature, about one-third of the total disulfide sulfur reacting at the higher temperature. They consider these groups to be the same that react with sodium bisulfite to give sulfhydryl and S — cysteine — sulfonate groups that are stable to water, since bisulfite no longer is capable of reacting with wool in this way after a preliminary treatment with F at 70°. Clearly wool protein presents important differences from collagen or gelatin, because of its high cystine content, and much further study is needed in order to elucidate the mechanism of the reactions involved.

6. ZEIN

Zein shows an interesting distinction from collagen, casein, or keratin in its reaction with F, since it contains no lysine and extremely little arginine (Cohn and Edsall, 1943, Chapter 15). Therefore, it cannot undergo

[25] Ratner and Clarke, in studies not included in their published paper, were unable to demonstrate the formation of acetyl thiazolidine carboxylic acid from the reaction of formaldehyde with acetyl cysteine. Not only could none be isolated, but the final rotation of the reaction mixture was observed to be -0.90° under conditions which would have given -2.95° if the acetyl thiazolidine derivative had been formed. The assumption that acylation of the nitrogen prevents thiazolidine formation rules out, as a reasonable hypothesis, the idea that thiazolidines can be formed from cysteine groups in which the nitrogen atom is in peptide linkage. The reaction would proceed only with terminal cysteine residues in which the NH_2 was free. (Personal communication from Professor H. T. Clarke.)

the same reactions that appear to underlie the tanning process in collagen or casein. Nevertheless, the action of F is capable of producing very marked increase in the strength of artificial films or fibers of zein. The study of the reaction is still in a relatively early stage, and much of the work has been carried out in unpublished studies in this Department.[26] In attempting to interpret the nature of the groups responsible for this reaction in zein, the large number of amide groups of the dicarboxylic acids is to be noted as a distinctive feature of its composition. Furthermore, the probability that these groups are involved in the reaction is strengthened by the fact that F has little action on zein in neutral or alkaline solutions, and shows its maximum effect on treatment in fairly acid solution near pH 4. This is comparable to the conditions found most favorable by Einhorn (1905, 1908) for the reaction of amides with F to form methylol derivatives and methylene diamides (Reactions 8 and 9). It would be premature to attempt today any final interpretation of the reactions involved, but there is fairly strong evidence that methylene bridges are formed, presumably between the nitrogen of a dicarboxylic acid amide group and that of a neighboring peptide linkage or perhaps of a second amide group; this reaction is the most important underlying process which increases the mechanical strength of zein films. Ammonium salts exert a marked accelerating effect on the reaction. The general character of the findings obtained in this laboratory is fully in accord with those recently mentioned in a brief note by Wormell and Kaye (1944).[27]

7. FORMATION OF TOXOIDS FROM BACTERIAL TOXINS

The action of dilute formaldehyde on solutions of soluble proteins leads to results which superficially, at least, are very different from the tanning reactions obtained with fibrous proteins. As far back as 1896, Blum observed that egg albumin, on treatment with dilute F, became resistant to heat coagulation, and a large number of similar scattered observations have been reported in the literature. These observations, however, have led to little theoretical study of the underlying mechanisms, but have led to important practical applications in the conversion of bacterial toxins to toxoids. The conditions for the most favorable course of the reaction have been determined by trial and error. Generally, the concentration of F employed is of the order of 0.12 to 0.16 per cent (0.3 to 0.4 per cent forma-

[26] These studies were carried out by C. C. Jensen, W. E. C. Yelland, C. R. Harmison, and Dexter French. Many other related studies on zein have been carried out at the laboratories of the Corn Products Refining Co., Argo, Illinois.

[27] The recent work of Fraenkel-Conrat, Cooper and Olcott (1945), discussed in the section on casein above, suggests that the terminal amide groups of asparagine and glutamine are much more reactive than the peptide groups, in F binding and methylene bridge formation. Wormell and Kaye (1945) express similar views.

lin).[28] The incubation is generally carried on at a temperature of 37 to 39 ° and over a period of several weeks. The reaction is therefore a very slow one and is essentially irreversible. Clearly, therefore, it cannot be comparable to the reaction of F with simple amino groups as in the formol titration, since the latter process is rapid and is readily reversible. No attempt will be made here to survey the earlier literature in this field. Careful and critical studies on preparations of diphtheria toxins that were still relatively impure were given by Hewitt (1930) and by Mudd and Joffe (1933), who give useful bibliographies of earlier work. The recent preparation of highly purified diphtheria toxins by Eaton (1937, 1938) and Pappenheimer (1938) has opened up the possibility of a far more precise study of the nature of the reaction. Most of the work on this problem remains for the future, but some significant observations have already been recorded by these authors. Eaton (1937) notes that much greater quantities of F are needed to convert toxin to toxoid in acid at pH 6 than in alkaline solutions near pH 9. In agreement with results of early workers, hexamethylenetetramine was found to produce unaltered toxoid from toxin at any pH value from 6 to 9. The conversion of toxin to toxoid involves no change in the ratio of protein nitrogen to the number of flocculating units (L_f) as determined by the reaction with diphtheria antitoxin. In alkaline solutions, the use of higher F concentrations than those needed to produce toxoid formation leads to marked impairment of the flocculating, combining, and immunizing properties of toxoid. The reaction is therefore very sensitive to conditions, but little is still known about the nature of the groups involved. Eaton finds that in the change from toxin to toxoid, about 30 per cent of the amino N of the toxin is slowly and irreversibly bound so that it can no longer be determined by the Van Slyke method. Pappenheimer (1938) similarly reports that 6.3 per cent of the nitrogen of toxin is free amino N, while in toxoid this has been reduced to from 2.8 to 3.3 per cent. The earlier studies of Hewitt (1930) on less highly purified toxin agree with these in showing that toxoid formation is complete when a large amount of free amino N still remains in the protein. The slow and irreversible nature of the disappearance of that amino N which is lost in the reaction suggests methylene bridge formation between amino groups and adjoining aromatic rings of tyrosine, tryptophan, or histidine residues. More exact interpretation of the nature of the reaction involves as a necessary preliminary an accurate knowledge of the amino acid composition of toxin.

[28] It should be noted that the statements in the literature on this point are frequently confusing. Many authors speak of F concentration when they actually mean formalin concentration. The true concentration of F is approximately 40 per cent of the concentration of formalin.

Diphtheria toxoid is much more stable to heat than is toxin and is less subject to irreversible alteration on precipitation in acid solution near pH 5 (Eaton, 1937). Toxoid precipitated by acetone at room temperature will readily redissolve and react with antitoxin as if it had been unchanged by the precipitation. Toxin, on the other hand, becomes almost completely insoluble after acetone precipitation under the same conditions, and the small residue that does redissolve no longer reacts with antitoxin as well as before.

If amino acids or peptones are present in the medium in which toxoid formation is occurring, the amount of F necessary to produce the reaction is increased, since some of the F is bound by the other substances in the medium which compete with the toxin for F. Wadsworth, Quigley, and Sickles (1937) have observed that a quantity of histidine, equivalent to the F present, almost completely inhibits toxoid formation. In view of the reaction of histidine with F already discussed in Part V, this inhibition is not surprising.

The reactions discussed in this section are drawn almost entirely from experiments with diphtheria toxins. The toxins of B. tetani and other bacteria presumably undergo reactions which are similar, but our knowledge of them is even more elementary than that of diphtheria toxin.

The effects of F on snake venoms are similar to those on toxins. Treatment at 38° for several days, at low F concentration, destroys or greatly reduces the toxicity of the venom, while leaving the antigenicity virtually unimpaired. The resulting products, frequently termed "anavenins," especially by the French workers in this field, are excellently adapted to the production of anti-venom on injection. For discussions and references in this field see Arthus (1930, 1931); Grasset and Zoutendyk (1933); Césari and Boquet (1939); Boquet and Vendrely (1943).

Few studies appear to have been carried out on plant toxins. Abrin (from Abrus precatorius) has been reported to become virtually non-toxic, while still remaining strongly and specifically antigenic, under conditions similar to those employed for the preparation of diphtheria toxoid (Ramon, 1925). On the other hand, the toxicity of ricin is not destroyed, although much diminished, by concentrations of F as high as 3.5 percent or even more, at temperatures of 38–50° C., acting over periods up to a month or more (Heymans, 1926). Moreover, ricin treated with F in this manner was found to have lost some of its immunological specificity as an antigen, in the process of reducing its toxicity.

8. TOBACCO MOSAIC VIRUS

A very thorough study of the inactivation of this virus by F was carried out by Ross and Stanley (1938). In most of their experiments they used

solutions containing approximately 2 per cent virus protein and 2 per cent F, the pH being held at 7 with phosphate buffer. They reported that a marked reactivation of virus activity could be obtained, after nearly all of the activity had been lost, if prolonged dialysis of the inactivated material at pH 3 was carried out. Reactivation under these circumstances (as measured by the infective power of the preparation for the tobacco plant) was never complete, but it was possible to increase the virus activity in many cases by a factor of 10 or more over that of the inactivated preparation before dialysis. Even preparations that were so completely inactivated that no infective power could be detected recovered an appreciable amount of virus activity on dialysis. The fully inactivated virus protein contains about 60 per cent of the amino N of the active protein. The rate of inactivation decreases with decreasing pH, being much lower at pH 6 than at 7. It was found that the amount of color obtainable when the virus protein is treated with ninhydrin or with Folin's phenol reagent was markedly reduced by the treatment with F which produced inactivation, and that definite increases in the amount of color obtained in both reactions were obtained after reactivation by dialysis. The reactions underlying the inactivation process, therefore, appeared to be at least partly reversible. Ross and Stanley inferred that the tryptophan residues in the virus protein might play an important part in the reaction. They carried out experiments with tryptophan, indole propionic acid, and glycyltryptophan, showing a marked effect of F on the capacity of these substances to react with Folin's phenol reagent, whereas no similar effects were observed with tyrosine or glycyltyrosine.

Kassanis and Kleczkowski (1944) were unable to find any reversal of F inactivation of virus activity, even after prolonged dialysis at pH 3; and they also found no completely regular parallelism between virus inactivation and changes in color produced with the phenol reagent. They suggest that the virus preparations employed by Ross and Stanley may have contained a virus inhibitor (other than F) which is removed by dialysis, giving apparent reactivation; or that the virus particles may have been reversibly aggregated and disaggregated under the conditions employed. Further investigation of these points is obviously desirable.

9. INFLUENZA AND OTHER VIRUSES

It has now been shown that many viruses, on treatment with F at low concentrations, lose most or all of their infectivity, while retaining full antigenicity, so that on injection the altered virus can produce a high degree of immunity to the unaltered virus. Such studies have been made with the viruses of typhus (Zinsser and Castaneda, 1931), equine encephalomyelitis (Beard, Beard and Finkelstein, 1939), malignant panleucopenia of cats

(Enders and Hammon, 1940) and recently with great success for influenza virus (Hirst, Rickard, Whitman and Horsfall, 1942; Stanley, 1945). The use of low F concentrations is very important; in the case of influenza virus, Stanley (1945) finds that the best results are obtained with concentrations of 0.01–0.10 per cent, in systems containing one to ten mg./cc. of virus material. Higher concentrations of F largely destroy antigenicity as well as virus activity, and render the material ineffective for immunization. The combination of F treatment, under carefully controlled conditions, with differential centrifugation, appears to have yielded concentrated preparations of modified influenza virus which can be used for large scale immunization of populations exposed to the disease.[29]

The studies discussed in this section represent only a small fraction of the vast number of reports on the action of F on various proteins. However, in the present fragmentary and preliminary state of our knowledge there would be little value in multiplying instances. The use of F as a reagent for modifying specific properties of proteins is only in its infancy. Certainly, it will be used very much in the future under more definitely controlled conditions and with a gradually increasing knowledge of the nature of the underlying processes involved in the reaction. Many of the reactions discussed in this paper are given by numerous other aldehydes as well. Formaldehyde, however, owing to the very compact structure of the molecule and its high reactivity, is a particularly versatile reagent with a vast range of possible reactions.

[29] Dr. John F. Enders has kindly supplied us with a number of references dealing with the formol treatment of viruses, in addition to those cited in the text. For the benefit of readers interested in this field, the other references are cited here.

Stuart-Harris, C. H., Andrewes, C. H., and Smith, W. (1938). *Med. Research Council, Special Report Series* No. 228.

Taylor, R. M., and Dreguss, M. (1940). *Am. J. Hyg.* **31**, 31–35.

Francis, T., Jr., Salk, J. E., Pearson, H. E., and Brown, P. N. (1944). Protective effect of vaccination against induced influenza A. *Proc. Soc. Exptl. Biol. Med.* **55**, 104–105.

Salk, J. E., Pearson, H. E., Brown, P. N., and Francis, T., Jr. (1944). Protective effect of vaccination against induced influenza B. *Proc. Soc. Exptl. Biol. Med.* **55**, 106–107.

Shahan, M. S., Giltner, L. T., and Schoening, H. W. (December, 1938). A review of the 1938 outbreak of infectious equine encephalomyelitis in the United States. *Proc. 42d Annual Meeting U. S. Live Stock Sanitary Assoc.*

Vaccination against influenza (August 15, 1944). *War Department Technical Bulletin, TB Med.* **85**.

Influenza vaccination program (October, 1944). *Bulletin of the U. S. Army Medical Department, No.* **81**, 25–26.

Enders, J. F., Kane, L. W., Cohen, S., and Levens, J. H. (1945). Immunity in Mumps. I. *J. Exp. Med.* **81**, 93.

REFERENCES

Arthus, M. (1930). *J. physiol. path. gén.* **28**, 529.

Arthus, M. (1931). *J. physiol. path. gén.* **29**, 256.

Auerbach. F., and Barschall, H. (1905). *Arb. kaiserl. Gesundh.* **22**, 584.

Balson, E. W., and Lawson, A. (1936). *Biochem. J.* **30**, 1257.

Baur, E. (1941). *Helv. Chim. Acta* **24**, 1018.

Bear, R. S. (1944). *J. Am. Chem. Soc.* **66**, 1297.

Beard, J. W., Beard, D., and Finkelstein. H. (1939). *Science* **90**, 215.

Bergmann, M., Jacobsohn, M., and Schotte, H. (1923). *Z. physiol. Chem.* **131**, 18.

Bergmann, M., and Miekeley, A. (1924). *Ber.* **57**, 662.

Bergmann, M., and Ensslin, H. (1925). *Z. physiol. Chem.* **145**, 194.

Bergmann, M., and Zervas, L. (1926). *Z. physiol. Chem.* **152**, 282.

Best, C. H., and McHenry, E. W. (1930). *J. Physiol.* **70**, 3.9.

Bielecki. and Henri, V. (1912). *Ber.* **45**, 2821.

Birch, T. W., and Harris, L. J. (1930a). *Biochem. J.* **24**, 564.

Birch, T. W., and Harris, L. J. (1930b). *Biochem. J.* **24**, 1080.

Blum, F. (1897). *Z. physiol. Chem.* **22**, 127.

Boquet, P., and Vendrely, R. (1943). *Compt. rend. soc. biol.* **137**, 179. See *Chem. Abst.* (1944) **38**, 2053.

Borsook, H., and Dubnoff, J. W. (1939). *J. Biol. Chem.* **131**, 163.

Bowes, J. H., and Pleass, W. B. (1939). *J. Intern. Soc. Leather Trades' Chem.* **23**, 365. 451, 453.

Cannan, R. K. (1942). *Chem. Revs.* **30**, 395.

Cannan, R. K., Kibrick, A. C., and Palmer, A. H. (1941). *Ann. N. Y. Acad. Sci.* **41**, 243.

Cannan, R. K., Palmer, A. H., and Kibrick, A. C. (1942). *J. Biol. Chem.* **142**, 803.

Carpenter, D. C., and Lovelace, F. E. (1942). *J. Am. Chem. Soc.* **64**, 2899.

Carpenter, D. C., and Lovelace, F. E. (1943). *J. Am. Chem. Soc.* **65**, 1181.

Césari, E., and Boquet, P. (1939). *Compt. rend. soc. biol.* **130**, 19.

Cherbuliez, E., and Stavritch, K. N. (1922). *Helv. Chim. Acta* **5**, 267.

Cherbuliez, E., and Feer, E. (1922). *Helv. Chim. Acta* **5**, 678.

Clarke, H. T., Gillespie, H. B., and Weisshaus, S. J. (1933). *J. Am. Chem. Soc.* **55**, 4571.

Cohn. E. J., and Edsall, J. T. (1943). "Proteins, Amino Acids, and Peptides." Reinhold Pub. Corp., New York.

Dickinson, R. G., and Raymond, A. L. (1923). *J. Am. Chem. Soc.* **45**, 22.

Dubuisson, M. (1941). *Arch. intern. physiol.* **51**, 133, 154. (See *Chem. Abst.* (1943) **37**, 3111.)

Duden, P., and Scharff, M. (1895). *Ann.* **288**, 218.

Dunn, M. S., and Loshakoff, A. (1936a). *J. Biol. Chem.* **113**, 359.

Dunn, M. S., and Loshakoff, A. (1936b). *J. Biol. Chem.* **113**, 691.

Dunn, M. S., and Weiner, J. G. (1937). *J. Biol. Chem.* **117**, 381.

Dybing, O. (1936). Konigl. Tierarztliche and Landwirtsch. Hochschule (Copenhagen), Jahrbuch, p. 49.

Eaton, M. D. (1937). *J. Immunol.* **33**, 419.

Eaton, M. D. (1938). *Bact. Revs.* **2**, 3.

Einhorn, A., and others (1905). *Ann.* **343**, 207–310.

Einhorn, A. (1908). *Ann.* **361**, 113.

Einhorn, A., and Prettner, A. (1904). *Ann.* **334**, 210.

Enders, J. F., and Hammon, W. D. (1940). *Proc. Soc. Exptl. Biol. Med.* **43**, 194.

Ewald, A. (1919). *Z. physiol. Chem.* **105**, 135.

Fraenkel-Conrat, H., Cooper, M., and Olcott, H. S. (1945). *J. Am. Chem. Soc.* **67**, 950.
Franzen, H., and Fellmer, E. (1917). *J. prakt. Chem.* **95**, 299.
French, D. (1945a). Unpublished measurements.
French, D. (1945b). Unpublished measurements.
Frieden, E. H. (1942). Ph.D. Thesis, Univ. of California, Los Angeles.
Frieden, E. H., Dunn, M. S., and Coryell, C. D. (1942). *J. Phys. Chem.* **46**, 215.
Frieden, E. H., Dunn, M. S., and Coryell, C. D. (1943a). *J. Phys. Chem.* **47**, 10.
Frieden, E. H., Dunn, M. S., and Coryell, C. D. (1943b). *J. Phys. Chem.* **47**, 20.
Frieden, E. H., Dunn, M. S., and Coryell, C. D. (1943c). *J. Phys. Chem.* **47**, 85.
Frieden, E. H., Dunn, M. S., and Coryell, C. D. (1943d). *J. Phys. Chem.* **47**, 118.
Grasset, E., and Zoutendyk, A. (1933). *Brit. J. Exptl. Path.* **14**, 308.
Gustavson, K. H. (1943). *Kolloid-Z.* **103**, 43.
Hahn, G., Bärwald, L., Schales, O., and Werner, H. (1935). *Ann.* **520**, 107.
Hegman, R. L. (1942). *J. Am. Leather Chem. Assoc.* **37**, 276.
Hewitt, L. F. (1930). *Biochem. J.* **24**, 983.
Heymans, M. (1926). *Arch. Internat. Pharm. et Thérap.* **32**, 101.
Hibben, J. H. (1931). *J. Am. Chem. Soc.* **53**, 2418.
Highberger, J. H., and O'Flaherty, F. (1939). *J. Intern. Soc. Leather Trades' Chem.* **23**, 549.
Highberger, J. H., and Retzsch, C. E. (1938). *J. Am. Leather Chem. Assoc.* **33**, 341.
Highberger, J. H., and Retzsch, E. W. (1939). *J. Am. Leather Chem. Assoc.* **34**, 131.
Hirst, G. K., Rickard, E. R., Whitman, L., and Horsfall, F. L., Jr. (1942). *J. Exptl. Med.* **75**, 495.
Hobohm, K. O. (1944). *Biochem. Z.* **316**, 202.
Holden, H. F., and Freeman, M. (1931). *Australian J. Exptl. Biol. Med. Sci.* **8**, 189.
Homer, A. (1912). *Biochem. J.* **7**, 101.
Jacobs, W. A., and Craig, L. C. (1936). *J. Biol. Chem.* **113**, 759.
Kahovec, L. (1939). *Z. Physik. Chem.* (B) **43**, 364.
Kassanis, B., and Kleczkowski, A. (1944). *Biochem. J.* **38**, 20.
Kauzmann, W., and Eyring, H. (1941). *J. Chem. Phys.* **9**, 41.
Kauzmann, W., Walter, J. E., and Eyring, H. (1940). *Chem. Revs.* **26**, 339.
Kekwick, R. A., and Cannan, R. K. (1936). *Biochem. J.* **30**, 235.
Knorr, L., and Matthes, H. (1901). *Ber.* **34**, 3484.
Kohlrausch, K. W. F., and Köppl, F. (1934). *Z. physik. Chem.* **24B**, 370.
Krishnamurti, P. (1931). *Indian J. Phys.* **6**, 309.
Küntzel, A. (1941). *Kolloid-Z.* **96**, 273.
Küntzel, A., and Prakke, F. (1933). *Biochem. Z.* **267**, 243.
Levy, M. (1933). *J. Biol. Chem.* **99**, 767.
Levy, M. (1934). *J. Biol. Chem.* **105**, 157.
Levy, M. (1935a). *J. Biol. Chem.* **109**, 361.
Levy, M. (1935b). *J. Biol. Chem.* **109**, 365.
Levy, M., and Silberman, D. E. (1937). *J. Biol. Chem.* **118**, 723.
Lichtenstein, I. (1940). *Biochem. Z.* **303**, 20.
Lillevik, H. A., and Sandstrom, W. M. (1941). *J. Am. Chem. Soc.* **63**, 1028.
Löb, W. (1913). *Biochem. Z.* **51**, 116.
Martin, A. J. P., and Synge, R. L. M. (1941). *Biochem. J.* **35**, 294.
Meyer, K. H. (1929). *Biochem. Z.* **208**, 23.
Middlebrook, W. R., and Phillips, H. (1942). *Biochem. J.* **36**, 294.
Mudd, S., and Joffe, E. W. (1933). *J. Gen. Physiol.* **16**, 947.
Neuberger, A. (1944). *Biochem. J.* **38**, 309.

Nicolet, B. H., and Shinn, L. A. (1941). *J. Biol. Chem.* **139**, 687.
Nitschmann, H., and Hadorn, H. (1941). *Helv. Chim. Acta* **24**, 237.
Nitschmann, H., Hadorn, H., and Lauener, H. (1943). *Helv. Chim. Acta* **26**, 1069.
Nitschmann, H., and Hadorn, H. (1943a). *Helv. Chim. Acta* **26**, 1075.
Nitschmann, H., and Hadorn, H. (1943b). *Helv. Chim. Acta* **26**, 1084.
Nitschmann, H., and Hadorn, H. (1944). *Helv. Chim. Acta* **27**, 299.
Pappenheimer, A. M., Jr. (1938). *J. Biol. Chem.* **125**, 201.
Pictet, A., and Spengler, T. (1911). *Ber.* **44**, 2030.
Ramon, G. (1925), *Ann. Inst. Pasteur* **39**, 1.
Ratner, S., and Clarke, H. T. (1937). *J. Am. Chem. Soc.* **59**, 200.
Ross, A. F., and Stanley, W. M. (1938). *J. Gen. Physiol.* **22**, 165.
Ryklan, L. R., and Schmidt, C. L. A. (1944). *Arch. Biochem.* **5**, 89.
Salcedo, J. S., and Highberger, J. H. (1941). *J. Am. Leather Chem. Assoc.* **36**, 271.
Schiff, H. (1900). *Ann.* **310**, 25.
Schiff, H. (1901). *Ann.* **319**, 53.
Schmitt, F. O. (1944). *Advances in Protein Chemistry* **1**, 25.
Schubert, M. P. (1935). *J. Biol. Chem.* **111**, 671.
Schubert, M. P. (1936). *J. Biol. Chem.* **114**, 341.
Sisco, R. C., Cunningham, B., and Kirk, P. L. (1941). *J. Biol. Chem.* **139**, 1.
Small, L. F. (1943). *in* Gilman, H. (editor) "Organic Chemistry," Vol. 2, Chapter **15**, John Wiley and Co., New York.
Smith, A. K., Handler, P., and Mrgudich, J. N. (1940). *J. Phys. Chem.* **44**, 874.
Sörensen, S. P. L. (1908). *Biochem. Z.* **7**, 45.
Sprung, M. M. (1940). *Chem. Revs.* **26**, 297.
Stanley, W. M. (1940). *Ann. Rev. Biochem.* **9**, 545.
Stanley, W. M. (1945). *J. Exptl. Med.* **81**, 193.
Staudinger, H. (1932). "Die Hochmolekularen Organischen Verbindungen." Julius Springer, Berlin.
Stoves, J. L. (1944). *Nature* **154**, 272.
Svehla, J. (1923). *Ber.* **56**, 331.
Theis, E. R. (1944). *J. Biol. Chem.* **154**, 87.
Theis, E. R. (1945). *J. Biol. Chem.* **157**, 7, 15.
Theis, E. R., and Jacoby, T. F. (1942). *J. Biol. Chem.* **146**, 163.
Theis, E. R., and Jacoby, T. F. (1943). *J. Biol. Chem.* **148**, 105.
Titherly, A. W., and Branch, G. E. K. (1913). *J. Chem. Soc.* **103**, 330.
Tomiyama, T. (1935). *J. Biol. Chem.* **111**, 51.
Traube, J. (1899). *Samml. chem. chem. tech. vortraege* **4**, 255.
Van Slyke, D. D., and Kirk, E. (1933). *J. Biol. Chem.* **102**, 651.
Van Veen, A. G., and Hyman, A. J. (1935). *Rec. trav. chim.* **54**, 493.
du Vigneaud, V., and Patterson, W. I. (1936). *J. Biol. Chem.* **114**, 533.
Wadano, M. (1934). *Ber.* **67**, 191.
Wadano, M., Trogus, C., and Hess, K. (1934). *Ber.* **67**, 174.
Wadsworth, A., and Pangborn, M. C. (1936). *J. Biol. Chem.* **116**, 423.
Wadsworth, A., Quigley, J. J., and Sickles, A. R. (1937). *J. Infectious Diseases* **61**, 237.
Walker, J. F. (1944). "Formaldehyde" 393 pp. Reinhold Pub. Corp., New York.
Wellisch, J. (1913). *Biochem. Z.* **49**, 173.
Wormell, R. L., and Kaye, M. A. G. (1944). *Nature* **153**, 525.
Wormell, R. L., and Kaye, M. A. G. (1945). *J. Soc. Chem. Ind.* **64**, 75.
Wyckoff, R. W. G., and Corey, R. B. (1934). *Z. Krist.* **89**, 462.
Zeleny, L., and Gortner, R. A., (1931). *J. Biol. Chem.* **90**, 427.
Zinsser, H., and Castaneda, M. R. (1931). *J. Exptl. Med.* **53**, 325.

Wheat Gluten

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CONTENTS

I. INTRODUCTION

The exclusiveness of wheat as a bread making cereal is accounted for by the special and distinctive characteristics of a protein substance that is intermixed with the starchy endosperm of the grain. It is only by virtue of the unique properties of this protein material that the carbon dioxide produced during dough fermentation is retained by the dough in a manner which provides the familiar porous and spongy structure of bread. This substance, recognized as the "gluten protein" of the wheat, can be readily and conveniently separated from the bulk of the wheat starch; the gluten, itself, being recovered as a coherent, extensible, and rubbery mass, merely by the thorough kneading (or similar physical manipulation) of flour dough under a stream of water. As isolated by this "washing-out" procedure, gluten has an average water content of about 65%, while its dry substance usually contains 75–80% protein, 5–15% residual carbohydrates (chiefly starch residues), 5–10% lipids, and a small quantity of mineral salts. Due to the presence of these substantial quantities of starch, fat, etc., which cannot be completely removed by the conventional washing procedure, the term "crude gluten" is commonly applied to the recovered proteinaceous material. By the use of exceedingly vigorous and prolonged mechani-

cal treatment during the washing operation crude glutens containing
85–90% protein (dry matter basis) can be obtained.

The protein substance of corn, as separated and recovered in the produc-
tion of corn starch, is also designated as "gluten." In contrast with wheat
gluten, corn gluten is a granular substance lacking in coherence, elasticity,
and extensibility.

Since wheat gluten exclusively provides the structural framework of
bread and of other baked food products its importance to civilization
equals that of bread itself. It might reasonably be expected that the
higher the gluten *content* of flour the better will be its baking value. This
does not always follow, however, because of variations in the physical and
mechanical character of glutens from different wheat types and varieties.
The nature and magnitude of these variations constitute the basis for the
most perplexing problems encountered in the broad field of cereal tech-
nology. Realization of this has been the factor chiefly responsible for
numerous and persistent scientific investigations of the fundamental struc-
ture, properties, and behavior of gluten. These researches have extended
over many decades and are still in progress.

II. HISTORICAL

The first recorded separation of gluten from the starch of flour by the
time-honored "washing-out" procedure was accomplished by Beccari, 1728,
and reported in 1745 (Beccari, 1745). Because of his observations and
conclusions as to the properties and behavior of gluten, Beccari is con-
sidered to have been among the first to recognize in plant material the
occurrence of a substance having characteristics in common with those of
animal protein.

In 1805, Einhof recorded the presence of an alcohol-soluble protein in
flour, and he considered this to be identical with Beccari's gluten (Einhof,
1805). Taddei (1820) announced his belief that gluten consists of two
distinct protein substances, and he named the alcohol-soluble component
"gliadin." During the next half century a number of workers became
interested in gluten protein, prominent among them being de Saussure,
Berzelius, Boussingault, Liebig, Dumas, and especially Ritthausen (1872).
In the aggregate, the published reports of these and of other contemporary
workers are characterized by confusion in terminology and by a lack of
agreement as to the number and nature of the individual components of
gluten.

A complete literature survey of the earlier studies on gluten protein was
prepared by Osborne (1907). For a more detailed account of the individual
researches cited by Osborne (1907) the recent book by Bailey (1944) is
recommended.

III. OSBORNE'S CHARACTERIZATION OF GLUTEN

It is appropriate to recognize, as is emphasized by Bailey (1944), that the "modern period" of gluten protein investigations begins with the comprehensive and systematic studies of Osborne and his associates. The results of these studies, which extended over a period of about 15 years, have been brought together in one inclusive report (Osborne, 1907).

Osborne's chief endeavor was to identify and characterize the protein constituents of wheat flour on the basis of solubility characteristics in conjunction with painstaking analyses to determine elementary and amino acid compositions of purified protein fractions successively and selectively extracted by appropriate solvents.

Osborne's essential conclusions are that gluten protein normally constitutes more than 80% of the total wheat flour protein, and that the actual protein substance of gluten is an intimate mixture of two distinct individual proteins, glutenin and gliadin,[1] present in nearly equal amounts. He found, moreover, that gluten, as prepared by the conventional "washing-out" procedure, contains, in addition to its 64–67% water of hydration, "more or less starch which cannot be wholly removed by washing, and also some fat, cholesterin, and lecithin. . . . The glutenin probably forms the nucleus to which the gliadin adheres, and this binds the gluten proteins into one coherent elastic mass." Incidentally, the *non-gluten* proteins of wheat flour were stated by Osborne to consist of an albumin (leucosin), a globulin (edestin), and a small quantity of proteose. These "soluble" proteins normally account for one-fifth to one-sixth of the total flour protein. Presumably they are not essential gluten components, and although they are substantially eliminated during the gluten-washing process, their removal is by no means complete.

The gross compositions of two samples of dry crude gluten, as reported by Dill (1925) are shown in Table I.

TABLE I

Composition of Dry Crude Gluten

	Gluten A (Soft Wheat)	Gluten B (Commercial Product)
	per cent	*per cent*
Protein (N×5.7)	72.7	81.0
Lipids (neutral extraction method)	7.1	11.6
Ash	0.6	0.9
Carbohydrates	18.8	4.9

[1] For an extensive discussion of various methods that have been proposed for the preparation and quantitative estimation of the gluten proteins, see Bailey's (1944) recent book.

Other analyses of samples of gluten might be cited here, but the values in Table I clearly show that crude glutens exhibit a wide range of variation as to content of total protein, carbohydrate, ash, and lipids, respectively. Differences in gross composition may be due to the type of wheat, or to the grade of flour, or both.

Numerous analyses intended to measure the gliadin content of gluten, based upon its extraction with alcohol, have been reported, and some of the reports indicate large variations in ratio of gliadin to glutenin, while others suggest that the range of variation is actually very small. It has become abundantly evident, however, that the conflicting opinions, interpretations, and conclusions are largely attributable to differences in the analytical methods employed (see Bailey, 1944).

In the light of relatively recent studies, and of modern criteria of protein homogeneity, the validity of Osborne's characterization of gluten as an intimate mixture of only two "individual" proteins is no longer acceptable, for reasons to be discussed later. Evidence will be cited in strong support of the view that gluten is composed of several, if not many, protein components, and also that Osborne's "glutenin" is a *derived*, not a *natural* protein. Until the true character of gluten is eventually disclosed, however, it is convenient and appropriate, for purposes of identification and discussion, to retain the terms *glutenin* and *gliadin* in referring to the later researches which have been based largely upon the distinguished work of Osborne. Of the two protein types, much more is known of the character and properties of gliadin — soluble in dilute alcohol and dilute acids — than of glutenin, which is far more insoluble than gliadin, and certainly more indefinite as to fundamental composition and properties.

IV. PHYSICAL PROPERTIES AND BEHAVIOR

When gluten is prepared from flour dough by the usual washing process, both the amount and the nature of the product will be influenced by a number of individual factors including the character of the flour itself or of the wheat from which it was milled. Not only do flours of higher total protein content yield the larger quantities of gluten, as expected, but also the ratio of gluten to non-gluten protein is usually larger as the total flour protein content increases. With flours of very low protein content, or of inferior grade, or both, it is frequently difficult to effect proper agglomeration of the gluten particles, and in such instances low or negligible yields of gluten are obtained unless special precautions and very careful handling are used.

Assuming a flour of satisfactory gluten content and character, several factors are known substantially to affect not only the yield of gluten but also its protein content and the percentage of total flour nitrogen recovered.

Soft water solublizes more gluten protein than hard water and gives a "weaker" gluten. Dill and Alsberg (1924) recommended for gluten-washing the use of a dilute sodium phosphate solution adjusted to pH 6.8, while various other workers have also used dilute salt solutions. Fisher and Halton (1936) propose a 0.1% NaCl solution in cases where sufficient hardness of the tap water is lacking. Other factors (see Fisher and Halton, 1936) include temperature of water, length of the "rest period" between preparation of the dough and the washing process, and personal peculiarities of the operator.

Familiarly known to milling and baking technologists is the fact that glutens washed from flours of different origin may exhibit conspicious variations in their physical properties, even though the flours are of equal grade and protein content. Merely a casual inspection will reveal pronounced differences in properties such as toughness, coherence, brittleness, elasticity, pliability, extensibility, etc. These properties, usually denoted as gluten "quality" manifestations, are considered to be related more specifically to varietal and genetic factors than to the soil and climate conditions under which the wheat was grown. To an experienced observer some of these physical gluten properties can be made to serve as useful criteria of bread making performance.

A variety of mechanical devices, intended for measuring and recording in numerical values the various physical characteristics (plasticity, extensibility, elasticity, etc.) of wet crude gluten, have been employed by different workers. Most of these contrivances have been described and discussed by Bailey (1940).

Water of hydration usually accounts for about two-thirds of the weight of wet crude gluten. Some variation in water content has been noted, however, and a high water absorbing capacity is likely to be associated with desirable physical and mechanical properties. When gluten is dehydrated it may or may not be reconstituted without loss of original physical properties, depending upon the temperature at which the drying operation was performed.

Gluten dried rapidly in a high vacuum, and without being subjected to elevated temperatures while wet, does not lose its hydration capacity and can be reconstituted without apparent change in original physical and colloidal properties. As the drying temperature of the wet gluten is gradually increased, however, there is a progressive loss of hydration capacity and of original "colloidal" properties including dispersibility in solvents. Sharp and Gortner (1922) noted a pronounced decrease in hydration capacity after drying at 45–50° C. Sharp and Gortner (1923) also presented conclusive evidence that the glutenin fraction is far more sensitive to heat "denaturation" than the gliadin portion.

Blish and Sandstedt (1926) found that the dehydration of gluten by prolonged exposure to 60–65° C. in a partial vacuum resulted in an almost complete denaturation of glutenin without significant effect on gliadin, and they made this operation the basis of a convenient laboratory method for the preparation of gliadin in quantity and in a high degree of purity.

Olcott and Blish (1944), using an arbitrarily chosen analytical method based upon changes in gluten solubility in a mixture of dilute acetic acid and alcohol, studied the comparative denaturing effects of various heat treatments, with results as shown in Table II.

TABLE II

Heat Denaturation of Gluten

Heat Treatment	"Soluble" N in Dried Gluten[1]
	per cent
Vacuum dried at 45° C.[2]	75
Vacuum dried at 100° C.[2]	75
Commercial "A," vacuum dried	65
Commercial "B," vacuum dried	53
Commercial "C," oven dried, no vacuum	23
Dried after one minute in boiling water	50
Dried after ten minutes in boiling water	33
Dried after thirty minutes in boiling water	10

[1] Method: 1 g. gluten, 5 ml. 95 per cent ethanol, 25 ml. 0.1 N acetic acid; let stand over night, filtered, and N determined in filtrate.

[2] These are *oven* temperatures, but under the high vacuum employed the gluten itself presumably remained at a temperature not above the low boiling point of water, until completely dry.

From the foregoing data (Table II) it is evident that gluten is rapidly denatured by drying at atmospheric pressure, and especially so when boiled in water.

Exposure of gluten protein to alcohol, in the presence of a significant quantity of water, effects a denaturation of glutenin that corresponds closely to that produced by heat. Kosutany (1903) found that the mere addition of alcohol to flour, followed by its removal by evaporation, caused a pronounced loss, both in yield and quality, of gluten recoverable. Sharp and Gortner (1923) and Sandstedt and Blish (1933) presented clear evidence of glutenin denaturation caused merely by exposure to alcohol.

It is noteworthy that although the manifestations of gluten "denaturation" closely resemble those that are characteristic of protein denaturation in general, there is at least one important respect in which gluten denaturation differs from the denaturation of globular proteins, such as hemoglobin and egg albumin. This is shown by the fact that various detergents known to denature most globular proteins do not correspondingly denature

gluten protein. Applying the currently accepted belief that the denaturation of globular proteins by detergents involves a molecular "unfolding" to give a random structure and an increased asymmetry, it does not appear that the gluten protein molecules undergo changes of this character. Accordingly, whereas hemoglobin is readily denatured by sodium salicylate (Anson and Mirsky, 1929) gluten protein can be alternately dispersed by, and salted out of sodium salicylate solution without significant change in original properties. The explanation probably has to do with difference in the molecular structure of the native proteins. Thus the asymmetry of hydrated gliadin, calculated by Neurath (1939), is 7.3, as compared to 1.5 for lactalbumin, suggesting that the gluten protein molecules are elongated or rodlike rather than essentially spherical.

It was previously stated that the physical properties of gluten can be influenced by the presence or absence of salts dissolved in the water used for separating it from starch by the usual washing procedure. Wood and Hardy (1908) were the first to undertake systematic studies of the colloidal properties and behavior of gluten, particularly as affected by relatively small quantities of acids, bases, and salts of different kinds and concentrations. It was observed that when gluten was suspended in some solutions of these substances it became rapidly hydrated to the point of almost complete dispersion, while in others the effect was to increase its coherence and toughness. In 0.0001 N HCl it became gradually dispersed, the rate of dispersion increasing with higher HCl concentrations up to 0.033N, beyond which the rate diminished until at about 0.08 N its original coherence was restored. With weaker acids, i.e., phosphoric acid and oxalic acid, dispersion was maintained over higher ranges of acid concentration. The addition of salts to dispersions in acid caused rapid coagulation of the gluten with restoration of original properties. Upson and Calvin (1915, 1916) quantitatively studied water imbibition of gluten as affected by acids, bases, and salts, and their findings substantiated those of Wood and Hardy. Upson and Calvin suggested that gluten "quality," as reflected in bread making value, is governed more by the kinds and amounts of acids and salts in the dough than by differences in chemical composition or in the relative quantities of the protein components of the gluten itself.

Many later investigations dealing with the effects of electrolytes and of hydrogen ion activity on gluten properties have been recorded, and in most of them the endeavor has been to disclose the underlying causes of differences in flour baking characteristics. A comprehensive, detailed review of the more significant of these studies has been presented by Sharp and Gortner (1923). They reported that glutens are decidedly variable in their imbibitional powers, that "strong" (in baking properties) flours are more nearly perfect colloidal gels — as evidenced by their greater hydration

capacities — than "weak" flours, and that these differences can be meas-
ured by properly performed viscosity determinations on acidified (lactic
acid) flour-water suspensions. They found that all acids produced maxi-
mum viscosity, with all flours, at a pH slightly above 3, but that different
acids do not produce their maximum effects to the same degree. "Salts . . .
divide themselves into sharply defined groups in respect to their ability to
inhibit imbibition produced by lactic acid . . . the maximum viscosity
produced by alkalies occurs at approximately a pH of 11. . . . Glutenin
is the protein mainly responsible for the marked imbibitional power of
flour and gluten."

The evidence that the *glutenin* is the all-important contributor to the
unique colloidal properties of gluten is highly significant when it is recalled
that, as compared to gliadin, glutenin is by far the more easily denatured by
heat and by alcohol. Gliadin presumably functions chiefly as a binding
agent or adhesive for the more labile glutenin particles or aggregates.

For a discussion of various data concerning isoelectric points, optical
rotation values, and refractive indices of gluten protein preparations, the
reader is referred to Bailey's (1944) book.

V. SOLUBILITY BEHAVIOR OF GLUTEN

The solubility characteristics of gluten present a serious stumblingblock
to anyone who wishes to study its fundamental properties by conventional
procedures. No solvent has been reported which will dissolve all of the
gluten protein so completely that it can be entirely dissociated from all non-
protein "impurities," either by filtration or by selective precipitation or
both, and recovered with all of its original properties intact.

It is of course possible to extract and isolate *gliadin* fractions by taking
advantage of its characteristic solubility in dilute (50–70%) alcohol.
Gliadin preparations thus isolated can be rendered free from starch and
lipids, and can be recovered in dry form without apparent alteration in
properties. Attempts to crystallize gliadin have not yet been successful.

After the removal of gliadin, insofar as possible, by repeated alcohol
extractions, the glutenin residue, which has been largely denatured during
exposure to the alcohol, is strongly resistant to nearly all solvents other
than caustic alkali. Osborne (1907) employed dilute KOH for dissolving
the glutenin from this residue, leaving the starch in suspension, from which
it could be eliminated by filtration of the alkaline solution through a thick
layer of paper pulp. The glutenin was precipitated by neutralization with
acid. For further purification it could be extracted with alcohol and ether,
then redissolved in dilute alkali and reprecipitated by neutralization with
acid.

Blish and Sandstedt (1929) found that "glutenin," when prepared by

methods employing dilute alkali as the dispersing agent, varies in chemical composition in accordance with the concentration of alkali used. They also showed that any glutenin thus prepared has undergone irreversible changes in its original physical properties (coherence, hydration), and that it should accordingly be regarded as a *derived* rather than a *native* protein. These findings are in conformity with those of Csonka and Horn (1931), in their study of the effect of alkalies on gluten proteins.

When freshly prepared wet gluten is exposed to the action of dilute acetic acid it becomes progressively and increasingly hydrated until it eventually disintegrates into a uniform, although extremely cloudy, "dispersion." Filtration of the dispersion is impossible, for the filter very quickly becomes clogged, due to the size and gelatinous character of the larger protein particles, or aggregates. By the addition of a neutral salt to such a dispersion, or by neutralization with alkali, the gluten can be rapidly coagulated and recovered without any apparent change in original properties, although after the dispersion has stood for a number of hours at room temperature a measurable (though slight) degradation, due to enzyme action, is detectable (Cook and Rose, 1935). Olcott, Sapirstein, and Blish (1943) found that this enzyme action can be prevented, and the dispersion stabilized without detectable alteration of original properties, by heating the acid dispersion for a few minutes at 95–100° C. The resistance of glutenin to heat denaturation, when in dilute acid dispersion, is striking, in contrast to its extreme lability to heat when in contact with water alone.

When a considerable volume of alcohol is added to a gluten-acetic acid dispersion (Blish and Sandstedt, 1929; Sandstedt and Blish, 1933; Blish, 1936) such a dispersion can be made a convenient starting point for the precipitation of "fractions" by adding salts of various kinds and amounts, at various temperatures, as will later be discussed in some detail. Starch can be satisfactorily separated from acid-alcohol dispersions by means of a Sharples supercentrifuge, at 40,000 r.p.m., but a substantial portion of the protein (12–14%) is also usually thrown out along with the starch.

Mangels and Martin (1935) found gluten to be readily dispersible in lactic, propionic, tartaric, citric, and oxalic acids, respectively. When dispersed in these acids the behavior of the gluten resembled its behavior in acetic acid, and over the range of 0.1 N to 2.0 N more protein was dispersed in the lower than in the higher acid concentrations.

Cook and Alsberg (1931) reported that gluten cain be dispersed in 30% urea. Cook and Rose (1934, 1935) discovered that it can almost be completely dissolved in 8–10% sodium salicylate solution, and that in such solution it does not undergo perceptible hydrolytic change on long standing. McCalla and Rose (1935) showed that gluten can be salted out of sodium salicylate dispersion without apparent loss of original physical properties,

and that a series of "fractions" can be progressively precipitated by adding successive increments of $MgSO_4$. Fractionation can also be accomplished by diluting sodium salicylate dispersions with water, alcohol and acetone (Blish, 1936). Sodium salicylate dispersion offers noteworthy advantages for purposes of gluten fractionation, but unfortunately most of the associated starch and lipids are dispersed along with the gluten, thereby preventing the isolation of protein fractions entirely uncontaminated with foreign substances.

VI. The "Individual" Protein Components of Gluten

The inadequacy of Osborne's characterization of gluten as a simple mixture of two individual proteins has been made clearly evident in the light of accumulated observations and data obtained in large part by the use of comparatively recent and specialized techniques, instruments, and criteria that were not available to Osborne. Among these are the ultracentrifuge, the electrophoresis procedure of Tiselius, and the diffusion measurement technique of Lamm. Additional evidence has been furnished by fractionation procedures with examinations of the properties of individual fractions.

The "glutenin" portion of the gluten protein has not satisfactorily lent itself to examination by ultracentrifugal, electrophoretic, or diffusion methods, largely because of failure thus far to find a suitable solvent in which it can be dispersed or "disàggregated" in a manner suitable for the requirements at hand. Studies involving these and related techniques have accordingly been restricted to the more soluble "gliadin" fractions, and even in these instances there is frequently good reason for doubt as to whether the protein is molecularly dispersed.

In all cases where solutions of gliadin preparations have been examined, as to molecular properties, by highly refined physical methods, it has been concluded that the preparations are inhomogeneous. Burk (1938) reached this conclusion from molecular weight determinations of gliadin by means of osmotic pressure measurements in several organic solvents. His values varied from 40,000 to 75,000, depending on the solvent used. Krejci and Svedberg (1935) reported evidence, from ultracentrifugal studies, of a mixture of whole- and half-molecules in a gliadin solution, stating that the former dissociated into the latter with increased temperature and acidity. The "whole" molecules showed a molecular weight of 34,500, the half-molecules 17,250.

Lamm and Polson (1936) found gliadin to be inhomogeneous, as shown[1] by differences in diffusion constants of several fractions, although the most soluble fraction appeared to be homogeneous. Schwert, Putnam, and Briggs (1944), using the moving-boundry electrophoretic method of Tiselius, found gliadin to be electrophoretically inhomogeneous. They were unable

to estimate the number of components because the components apparently did not migrate as independent entities, but rather as complexes resulting from component interaction.

McCalla and Gralen (1940, 1942) undertook an investigation of the molecular characteristics of gluten in sodium salicylate solution, using methods of sedimentation (ultracentrifuge) and diffusion. As the sodium salicylate concentration was increased there was a corresponding increase in molecularly dispersed protein, a maximum of about 25% of the protein being regarded as molecularly dispersed in a 12% solution of salicylate. In the molecularly dispersed fractions sedimentation constants increased slightly as solubility decreased, while diffusion constants decreased accordingly. All fractions contained aggregates of varying size. A negative correlation between length and solubility of molecules was indicated. It was concluded that gluten is a protein system of innumerable components which vary progressively in solubility and other physical properties, and that it is therefore incorrect to regard it as being composed of 2 or even 3 or 4 individual components. The estimated minimal molecular weight of the most soluble fraction was 35,000.

It has been found possible to isolate a large number and variety of "fractions" from gluten protein by any of several procedures, none of which involve dispersion in alkali. One of the simplest methods (Blish, 1936) is to add increments of very small quantities of salt to a gluten dispersion in dilute acetic acid. Factors influencing the amount of protein precipitated are, respectively, pH, quantity of salt, protein concentration, and time.

Series of protein fractions can be successively precipitated from a dispersion of gluten in acetic acid dispersion to which approximately an equal volume of alcohol has been added. When ethyl alcohol is used, the addition of NaCl causes little or no precipitation at room temperature, but fractional precipitation occurs as the temperature is progressively lowered. When methanol, instead of ethanol is used, in conjunction with added salt, fractional precipitation starts at higher temperature. According to Blish and Sandstedt (1933): "There is no established limit as to minor variations in the number, quantities, and character, respectively, of protein fractions that can be isolated from gluten by modifications of this type of method. Nevertheless, certain trends of properties and behavior indicate that these fractions may be conveniently classified into 3 main groups . . . designated, for convenience as the 'glutenin' group, the 'gliadin' group, and a group to which the name 'mesonin'[2] has been tentatively assigned. The name 'mesonin' is intended to convey the idea that the protein components of that group have properties which are *intermediate* with respect to . . .

[2] Suggested by C. H. Bailey.

glutenin and gliadin." For detailed evidence (which is admittedly circumstantial rather than direct) in justification of this suggested classification, the paper of Blish and Sandstedt (1933) may be consulted.

Stockelbach and Bailey (1938) prepared mesonin and glutenin by the foregoing type of fractionation, and studied the distribution of amino acids in the two fractions by a modification of the Brazier method (1930). Substantial differences in amino acid composition were found. Harris and Bailey (1937) found significant differences in the proportions of glutenin, mesonin, and gliadin among 20 flour samples of widely varying characteristics.

Reference has been made to the fractionation experiments of McCalla and Rose (1935) in which successive fractions were obtained by varying quantities of $MgSO_4$ added to gluten dispersions in 8–10% sodium salicylate. When individually isolated, hydrolyzed, and analyzed for amide-N and arginine-N, a series of these fractions showed progressive and systematic variations in chemical composition. It was concluded that gluten is "a single protein complex which may be separated into a great many fractions which differ progressively and systematically in both physical and chemical properties." Further support for such a conclusion was afforded by the work of Spencer and McCalla (1938) in which fractional *solubility* in sodium salicylate was employed.

Haugaard and Johnson (1930) obtained a thermal fractionation of gliadin, starting with a 10% gliadin solution in 60% ethanol at room temperature. Upon lowering the temperature to 0° C., a considerable portion of the gliadin precipitated after several days standing. The fraction was isolated and dried with absolute alcohol and ether. When the clear mother liquor was cooled to −11° C., another large fraction deposited, while a smaller fraction remained in solution. When studied individually the several fractions showed significant differences in viscosity and in optical rotation. When subjected to acid hydrolysis and analysis for amino acid make-up, however, the fractions showed no appreciable differences other than in content of tryptophan and tyrosine. Haugaard and Johnson concluded that gliadin is inhomogeneous.

It is apparent that fractional precipitation procedures have not provided trustworthy evidence as to the number and character of the individual components of gluten. The successive precipitation of many fractions, differing progressively in composition and properties, might well be interpreted to mean that gluten is a system consisting of innumerable components, as is believed by McCalla and his associates. Nevertheless, it is quite conceivable that a series of systematically varying fractions could result from *component interaction*, and that relatively few components might precipitate as mixtures in which the components occur in progres-

sively varying proportions. This type of interaction was, in fact, demonstrated by Bungenberg de Jong (1933) with purified preparations of glutenin and gliadin. de Jong found that interaction between his two "components" resulted in the formation of complexes, by reason of differences in electrical charges on the protein particles. The nature and degree of interaction varied with the pH and with the concentrations of the two proteins. As would be expected, maximum interaction occurred in the region between the isoelectric points of the two proteins, in which the gliadin carried a positive and the glutenin a negative charge. de Jong suggests that the formation and stability of *natural* gluten is dependent upon complex formation of this character. This reviewer subscribes to the belief that the principal components of gluten are limited and relatively few in number, but that component interaction, or "complex formation" is frequently a complicating factor which makes any rational interpretation of results extremely difficult.

Summarizing the evidence as to the individual protein components and homogeneity of gluten protein, the following conclusions seem warranted:

1. Gluten protein is definitely inhomogeneous and probably consists of several, if not many components, instead of only two, as postulated by Osborne (1907).

2. Non-homogeneity appears to increase with a decrease in solubility of the various protein fractions.

3. Evidence of non-homogeneity may however be due, in considerable measure, to aggregation, and to component interaction with "complex formation," rather than to the actual existence of numerous individual components.

4. The solubility characteristics of gluten present unique difficulties and complexities when attempts are made to apply and interpret modern physical methods for studying protein individuality and molecular properties.

5. Convincing solution of the problem of gluten structural composition and homogeneity apparently must await discovery and application of appropriate solvents, or of new methods and criteria, or a combination of these developments.

VII. ELEMENTARY AND AMINO ACID COMPOSITION OF GLUTEN PROTEINS

Since gluten, as prepared by the conventional method, always contains more or less associated carbohydrate and lipid substances, it logically follows that efforts to study its elementary and amino acid composition have usually been confined to purified gliadin and, to lesser degree, to glutenin preparations. Osborne's determinations of the ultimate composi-

tions of gliadin and glutenin (as shown in Table III) are doubtless reliable and authoritative.

TABLE III

Elementary Analysis of Gliadin and Glutenin (Osborne 1907)

	Gliadin	Glutenin
	per cent	*per cent*
Carbon	52.72	52.34
Hydrogen	6.86	6.83
Nitrogen	17.66	17.49
Oxygen	21.73	22.26
Sulfur	1.03	1.08

In consideration of the close agreement between total N values for gliadin and glutenin (17.66 and 17.49%) it follows that total N multiplied by the factor 5.7 will serve satisfactorily as a close approximation of the quantity of gluten protein in gluten or in various protein fractions prepared from it.

Osborne and his coworkers were the first to undertake extensive analytical studies of the amino acid constituents of purified samples of gliadin and glutenin. Applying the Fischer ester method for the monoamino acids and the familiar technique of Kossel and Kutscher for the basic amino acids, Osborne's analyses accounted for considerably more than half of the total quantity of amino acids in his purified gliadin and glutenin preparations.

Later analyses, with improved and refined methods, have made it possible to present a more complete picture as to content and distribution of amino acids in gluten protein. In this, as in other respects, gliadin has received far more attention than glutenin, doubtless because of the greater ease and confidence with which it can be prepared in a highly purified state and recovered without having undergone obvious and irreversible alterations in original properties.

In Table IV are shown the amino acid compositions of gluten, gliadin, and glutenin, respectively, the values given being as authoritative and up-to-date as the reviewer has been able to assemble from available sources. Most of the figures appearing in Table IV have been selected from tables recently compiled and published by Block and Bolling (1945). The values have been calculated to a total N content of 17.5%, and in some instances, notably of glycine and valine, they represent a compromise among relatively few discordant figures reported.

The outstanding feature of gluten protein composition is the extraordinarily high content of glutamic acid, especially in gliadin, where glutamic acid constitutes nearly half of the entire protein substance. Noteworthy

TABLE IV

Amino Acid Composition of Gluten Proteins

	Gluten	Gliadin	Glutenin
	per cent	per cent	per cent
Arginine	4.3	3.2	4.7
Lysine	2.1	0.6	1.9
Histidine	2.4	2.1	1.8
Tyrosine	4.2	3.1	5.1
Tryptophan	1.1	0.9	1.8
Phenylalanine	2.0	2.5	2.0
Cystine	1.9	2.3	1.7
Methionine	3.3	2.3	—
Serine	—	0.1	0.7
Threonine	2.5	3.0	—
Leucine and Isoleucine	6.0	6.0	6.0
Valine	3.0	3.0	1.0
Glutamic Acid	36.0	46.0	27.2
Aspartic Acid	—	1.4	2.1
Glycine	—	1.0	1.0
Alanine	5.5	2.5	4.4
Proline	11.0	13.2	4.4
Hydroxyproline	—	—	—
Ammonia	4.5	5.10	4.0

also are the large proline content and the relatively small amounts of basic amino acids and of tryptophan. The obvious lysine and tryptophan deficiencies indicate a limited nutritional value, an indication which has been abundantly confirmed experimentally. The presence of hydroxyglutamic acid, in both glutenin and gliadin, was reported by Dakin (1919), but this must now be considered doubtful, in the light of the findings of Nicolet and Shinn (1942).

VIII. Technology of Gluten in the Bread Industry

Admittedly our knowledge of the chemistry of gluten and of its components is wholly inadequate either to account for its peculiar physical properties or to explain the manner and degree in which these properties vary among the glutens of different wheats. This is a technological disadvantage when it is realized that variations in physical properties are factors chiefly responsible for differences in specific industrial utility. Thus, for example, a quality of gluten best adapted for current types of commercial bread production is quite different from that which is desired for the baking of crackers or biscuit.

In numerous endeavors to discover correlations between chemical and physical properties of gluten the main objective has been to establish reliable testing methods for the identification and classification of raw

material (wheat) in terms of those gluten characteristics that are most significant in the control of industrial processing operations (milling and baking). These endeavors have not yet succeeded in providing satisfactory methods of *chemical analysis* that will permit quantitative measurement and recording of industrially important gluten properties.

The general acceptance of the findings and conclusions of Osborne, as first announced about fifty years ago, at once led to the supposition that differences in gluten "quality" might be attributed to variations in the quantity of either gliadin or glutenin, or to differences in the ratio of one of the other. Accordingly many workers, among the first of whom were Fleurent (1896), Guthrie (1896), and Teller (1896), proposed methods for the quantitative estimation of gliadin and glutenin, and these or similar procedures were applied to flours of different types and grades. A variety of methods and of conflicting results and interpretations were reported from time to time during the next 20 or 25 years, but no clearly discernible correlations with baking properties were established. Since it has become increasingly probable that neither glutenin or gliadin is a homogeneous protein, efforts to evaluate glutens or flours on the basis of alleged variations in glutenin or gliadin content, or of differences in glutenin-gliadin ratios, have been largely abandoned.

The possibility that variations in gluten qualities might be due to differences in amino acid composition was examined by Blish (1916) and by Cross and Swain (1924). In these investigations the Van Slyke nitrogen distribution method, as applied to glutenin and gliadin preparations from different flours, failed to show any convincing evidence of differences in amino acid make-up among the various preparations of gliadin, on the one hand, and of glutenin, on the other.

Failure to discover technologically significant relationships between chemical and physical properties of gluten has led to numerous investigations of the merits of various methods for the measurement and classification of the physical properties themselves. Several mechanical contrivances, designed for the measurement of plasticity, tensile strength, elasticity, extensibility, etc., are applicable directly to the wet crude gluten, while others are intended for doughs, flour pastes, or suspensions. In any case the variations measured are considered to reflect corresponding differences that are attributable exclusively to the gluten constituents and not to starch or other associated substances.

One of the most widely used and currently popular among these physical testing procedures measures and records progressive changes in plasticity which a dough undergoes when subjected to a certain type of prolonged mechanical mixing. During the first part of the mixing period there is a gradual increase in plasticity until a maximum is reached, beyond which

further mixing causes a pronounced breakdown of structural properties, which, in turn, is denoted by a corresponding *decline* in plasticity. A point is finally reached where the dough becomes slack and sticky, and will not yield gluten by the customary washing process. When plasticity values are plotted against mixing time — as is accomplished by an automatically recording "plastometer" — the result is a curve (called a *"mixogram"*) that indicates both rate and magnitude of the changes in plasticity during the entire mixing operation. The patterns of the mixograms vary significantly and characteristically among wheat varieties, and are believed to indicate inherent differences in gluten properties. This method of recording distinctive physical peculiarities of various glutens is adequately described by Swanson and Working (1933) and has been studied in great detail by many workers.[3]

Although there are several types of methods whereby wheats may be classified and evaluated on the basis of variations in the physical and mechanical properties of their glutens, the technological implications of these differences are not always apparent unless the differences are of considerable magnitude. Physical testing procedures are especially useful as a convenient means for the identification of particular varieties of wheat which have notoriously inferior gluten quality, but which cannot be reliably distinguished by visual inspection. Accordingly such tests are frequently specified and used in connection with importations of wheat from one country or continent to another.

Since the chief problems of milling and baking technology are attributable to peculiarities of gluten properties and behavior, and because these gluten properties are, in turn, predominantly *inherent* and *varietal* in character, it follows that problems of processing control would be greatly simplified if the processor could select and purchase his wheat according to variety. This however is seldom possible because bulk shipments are more than likely to be mixtures of which cannot be distinguished by inspection. The difficulty can be largely overcome if adequate storage facilities are available, whereby various lots of wheat are segregated and held until their individual gluten properties can be determined and classified by means of properly conducted laboratory milling and baking tests. Having been thus classified, different lots may be blended in such a manner that the deficiencies of one are supplemented or offset by the desirable properties of another.

An interesting and highly effective method of processing control now available to the cereal technologist depends upon the fact that gluten properties and behavior can be profoundly and usefully altered by means

[3] Numerous reports of studies by Dr. Swanson and associates, and by others, have been published in *Cereal Chemistry*, during the past ten years.

of treatment with very small quantities of effective oxidizing agents. A foundation for this type of treatment was laid by Kohman, Irvin, and Cross (1920), who patented a salt mixture containing a small quantity of potassium bromate, and found that its use in the baking of freshly milled winter wheat flours produced at once a favorable result that otherwise would be obtained only by permitting the flour to undergo weeks or months of natural aging in storage. The significance of this "artificial maturing" by means of potassium bromate was not generally appreciated at first, but became increasingly apparent after the introduction of nitrogen trichloride as a flour bleaching agent, by Baker (1921), for it was promptly observed that very small amounts of nitrogen trichloride produced a powerful "maturing" effect in addition to its bleaching action.

By means of controlled utilization of an oxidizing agent (such as chlorine, nitrogen trichloride, potassium bromate, potassium iodate, ammonium persulfate, sodium chlorite) for modifying the gluten properties of certain flours, it is possible to effect a remarkable improvement both in baking performance and in the character of the final product. Gaseous oxidizing agents are applied to the flour in the milling process while salts such as potassium bromate are more conveniently employed in the bakeshop.

The modifying action of oxidizing agents on gluten is not instantaneous, but develops gradually during dough fermentation, the rate of its progress varying with the kind and quantity of the agent used. The degree of effect is usually measurable only in terms of dough behavior and of the character of the baked loaf of bread. The treatment has a toughening or "strengthening" effect on the gluten. When properly applied, it favorably affects the gas retaining properties of the dough, and produces an astonishing improvement both in volume and texture of the loaf. That the quantity of oxidizing agent required is remarkably small in proportion to the magnitude of the effects achieved is evidenced by the fact that one or two thousandths of one per cent of potassium bromate (based upon the weight of flour) is ordinarily all that is required for optimum results. Merritt, Blish, and Sandstedt (1932) found that, under comparable conditions, potassium iodate has even a more powerful action than potassium bromate. Overtreatment is disastrous, for it tends to destroy the elasticity of the gluten so that the dough tears easily, its gas retaining capabilities are injured, and the final product is inferior.

The artificial "maturing" of wheat gluten by means of chemical oxidizing agents has not only greatly facilitated the industrial processing of wheat in a technological sense but it has also benefited agriculture in several important wheat growing areas. It has largely removed prejudice against several wheat varieties which are agronomically best adapted for their environments but which were *formerly* discounted because their flours did

not give satisfactory baking performance until they had been *naturally* matured or "oxidized" by means of long periods of aging during storage in warehouses. It is a varietal and inherent characteristic of such wheats that *when freshly milled* into flour their gluten is relatively soft, extensible, and lacking in elasticity and "toughness." Oxidation is the only known method of treatment which will produce the improvement necessary for proper functioning in the bakeshop. "Response to oxidation" as measured by increased loaf volume, becomes progressively greater as flour protein *content* increases. Some types of wheat obviously require no oxidizing treatment for optimum baking performance and in those instances artificial "maturing" by oxidizing agents is likely to be detrimental rather than otherwise.

In considering the striking effect of minute quantities of oxidizing agents on the physical and mechanical properties of gluten it is important also to note the action of various *reducing* agents, including soluble sulfides, sulfites, sulfur dioxide, cysteine, and glutathione. These substances when applied in quantities of the order of a few thousandths of one per cent, also act as powerful modifying agents, and, as might be expected, their effect is opposite to that produced by oxidizing agents. The reducing agents cause increased extensibility, fluidity, and stickiness of the gluten and dough, this "weakening" action being accompanied by a marked lowering of gas-retaining power.

The true explanation of the effects of such minute quantities of oxidizing and reducing agents on the structural properties of gluten constitutes one of the foremost current problems in the field of cereal technology. One group of workers, including Jorgensen (1935), Balls and Hale (1936, 1938), and Elion (1943) has subscribed to the belief that the phenomena are best explained on the basis of an inhibition of proteinase activity by the oxidizing agents, in contrast to an activation of proteinases by the reducing agents, as is known to be the case with papain. This viewpoint necessarily assumes that the properties of wheat proteinase closely resemble those of papain, although there are some who disagree with that assumption.

Many investigators consider that the enzyme theory is entirely inadequate to explain the observed types and peculiarities of behavior, and they believe that other factors are of far greater importance. Read and Haas (1939) observed that the proteinase of malted wheat flour is much less responsive to inhibition by potassium bromate than is papain, and that "when applied in quantities applicable to commercial baking practice no significant repression of wheat proteinase was apparent." Ford and Maiden (1938) found the softening action of glutathione on dough to be much more rapid than that of papain, showing that the effect of glutathione was directly on the gluten and not merely a matter of proteinase activation. Sullivan,

Howe, Schmalz, and Astleford (1940) expressed the opinion that "all the work indicates that changes in the sulfur linkages of the gluten proteins are responsible for many of the effects described." Sandstedt and Fortmann (1943) were able to reverse the action of reducing agents in flour by subsequent oxidation, and they regard this as strong evidence against the enzyme theory. Olcott, Sapirstein, and Blish (1943) found that the viscosity of gluten dispersions in dilute acetic acid was greatly lowered by reducing agents even though all enzymic activity had previously been destroyed by heat.

This reviewer is unable to believe that the drastic alterations in gluten properties produced by seemingly insignificant quantities of oxidizing and reducing agents can satisfactorily be accounted for on the basis of proteinase inhibition or activation. The actual proteinase activity of sound wheat flour, as measured by gelatin liquefaction (Landis and Frey, 1938), is of an exceedingly low order. Moreover no one has been able to demonstrate that any substantial hydrolytic degradation of gluten protein occurs during a normal process of dough fermentation unless malt or other enzyme preparation is added. Efforts to obtain direct and positive evidence as to the mechanism by which the effects are produced have thus far failed to produce convincing results. A satisfactory solution of the problem will doubtless have implications extending well beyond the field of milling and baking technology.

IX. COMMERCIAL PRODUCTION OF GLUTAMIC ACID AND SODIUM GLUTAMATE FROM WHEAT GLUTEN

Because of its extraordinarily high glutamic acid content (about 36% on a protein basis) wheat gluten takes precedence, among the several available protein substances, as a raw material for the commercial production of monosodium glutamate, a meat-flavoring condiment first introduced in the Orient by the Japanese about 30 years ago. Currently, monosodium glutamate is in large and rapidly increasing demand in the United States as an indispensable ingredient of canned and dehydrated soups. The annual production and consumption of monosodium glutamate in this country now amounts to approximately 4,000,000 pounds. Availability of gluten for this type of use is, however, dependent largely upon the market demand for wheat *starch*, for only as a by-product of wheat starch manufacture can gluten be produced cheaply enough to meet economic requirements.

Methods for large scale production of sodium glutamate from gluten necessarily involve hydrolysis by acid, followed by precipitation of glutamic acid or glutamic acid hydrochloride, from the hydrolyzate. After suitable purification operations, the glutamic acid is converted to the monosodium salt. Essential steps in the process have recently been described by Geddes

(1945), and statements of various modifications of procedure are to be found in several United States patents.

X. Industrial Non-Food Uses for Wheat Gluten

The fact that until recently wheat was generally regarded as one of the more serious *surplus commodity* problems has stimulated considerable inquiry into the potentialities of gluten protein for adaptability to non-food industrial utilization. Laboratory investigations in this field have thus far failed to produce any announced results having important industrial significance (Olcott and Blish, 1944). Although plastics, fibers, film, and adhesives can be made from gluten, no means have been found for overcoming serious disadvantages that are both technological and economic in nature. Plastics and fibers are characterized by inherent tendencies toward brittleness and low water resistance, and no methods of overcoming these handicaps have yet been disclosed. There are strong indications that excellent adhesives can be made from gluten protein, but it is to be doubted that these adhesives can successfully cope with competitive products made from cheaper raw materials.

The probability of competition with cheaper proteins that are more abundantly available as processing by-products (cottonseed, soybean, and peanut proteins) suggest that any successful industrial application for gluten must of necessity be one that is unique and exclusive. An interesting example of such an application has been reported by Reitz, Ferrel, and Olcott (1944), who have described a "gluten sulfate" which has extraordinary gel-forming properties. This gluten protein derivative almost instantaneously absorbs "one hundred to three hundred times its weight of cold water to form a firm, odorless, tasteless, and non-toxic gel." Attempts to produce the same product from other proteins were not successful.

With an anticipated expansion in commercial uses for wheat starch, the gluten by-product will become more abundantly and cheaply available, and the search for new industrial applications for gluten protein will be correspondingly intensified.

REFERENCES

Anson, M. L., and Mirsky, A. E. (1929). *J. Gen. Physiol.* **13**, 121.

Bailey, C. H. (1944). The Constituents of Wheat and Wheat Products. New York.

Bailey, C. H. (1940). Wheat Studies of the Food Research Institute. **16**, No. 6. Stanford University.

Baker, J. C. (1921). U. S. Pat. 1,367,530.

Balls, A. K., and Hale, W. S. (1936). *Cereal Chem.* **13**, 656.

Balls, A. K., and Hale, W. S. (1938). *Cereal Chem.* **15**, 622.

Becarri. (1936). De Bononiensi Scientiarium et Artium Instituto atque Academia Commentarii 2, Part 1, 122.

Blish, M. J. (1936). Supplement to *Cereal Chem.* **13**, No. 5.

Blish, M. J. (1916). *Ind. Eng. Chem.* **8**, 138.

Blish, M. J., and Sandstedt, R. M. (1926). *Cereal Chem.* **3**, 144.

Blish, M. J., and Sandstedt, R. M. (1929). *J. Biol. Chem.* **85**, 195.

Block, R. J., and Bolling, D. (1945). The Amino Acid Composition of Proteins and Foods. Springfield, Ill.

Brazier, M. A. B. (1930). *Biochem. J.* **24**, 1188.

Burk, N. F. (1938). *J. Biol. Chem.* **124**, 49.

Cook, W. H., and Alsberg, C. L. (1931). *Can. J. Research* **5**, 355.

Cook, W. H., and Rose, R. C. (1934). *Nature* **134**, 380.

Cook, W. H., and Rose, R. C. (1935). *Can. J. Research* **12**, 248.

Cross, R. J., and Swain, R. E. (1924). *Ind. Eng. Chem.* **16**, 49.

Csonka, F. A., and Horn, M. J. (1931). *J. Biol. Chem.* **93**, 677.

Dakin, H. D. (1919). *Biochem. J.* **13**, 398.

Dill, D. B. (1925). *Cereal Chem.* **2**, 1.

Dill, D. B., and Alsberg, C. L. (1924). *Cereal Chem.* **1**, 222.

Einhof, H. (1805). *J. der Chemie (von Gehlen)* **5**, 131.

Elion, E. (1943). *Cereal Chem.* **20**, 234.

Fisher, E. A., and Halton, P. (1936). *Cereal Chem.* **13**, 575.

Fleurent, E. (1896). *Compt. Rend.* **123**, 755.

Ford, W. P., and Maiden, A. M. (1938). *J. Soc. Chem. Ind.* **57**, 278.

Geddes, W. F. (1944). The Chemistry and Technology of Food Products. Vol. II, Part XV, 496. New York.

Guthrie, F. B. (1896). *Agr. Gaz. N. S. Wales* **7**, 583.

Haugaard, G., and Johnson, A. H. (1930). *Compt. rend. trav. lab. Carlsberg* **18**, 1.

Harris, R. H., and Bailey, C. H. (1937). *Cereal Chem.* **14**, 182.

Jong, H. L. Bungenberg de (1933). *J. Soc. Chem. Ind.* **52**, 391 T.

Jorgensen, H. (1935). *Biochem. Z.* **280**, 1.

Jorgensen, H. (1935). *Biochem. Z.* **283**, 134.

Kohman, H., Irvin, R., and Cross, R. J. (1920). U. S. Pat. 1,325,327.

Kosutany, T. (1903). *J. Landw.* **51**, 139.

Krejci, L., and Svedberg, T. (1935). *J. Am. Chem. Soc.* **57**, 946.

Lamm, O., and Polson A. (1936). *Biochem. J.* **30**, 528.

Landis, Q., and Frey, C. N. (1938). *Cereal Chem.* **15**, 91.

Mangels, C. E., and Martin, J. J. (1935). *Cereal Chem.* **12**, 149.

McCalla, A. G., and Gralen, N. (1942). *Can. J. Research (Sec. C)* **20**, 130.

McCalla, A. G., and Rose, R. C. (1935). *Can. J. Research* **12**, 346.

Merritt, P. P., Blish, M. J., and Sandstedt, R. M. (1932). *Cereal Chem.* **9**, 175.

Neurath, H. (1939). *J. Am. Chem. Soc.* **61**, 1841.

Nicolet, B. H., and Shinn, L. A. (1942). *J. Biol. Chem.* **142**, 139.

Olcott, H. S., and Blish, M. J. (1944). *Trans. Am. Assoc. Cereal Chemists* **2**, 20.

Olcott, H. S., Sapirstein, L. A., and Blish, M. J. (1943). *Cereal Chem.* **20**, 87.

Osborne, T. B. (1907). The Proteins of the Wheat Kernel. *Carnegie Inst. Wash. Pub.* No. 84.

Read, J. W., and Haas, L. W. (1939). *Cereal Chem.* **16**, 60.

Reitz, H. C., Ferrel, R., and Olcott, H. W. (1944). *Ind. Eng. Chem.* **36**, 1149.

Ritthausen, H. (1872). *J. prakt. Chem.* [2] **5**, 215.

Sandstedt, R. M., and Blish, M. J. (1933). *Cereal Chem.* **10**, 359.

Sandstedt, R. M., and Fortmann, K. (1943). *Cereal Chem.* **20**, 517.

Schwert, G. W., Putnam, F. W., and Briggs, D. R. (1944). *Arch. Biochem.* **4**, 371.

Sharp, P. F., and Gortner, R. A. (1923). *Minn. Agr. Expt. Sta. Tech. Bull.* **19.**

Spencer, E. Y., and McCalla, A. G. (1938). *Can. J. Research* (Sec. C). **16,** 483.

Stockelbach, L. S., and Bailey, C. H. (1938). *Cereal Chem.* **15,** 801.

Sullivan, B., Howe, M., Schmalz, F. D., and Astleford, G. (1940). *Cereal Chem.* **17, 507.**

Swanson, C. O., and Working, E. B. (1933). *Cereal Chem.* **10,** 1.

Taddei, G. (1820). *Annals of Philosophy* **15,** 390.

Teller, G. L. (1896). *Ark. Agr. Expt. Sta. Bull.* **42,** 81.

Upson, F. W., and Calvin, J. W. (1915). *J. Am. Chem. Soc.* **37,** 1295.

Upson, F. W., and Calvin, J. W. (1916). *Nebr. Agr. Expt. Sta. Research Bull.* No. **8.**

Wood, T. B., and Hardy, W. B. (1908). *Proc. Roy. Soc. (London)* (B) **81,** 38.

Protein Denaturation and the Properties of Protein Groups

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CONTENTS

I. INTRODUCTION

If a solution of a protein like egg albumin is heated in the neighborhood of its isoelectric point, the protein is coagulated. This heat coagulation of isoelectric protein takes place some 600 times faster when the temperature is raised ten degrees. In contrast, temperature has a much smaller effect on the rate of ordinary chemical reactions. Acid and alkali dissolve heat coagulated protein, and heat coagulated protein can be dissolved even

at the isoelectric point by a number of substances such as urea, guanidine hydrochloride, detergents, and salicylate. The conversion of native protein which is soluble at its isoelectric point to denatured protein which is insoluble at the isoelectric point in the absence of reagents such as urea, can be brought about not only by heat but by surface action, by ultra-violet light, by high pressure, by organic solvents such as alcohol and, in general, by those reagents such as acid, alkali, urea, guanidine hydrochloride, detergents, and salicylate which can dissolve coagulated protein.

A protein denatured so that it becomes insoluble at its isoelectric point in pure water or dilute salt solutions, is also changed in a number of other properties. Groups, such as SH, S-S, and tyrosine groups, give certain characteristic reactions more readily in the denatured than in the native form of many proteins. Some of the specific properties of individual native proteins are lost when the protein is denatured. For instance, enzymes become inactivated and hemoglobin changes color and no longer can combine loosely with oxygen. Some proteins which in their native state are not digested by enzymes become readily digestible after denaturation. As we have seen, a conjugated protein such as hemoglobin loses some of its specific properties on being denatured. There also is a change in the nature of the combination between prosthetic group and protein. Native hemoglobin is a tight combination between native globin and reduced heme. Denatured hemoglobin, or globin hemochromogen, is a loose combination between denatured globin and reduced heme. A number of protein properties which depend on the shape of the molecule change when the protein is denatured and changes its molecular shape. A urea solution of a corpuscular protein, such as egg albumin or hemoglobin, becomes more viscous as denaturation takes place and a protein is formed which precipitates on removal of the urea. Furthermore, the rates of diffusion and sedimentation of a protein are decreased when the protein is denatured in urea solution, except in those cases in which the denaturation causes dissociation of the protein into smaller units. Finally, although many native proteins are known in crystalline form, no denatured protein has been crystallized out of solution. Thus denaturation can now be followed, in many cases quantitatively, in a great variety of ways. One is no longer limited, in measuring the rate or the extent of denaturation, to measuring the formation of insoluble protein.

When a molecule of protein is denatured it opens up and, as a result, changes its shape radically. Although this general change in structure is now well established, it is not possible at the present stage of knowledge of protein structure to define denaturation exactly by means of a detailed description of the change in structure of the protein molecule. One can as yet define denaturation only in terms of the whole characteristic group

of changes in properties which are known to be brought about by certain types of procedures. For the present it is desirable to discuss denaturation primarily in terms of these observed changes in properties rather than in terms of some dogmatic definition of the word or some hypothetical theory of structural change.

Despite the fact that it is not yet possible to define denaturation exactly, it is possible to separate denaturation, with its characteristic group of changes in observed properties, from other changes in observed properties of a protein which definitely are different from denaturation. Thus, the combination of native proteins with various salts or certain chemical modifications of the groups of native proteins such as those which result from reactions with amino groups (to be reviewed in a later volume) are quite clearly not denaturation. Similarly, the dissociation of proteins into smaller units such as can take place with tobacco mosaic virus and hemocyanin (also to be reviewed in a later volume), the hydrolysis of proteins and the splitting off of ammonia, can and should be separated from denaturation. A more precise understanding of what happens to the large complicated molecule of protein upon denaturation is not promoted by lumping together with denaturation all the miscellaneous modifications which protein molecules can undergo.

The purpose of this review is to discuss (1) the properties of protein groups, how they are influenced by denaturation and other factors, and how these influences can be explained, (2) the reversibility of denaturation, and (3) the all-or-none character of denaturation.

Previous reviews. A brief and more general review of the subject of denaturation with references to the earlier literature has already been written for Schmidt's book on proteins (Anson, 1944). A much more extensive review has recently been presented by Neurath, Greenstein, Putnam, and Erickson (1944). Denaturation by ultra-violet light has been reviewed by Arnow (1936). The present volume contains a review by Fankuchen (1945) on X-ray studies of native and denatured proteins. Protein films, protein fibers, and the reactions of native and denatured proteins with detergents will be discussed in later volumes. Matthews, Dow, and Anderson (1940) give references to papers on denaturation by high pressure. Barron and Singer (1943) enumerate those enzymes whose activities are destroyed by reagents which eliminate free reactive SH groups.

II. SULFHYDRYL GROUPS

SH groups of coagulated egg albumin. It was observed a long time ago that denatured but not native egg albumin gives the nitroprusside color test for SH groups (Heffter, 1907; Arnold, 1911). The SH groups of coagulated egg albumin were first studied quantitatively by measuring how much

cystine is reduced by a suspension of the precipitated protein (Mirsky and Anson, 1935). $0.05mM$ SH groups were found per gram of protein. A similar figure was obtained when the much stronger oxidizing agent porphyrindin was used (Kuhn and Desnuelle, 1938).

SH groups of denatured egg albumin in solution. Greenstein (1938) made an important step forward by applying the porphyrindin titration not to a suspension of coagulated egg albumin but to a solution of denatured egg albumin in guanidine hydrochloride solution, and by using as the end point of the titration the disappearance of the nitroprusside test. The number of SH groups titrated in the guanidine hydrochloride solution was double that found by oxidation of the coagulum. In urea solution 80% as many groups were titrated as in guanidine hydrochloride solution. Greenstein concluded from his observations that guanidine hydrochloride liberates all the SH groups of egg albumin, whereas heat denaturation liberates only half, and urea solution liberates only 80% of the SH groups. Although Greenstein's discovery that egg albumin contains twice as many SH-groups as had been previously observed in coagulated egg albumin has been amply confirmed, his conclusion that heat and urea do not "liberate" all the SH groups of egg albumin has since, in my opinion, been experimentally disproven.

The titration in guanidine hydrochloride solution can be carried out not only with porphyrindin, which is hard to prepare, unstable, and an unnecessarily strong oxidizing agent, but by the convenient oxidizing agents ferricyanide and tetrathionate and by *p*-chloromercuribenzoate, which is not an oxidizing agent at all and hence is a good reagent for testing the SH specificity of the titration (Anson, 1941).

The titrations are carried out in neutral solution and the nitroprusside test in alkaline solution. To make sure that the oxidation takes place in the neutral solution and not under the alkaline end point conditions, one can observe the disappearance of the color of the porphyrindin in the neutral solution or test the end point of the oxidation by precipitating the protein with trichloroacetic acid and removing any excess of the oxidizing agent before making the nitroprusside test or seeing whether the protein reduces ferricyanide.

Another precaution which must be taken with the nitroprusside test is to avoid the presence of minute traces of heavy metals which can prevent the appearance of part or all of the nitroprusside color even when some SH groups are still intact before the nitroprusside test (Anson, 1941). Either the guanidine hydrochloride should be purified (Greenstein and Jenrette, 1942) or the guanidine hydrochloride should be prepared from purified guanidine carbonate which can be prepared quite readily (Anson, 1941) If the heavy metal impurity is in the protein preparation, as it is in some

preparations of tobacco mosaic virus, the SH protein may give no nitro-prusside test at all. Then a small amount of cyanide should be added before the nitroprusside test to combine with heavy metal impurities, an amount of cyanide insufficient to cause reduction of the S-S groups (Anson and Stanley, 1941). Cyanide must not be present during oxidation of the SH groups by the titrating agent because the oxidation of SH groups by common oxidizing agents such as ferricyanide is catalyzed by copper salts and inhibited by cyanide just as is the oxidation by oxygen (Anson, 1942). It is advisable, however, to add the titrating agent before the denaturing agent so that the SH groups of the denatured protein should not be exposed to oxygen before they are exposed to the titrating agent.

The titrations can be carried out in urea solution and in a solution of long chain alkyl sulfates (Duponol PC) as well as in guanidine hydro-chloride solution (Anson, 1941, 1942). The nitroprusside test for the end point is carried out after precipitation of the protein with trichloroacetic acid and resolution of the protein with guanidine hydrochloride. The fact that a negative nitroprusside test in guanidine hydrochloride solution is obtained after oxidation in urea or Duponol solutions shows that all the SH groups which are "liberated" by the guanidine hydrochloride are also "liberated" by urea and Duponol.

The SH groups of denatured egg albumin can be estimated by measuring the amount of ferricyanide reduced in solutions of Duponol, urea, and guanidine hydrochloride (Anson, 1939a, b, 1941; Mirsky, 1941a). The Duponol solution is the most convenient for the estimation of ferrocyanide formed as Prussian blue. Under the conditions used for the ferricyanide reduction, cysteine is the only amino acid which reduces ferricyanide. Denatured proteins which do not contain cysteine do not reduce ferri-cyanide (Anson, 1939b). Denatured egg albumin which has been treated with the SH reagents formaldehyde, iodoacetamide (Anson, 1939b), mercuribenzoate (Anson, 1941), mercuric chloride or iodoacetate(Mirsky, 1941a), likewise does not reduce ferricyanide.

One can also measure the amount of Folin's Uric Acid Reagent reduced by denatured egg albumin in urea solution. This procedure for estimating the SH groups is very convenient since the reduced reagent has a blue color by which it is readily estimated (Anson, 1942).

Iodosobenzoate has been used as an oxidizing agent for the SH groups of egg albumin (Hellerman, Chinard, and Ramsdell, 1941). The iodine liberated is estimated by titration in acid solution. There is the danger in this procedure of the liberated iodine itself taking part in some reactions but this does not seem to have taken place in the titration used by Heller-man *et al.*

Finally, the SH groups of egg albumin have been estimated by iodine titration (Anson, 1940, 1942; Hess and Sullivan, 1943).

0.1 mM of SH per gram of egg albumin (corresponding to 1.2% cysteine) have been found whether the titration is carried out with various oxidizing agents or with mercuribenzoate, whether the nitroprusside test or ferricyanide reduction is used as an end point, whether the titration is carried out in guanidine hydrochloride, urea, or Duponol solution, whether one uses a titration method or measures the amount of oxidizing agent reduced in guanidine hydrochloride, urea, or Duponol solution. This general agreement between the results obtained by many different methods indicates that the same groups are being estimated by the different methods and that all the groups estimated by one method are also estimated by the other methods.

Mirsky (1941a) reported some 20% less SH in denatured egg albumin than other investigators. His methods were not especially different from those used by others so it is difficult to ascribe this difference to any methodological reason. I have found an occasional sample of recrystallized egg albumin which had a low SH content. Egg albumin is a mixture of proteins (Longsworth, 1941) and so not entirely reproducible in composition. It has recently been stated that old samples of egg albumin are changed in composition (MacPherson, Moore, and Longsworth, 1944). Dr. M. X. Sullivan has written me that he observed some years ago that samples of egg albumin change in cysteine content on storage.

In comparing different methods for the estimation of an amino acid, one should either apply the different methods to a single sample of protein or use a protein, such as chymotrypsinogen, which is a pure, one component, reproducible protein. Unfortunately, there is not readily available a one component protein which contains cysteine in amounts convenient for estimation.

In the case of tobacco mosaic virus the SH content found by titration (Stanley and Lauffer, 1939; Anson and Stanley, 1941) corresponds to the total sulfur of the protein (Ross, 1940) which is a good check on the correctness of the titration.

Aggregation. Aggregation of denatured egg albumin decreases the reactivity of its SH groups (Anson, 1939a). When the SH groups of denatured egg albumin were estimated with ferricyanide, using not a suspension of coagulated egg albumin, but a solution of denatured egg albumin in which the protein was still somewhat aggregated, then 64% of the SH groups were oxidized by twice the stoichiometric amount of ferricyanide under the conditions used, and 94% of the SH groups were oxidized by fifty times the stoichiometric amount of ferricyanide (Anson, 1939b). When still greater precautions were taken to avoid aggregation, then all

the SH groups were oxidized by a stoichiometric amount of ferricyanide in the complete absence of reagents such as guanidine hydrochloride, urea, and Duponol (Anson, 1942). This shows that all the SH groups of egg albumin can be "liberated" by heat denaturation alone.

The effect of aggregation can also be demonstrated by coagulating egg albumin by shaking in the presence of a large excess of ferricyanide. Under these conditions all the SH groups are oxidized by the ferricyanide, which reacts before the egg albumin is aggregated despite the fact that ferricyanide does not oxidize all the SH groups if it is added to the surface coagulated egg albumin after the coagulation (Mirsky, 1941b). If a solution of denatured egg albumin in urea is diluted so that aggregation takes place, the reactivity of the SH groups is decreased (Mirsky, 1941b).

Although all the SH groups of coagulated, aggregated egg albumin are not oxidized by ferricyanide they are all oxidized by the stronger oxidizing agent, porphyrindin, in sufficiently high concentration (Brand and Kassel, 1940).

Different solvents. The conditions for the estimation of SH groups already described were deliberately made favorable in order that the SH reagent should react with all the SH groups. Under such favorable conditions all the procedures, as we have seen, give the same result for the number of SH groups and, at the most, the rate of reaction varies. That the reactivity of protein SH groups, however, is quite different in different solvents can readily be shown by making the conditions of reaction less favorable. This can be done by using a weaker oxidizing agent, by making the solution more acid, and by varying the protein (Anson, 1942).

Although ferricyanide can oxidize the SH groups of denatured egg albumin even in plain water solution, the Uric Acid Reagent, a weaker oxidizing agent, cannot oxidize these SH groups either in water solution or even in Duponol solution, but it does oxidize the SH groups in urea solution.

Ferricyanide which oxidizes the SH groups of denatured egg albumin in both Duponol and guanidine hydrochloride solution, oxidizes the SH groups of denatured tobacco mosaic virus completely in guanidine hydrochloride solution but only incompletely in Duponol solution.

At a slightly acid pH, ferricyanide oxidizes the SH groups of denatured egg albumin more completely in guanidine hydrochloride solution than in urea solution.

Denatured egg albumin gives a strong nitroprusside test in ·guanidine hydrochloride solution, a weaker test in urea solution and a still weaker test in Duponol solution (Anson, 1941). The weak test in urea solution may be the reason why Greenstein got a low result when titrating the SH groups of denatured egg albumin in urea solution although the observations of Burk (1940) indicate that the nitroprusside test in urea solution is

still sufficiently sensitive for titration purposes. Some proteins, such as serum albumin, which give no nitroprusside test in urea solution, give a weak nitroprusside test in guanidine hydrochloride solution (Greenstein, 1940).

Thus, the few experiments which have been done indicate that the SH groups of denatured protein are more reactive in a solution of long chain alkyl sulfates than in water, more reactive in urea solution than in the detergent solution and more reactive in guanidine hydrochloride solution than in urea solution. Nevertheless, under favorable conditions, all the SH groups of denatured egg albumin are accessible to SH reagents in all these solutions.

Hydrolysis. The SH groups of denatured egg albumin can be made more reactive by partial digestion to the point of no precipitation by trichloroacetic acid (Anson, 1942). Thus, the Uric Acid Reagent oxidizes the SH groups of a neutralized digest although it does not oxidize the SH groups of undigested denatured egg albumin in plain water or in Duponol solution. At pH 4.8 under the conditions chosen, ferricyanide oxidizes 15% of the SH groups of denatured egg albumin in Duponol solution but 83% of the SH groups of the digested protein.

We have seen that the SH groups of a protein can be readily estimated by a variety of procedures which take only a few minutes and require only a few milligrams of protein. Even when partial enzymatic digestion is resorted to, the digestion is very convenient, takes only a short time, and does not involve any of the problems of humin formation, racemization, and decomposition which one encounters when one uses complete acid hydrolysis. In general, it should be possible to develop similar convenient methods for the estimation of all amino acids with characteristic groups without using the usual complete acid hydrolysis but by taking advantage of a knowledge of the factors which increase the reactivity of protein groups. It should be further possible to estimate all the amino acids which do not have characteristic groups by specific enzymatic methods, applied to hydrolysates, as will be discussed in a later volume.

SH groups of native proteins. SH groups of very different degrees of reactivity are found in different native proteins. Although all the SH groups of denatured egg albumin can be titrated in guanidine hydrochloride solution and in other solutions, native egg albumin does not give a nitroprusside test, and does not reduce ferricyanide or porphyrindin. Tobacco mosaic virus in these respects is a SH protein of the egg albumin type (Stanley and Lauffer, 1939). In contrast, all the SH groups of tissue nucleoproteins in the form isolated by Greenstein and Jenrette (1940), are accessible to titration. Lens protein and myosin contain some SH groups which can be detected by ordinary procedures in the native protein and others

which become detectable only when the protein is denatured (Mirsky and Anson, 1935; Mirsky, 1936; Greenstein and Edsall, 1940). Many other tissue proteins behave in the same way as myosin (Greenstein, Thompson, and Jenrette, 1940; Greenstein, Jenrette, and White, 1941).

Urease has two kinds of SH groups. The first kind gives a nitroprusside test and can be readily oxidized by porphyrindin. These SH groups can be oxidized without an effect on the activity of the urease. Urease SH groups of the second kind do not give a nitroprusside test and when they react with mercuribenzoate the activity of the urease is destroyed (Hellerman, Chinard, and Deitz, 1943). Isolated papain does not give the nitroprusside test, is inactivated by iodoacetate, and is not inactivated by porphyrindin (Balls and Lineweaver, 1939a, b). A myosin preparation with an initial adenosinetriphosphatase activity of $Qp = 1150-1300$ in carbonate buffer, pH 8.52 at 38° C. was not inactivated by $3 \times 10^{-3} M$ of iodoacetamide while it was completely inactivated by $1.3 \times 10^{-5} M$ of p-chloromercuribenzoate (Singer and Barron, 1944).

None of the SH groups of native hemoglobin are oxidized by ferricyanide in neutral solution. 65% are oxidized at pH 9.5 (Mirsky and Anson, 1936). It would be desirable to repeat these experiments with the newer SH methods, to extend the experiments to other proteins, and to test the effects of denaturants such as urea in concentrations too low to cause denaturation, and also of temperatures too low to cause denaturation.

SH groups of native egg albumin. The SH groups of native egg albumin which do not react with ferricyanide or porphyrindin can be readily oxidized by iodine (Anson, 1941). If 1.5 times the stoichiometric amount of iodine is added at pH 3.2, all the iodine is absorbed and no SH groups can be detected after denaturation of the protein. At pH 3.2, iodine does not react with tyrosine, and although it reacts with tryptophan it does not react with native chymotrypsinogen which is rich in tryptophan but does not contain cysteine.

The SH groups of native egg albumin can be completely oxidized by a stoichiometric amount of iodine if the oxidation is carried out at zero degrees in 1 N KI (Anson, unpublished experiments mentioned in Anson and Stanley, 1941). 1 N KI prevents the reaction of iodine with tyrosine.

When the SH groups of native egg albumin are oxidized by a stoichiometric amount of iodine in 1 N KI there is no change in the sedimentation rate (Anson, 1942). This indicates that iodine oxidizes pairs of neighboring SH groups, each pair in a single molecule, and does not bring together SH groups from different molecules.

There is evidence that the first step in the oxidation of SH groups by iodine involves the reaction between one iodine molecule and one SH group to give free radicals from both the iodine and the SH groups. Two free

radicals from two SH groups then combine to give one S-S group (Anson, 1940).

Iodoacetamide can react with 40% of the SH groups of native egg albumin at pH 9 (Anson, 1940).

In summary, whether or not SH groups of a native protein are detected depends on which protein is being examined, on which SH groups in a particular protein are being examined, on which SH reagent is used, and on the composition of the solution.

Differences between different native proteins have also been observed in the reactions of S-S groups and in digestibility and similar differences undoubtedly exist in many other properties. As will be described later, the S-S groups of some native proteins are reducible by cysteine, whereas the S-S groups of other native proteins do not react with cysteine at all. Native hemoglobin is not digested at all even by pure trypsin in high concentration (Anson and Mirsky, 1934b). Native edestin, in contrast, is digested very rapidly by trypsin in low concentration (Anson, 1944). Some plant proteinases digest even native hemoglobin, but very much more slowly than they digest denatured hemoglobin (Winnick, Davis, and Greenberg, 1940; Lineweaver and Hoover, 1941).

SH groups of tobacco mosaic virus. After it was found that the SH groups of native egg albumin can be oxidized by iodine and that the tobacco mosaic virus is a SH protein of the egg albumin type, a preliminary experiment showed that iodine can be absorbed by tobacco mosaic virus without inactivation of the virus (Anson, 1940). In all previous experiments, whenever a virus was shown to be chemically modified, the virus was inactivated. A more detailed study (Anson and Stanley, 1941) showed that iodine abolishes the SH groups of native tobacco mosaic virus as it does the SH groups of native egg albumin, that tobacco mosaic virus with all its SH groups destroyed retains quantitatively its original infectivity, and that plants infected with virus whose SH groups have been destroyed produce virus with the normal number of SH groups.

Although the SH groups of native egg albumin are oxidized by iodine even in the presence of 1 N KI, KI prevents altogether the reaction of iodine with tobacco mosaic virus (Anson and Stanley, 1941).

It is possible that oxidized virus is reduced by the living plant to normal SH virus and that this normal virus in turn starts the infection. When, however, oxidized virus was incubated with tobacco tissue and the virus was isolated again, the virus was still found to be without any SH groups.

Tobacco mosaic virus can also be modified by reactions with its amino groups without inactivation of the virus (Schramm and Miller, 1940; Agatov, 1941; Miller and Stanley, 1941, 1942). Miller and Stanley showed that plants infected with virus modified by amino group reagents produce normal virus just as plants do when they are infected by virus whose SH

groups have been oxidized by iodine. Some of the amino group reactions they used could hardly be reversed by ordinary tissue reactions. It may, therefore, be safely concluded that viruses, like enzymes, can undergo some chemical modifications without loss of biological activity. Unfortunately, the production of a virus mutant has not yet been achieved by direct chemical modification of a virus. In order to synthesize a mutant, it will probably be necessary to create the kind of chemical modification, such as change in amino acid composition (Knight and Stanley, 1941), which is normal to the living cell. The technique of producing such modifications is not yet known.

III. Disulfide, Tyrosine, and Tryptophan Groups

The SH groups of proteins have been studied much more than other groups because they are the easiest groups to study. So far as is known, denaturation and some other factors such as the addition of urea affect other protein groups in much the same sort of way as they affect SH groups. It would clearly be desirable to have as many protein groups as possible studied in a variety of native and denatured proteins with many different reagents used under many different conditions. If the SH results turn out to be applicable to protein groups in general, it will be necessary to explain the changes in protein groups on denaturation on the basis of general considerations of protein structure.

S-S groups. Reactive S-S groups may appear on the denaturation of certain proteins just as reactive SH groups appear. Thus, an S-S protein which does not contain cysteine may give a nitroprusside test in the presence of cyanide, which reduces S-S partially to SH, only when the protein is denatured (Walker, 1925). Thioglycolic acid reduces more S-S groups of serum albumin to SH when the protein is denatured than when the protein is native (Mirsky and Anson, 1935).

Tyrosine groups. Folin's Phenol Reagent oxidizes more tyrosine groups in denatured pepsinogen and egg albumin than in the native forms (Herriott, 1935, 1938, unpublished results). When native egg albumin is titrated with alkali the tyrosine phenolic groups are not titrated at the pH at which the phenolic groups of pure tyrosine react with hydroxyl groups (Cannon, Kibrick, and Palmer, 1941). This has been confirmed by the failure to find the change in absorption of ultraviolet light which tyrosine phenolic groups undergo when their ionization changes (Crammer and Neuberger, 1943). The optical method, however, showed that the phenolic groups of egg albumin are titrated at the usual pH when the protein is denatured.

In the experiments of Crammer and Neuberger only 20% of the tyrosine groups of native egg albumin were found to react with the small hydroxyl ions at pH 12. In the experiments of Herriott carried out under similar

conditions, 60% of the tyrosine groups were found to react with the Phenol Reagent, which has a molecular weight of about 4100 (Wu, 1920). Here, as has been found to be the case with SH groups, a particular group of a native protein fails to react with one reagent and nevertheless does react with a reagent of larger size. This suggests that factors other than location of the groups may influence the reactivity of the groups of a native protein.

Tryptophan groups. There have been no studies of the tryptophan groups of both native and denatured proteins with reagents specific for tryptophan. In 1 N potassium iodide solution iodine reacts with free tryptophan although it does not react with pure tyrosine. Yet chymotrypsinogen, which is rich in tryptophan and free of SH, does not react at all with iodine in 1 N KI (Anson, 1940). It would be interesting to follow the reactions of iodine in 1 N KI and of other tryptophan reagents with various native and denatured proteins.

Amino and carboxyl groups. It is well known that amino and carboxyl groups of native proteins react with many different reagents and hence are undoubtedly accessible in the native protein. There has been no systematic comparison, however, of the rates of these various reactions with amino and carboxyl groups in native and denatured proteins and of the effects of reagents such as guanidine hydrochloride on these rates. Perhaps a general effect of denaturation on the rates of reactions of protein groups can be shown even when the groups of the native protein are all finally detectable with the reagent used. Furthermore, there has been no attempt to discover those unfavorable conditions which just prevent the reactions of native proteins with amino and carboxyl reagents. Perhaps under these unfavorable conditions the amino and carboxyl reagents would still react with denatured protein.

Other groups. Most of the specific reactions for particular protein groups have been reactions which yield colored products. Most of these color producing reactions, unfortunately, are carried out under conditions which result in denaturation, so there has been no comparison of the behavior of the native and denatured forms of the protein. Many amino acids, however, have such special structures and properties that it ought to be possible to find reactions, color producing or not, which take place at a pH and a temperature which permit the protein to remain native. The further development of such reactions would be useful not only for the theoretical study of the factors which influence the properties of the protein groups but also for the preparation of modified native proteins to be used in immunological experiments and for the characterization and identification of proteins.

The kind of optical method used by Crammer and Neuberger in their

study of tyrosine groups could perhaps be applied to tryptophan and phenylalanine groups by the use of proteins which are particularly rich in these amino acids.

Biological reactions of protein groups. It is well known that the buffer action of the amino and carboxyl groups of proteins is an important biological reaction. It is not known whether other protein groups are likewise involved in the normal reactions of the living cell, although it is likely that the properties of special protein groups influence the reversible combination between specific proteins and prosthetic groups to form enzymes. When glutathione was discovered, it was assumed that any substance possessing such reactive SH groups and present in such significant concentration must surely take part in some biological SH reactions. Yet native muscle protein is a much larger source of reactive SH groups and there has been little discussion of the possibility of muscle protein SH groups taking part in any normal cellular processes.

So far as is known, the usual amino acid enzymes, such as tyrosinase, do not bring about any amino acid reactions in intact proteins. In general, no case has as yet been discovered in which an enzyme increases the reactivity of any group in native protein.

IV. HYPOTHETICAL STRUCTURAL MECHANISMS

All present-day theorizing about protein structure attempts to include an explanation of why the groups of the native and denatured forms of a protein behave differently. Although the newer methods of studying the shapes of protein molecules have made some progress possible, the knowledge of the structures of the native and denatured forms of protein is still not adequate to account completely for the changes in properties when a protein is denatured. What are lacking, in particular, are experimental methods of studying the bonds between polypeptide chains in native protein and their effects on protein properties.

The compound theory. Three types of explanation have been suggested for the appearance of reactive SH groups when egg albumin is denatured. All three have serious weaknesses. The compound theory assumes that the SH groups in native albumin are involved in some kind of specific compound formation, the compound being broken on denaturation to release free SH groups. For instance, Linderstrom-Lang and Jacobsen (1940) have suggested that the unreactive SH groups of native protein are combined in thiazolidine rings which open up on denaturation of the protein. There are two objections to this type of theory. First, there is no positive evidence for the existence of such compounds in proteins. Secondly, if one assumes SH groups to be involved in specific compounds in native proteins, one must also assume specific compounds for S-S,

tyrosine, and perhaps many other groups. The correctness of so complex a theory is highly improbable. It seems better to seek the explanation of the changes which occur in many different protein groups when the protein is denatured in terms of some general change in protein structure.

The fact that iodine oxidizes the SH groups of native egg albumin cannot be considered an argument against the compound theory. SH groups combined with aldehyde (Schubert, 1936) or with p-chloromercuribenzoate (Anson, 1941) still are oxidized by iodine although they do not give a nitroprusside test and, in the case of the mercuribenzoate compound, have been shown not to be oxidized by ferricyanide.

Denaturation and change in shape. It is now well established that the protein molecule opens up when the protein is denatured. The evidence from X-ray studies (Fankuchen, 1945) is described elsewhere in this volume. The evidence from studies of protein films will be described in a later volume. There is also evidence from measurements of viscosity, diffusion, and sedimentation which will now be summarized.

The long known increase in viscosity when a protein solution is heated is usually due to aggregation of the molecules of denatured protein, but there is a definite increase in viscosity even when the denaturation is brought about by urea and aggregation is avoided (Anson and Mirsky, 1932). This increase in viscosity has been attributed to an uncoiling of the molecule (Mirsky and Pauling, 1936). Recently Neurath and coworkers (reviewed in Neurath, Greenstein *et al.*, 1944) have made careful measurements of the effect of denaturation on viscosity and have calculated the change in asymmetry from the latest equations relating viscosity to molecular shape as derived for ellipsoids of revolution by F. Perrin and others. Neurath and coworkers have also measured the decrease in the diffusion constant on denaturation and have calculated the increase in asymmetry from the Perrin equation. Finally, Rothen (1942) has demonstrated the opening up of the protein molecule in denaturation by the classical Svedberg technique involving measurements of diffusion and sedimentation.

The accessibility theory. It has been suggested that the opening up of the protein molecule accounts for the appearance of active groups on denaturation (Mirsky and Pauling, 1936; Mirsky, 1941a, b). On this basis, the groups are inaccessible in the interior of the native, coiled molecule, and become accessible when the molecule is opened up.

The accessibility theory, despite its obvious advantages, has several weaknesses. Although the SH groups of native egg albumin do not react with nitroprusside, ferricyanide, porphyrindin, oxygen, or dilute hydrogen peroxide, they do react with iodine, iodoacetamide, permanganate, and concentrated hydrogen peroxide. The SH groups of denatured egg

albumin do not react at all with some oxidizing agents under certain conditions but react readily with stronger oxidizing agents. These results are difficult to explain on the basis of the accessibility theory. They suggest that denaturing agents make already accessible groups more reactive or destroy some protective structure.

As has already been stated, the tyrosine phenolic groups of denatured but not of native egg albumin react with hydroxyl groups at pH 12. Crammer and Neuberger (1943) have pointed out it is unlikely that the interior of the native molecule is inaccessible to the small hydroxyl ions. Furthermore, some of the phenolic groups of native egg albumin react with Folin's Phenol Reagent.

As is well known, many reagents, some of them of large molecular size compared with hydrogen and hydroxyl ions, can combine with the free amino groups of proteins. Recently, the same has been shown to be true of protein carboxyl groups (Herriott, unpublished results). There is nothing in the present knowledge of protein structure which suggests why amino and carboxyl groups should be on the outside of the native protein molecule and SH, S-S and tyrosine groups in the interior.

The bonding theory. If the SH groups of native egg albumin are unreactive but not tied up in specific compounds and not inaccessible, there remains to consider what kinds of structural change could possibly make them reactive. In some cases it is known that amino acid groups are more reactive in the free amino acid than in a peptide, and more reactive in the peptide than in denatured protein. Furthermore, there are big differences between different denatured proteins. It is possible that this effect of neighboring peptide bonds and protein groups is more pronounced in native than in denatured protein, since any protein group has more and closer neighbors in the coiled native molecule than in the extended denatured molecule. But although this kind of effect must influence the reactivity of protein groups to some extent, it is doubtful whether by itself it can account for the extreme and general differences between native and denatured protein.

It has been suggested that the bonds between polypeptide chains in native protein are hydrogen bonds (Mirsky and Pauling, 1936; Jordan Lloyd, 1938), and that it is hydrogen bonds which are broken by denaturation procedures. The hydrogen bond theory is plausible on general chemical grounds. It has not as yet any direct experimental basis. Whatever the nature of the non-peptide bonds in proteins, their rupture may well cause a general change in the reactivity of protein groups apart from any bringing of groups from the interior of the molecule to the exterior. The bonding theory avoids the difficulties of the accessibility theory but has no positive experimental basis and therefore is too vague to be of much practical use, although it was of some use in suggesting the experiment of oxidizing the

SH groups of native egg albumin with iodine. In fact, the bonding theory (Brand and Kassell, 1942; Crammer and Neuberger, 1943) or more generally the theory of a change in reactivity not due to the breaking of a specific compound or the bringing of a group from the interior to the exterior of the protein molecule (Anson, 1940) is merely a statement of the type of theory which remains if one rejects the specific compound and the accessibility theories. And it has the advantage of suggesting that all the groups even of a native protein can be made to react by a suitable choice of reagent and conditions.

In summary, it may be said that the increase in reactivity of protein groups when a protein is denatured must be explained by some general structural theory and not by a mass of specific structural theories like that of Linderstrom-Lang and Jacobsen. The increase in reactivity is probably connected in some way with the breaking of the bonds between the polypeptide chains. To what extent the breaking of these bonds merely brings the groups to the exterior of the molecule and to what extent it increases the reactivity of the groups in other ways which may or may not involve spatial considerations cannot be decided on the basis of the present limited knowledge.

Aggregation. Mirsky (1941b) has suggested that some groups of coagulated egg albumin do not react with SH reagents because they are inaccessible in the interior of the coagulum, as he supposes all the SH of native egg albumin to be inaccessible in the interior of the individual native molecule. Just as the accessibility theory applied to denaturation has to account for the fact that the SH groups of native egg albumin react with some reagents and not with others, so the accessibility theory applied to aggregation has to account for the fact that all the SH groups of coagulated egg albumin can be oxidized by concentrated porphyrindin but not by concentrated ferricyanide, a weaker oxidizing agent (Brand and Kassel, 1940).

Although a considerable part of the protein of the living cell is in the precipitated form, there has unfortunately been relatively little study of the effect of precipitation on the reactions of proteins. A priori, there are three ways in which precipitation can change the properties of any protein and, in particular, the reactivity of the groups of denatured protein. (1) Certain parts of the protein may become completely inaccessible to the test reagent, as suggested by Mirsky. (2) Precipitation may change the rate at which the test reagent diffuses into contact with the group being tested. (3) The bonding between the protein molecules which brings about the precipitation and the proximity of many groups from different molecules in the precipitate may change the reactivity of the group being tested.

There is no way of telling from present data the relative importance of these three factors.

So far as denaturation itself is concerned, it has at least been shown in a preliminary experiment that the temperature coefficient of denaturation is roughly the same whether the protein is precipitated or in solution (Anson, 1944). This result, if confirmed, would indicate that the unit which undergoes denaturation is the same whether the protein molecules are dissolved or precipitated.

It would be desirable to have structural theories to account not only for the effects of denaturation and aggregation but for the different effects of different denaturing agents and for the effect of partial digestion of already denatured protein. Unfortunately there is little basis for speculation.

In considering possible mechanisms for the effect of reagents such as urea on protein groups the possibility should not be neglected that urea and similar reagents affect protein groups directly apart from any effects they may have on protein structure. Some preliminary experiments carried out by Dr. V. Hollander in the writer's laboratory indicated that even free tyrosine is oxidized more readily in urea solution than in plain water.

V. Reversibility of Denaturation

Evidences of reversibility. Hemoglobin which has been denatured in a variety of ways can be converted back into native protein which has the same solubility as the original protein, is crystallizable, has the characteristic spectrum of native hemoglobin, can combine loosely with oxygen has the same relative affinities for carbon monoxide and oxygen, and is not readily digested by trypsin. Similarly denatured serum albumin can be converted back into protein which has many of the properties of the original native protein. The "reversed" protein is soluble (Spiegel-Adolph 1926; Anson and Mirsky, 1931), crystallizable (Anson and Mirsky, 1931) and has very nearly the normal molecular size and shape (Neurath, Cooper, and Erickson, 1942). It has S-S groups which are not reducible by cysteine as are those of denatured serum albumin (Mirsky and Anson, 1931). The original serological specificity is restored by the reversal process (Miller, 1933; Erickson and Neurath, 1943). The proteinases trypsin and chymotrypsin become inactive when denatured and recover their enzymatic activity when the denaturation is reversed. When the denaturation of the proteinase precursors, trypsinogen, chrymotrypsinogen, and pepsinogen is reversed, soluble proteins are obtained which can again be converted into the active proteinases (Northrop, 1939). There is thus no doubt that the major changes involved in the denaturation of hemoglobin, serum albumin, and several proteinases and proteinase precursors can be reversed.

Equilibrium between native and denatured proteins. In some cases only

a part of the denatured protein can be converted back into protein with some, at least, of the characteristics of the original native protein. For instance, if acid denatured hemoglobin is neutralized only about 75% is changed back into native protein by the neutralization, whereas the rest is precipitated as still denatured protein (Mirsky and Anson, 1930). In other cases, all of the denatured protein can be converted into soluble native protein again. For instance, if an acid solution of trypsin is heated to denature the protein, then all of the trypsin is converted into native, active trypsin again by cooling (Northrop, 1932; Anson and Mirsky, 1934a). or if hemoglobin is denatured by salicylate under special conditions, then on removal or mere dilution of the salicylate all the hemoglobin is made native again (Anson and Mirsky, 1934b).

Similar results have been obtained with chymotrypsinogen (Kunitz and Northrop, 1935), pepsinogen (Herriott, 1938), and a number of other proteins. In those cases in which all the denatured protein can be converted back into soluble, native protein again, it is possible to study the equilibrium between native and denatured protein.

As has long been known, the *rate* of heat denaturation of protein at its isoelectric point is extremely sensitive to the temperature. With trypsin it was possible to show that the *equilibrium* between native and denatured trypsin is likewise extremely sensitive to temperature (Anson and Mirsky, 1934a). The heat of denaturation of trypsin, calculated from the effect of temperature on the equilibrium, is 67,000 calories. In contrast, temperature has practically no effect on the equilibrium between native and denatured hemoglobin in salicylate solution (Anson and Mirsky, 1934b).

The following explanation was given for the apparent discrepancy between the great effect of temperature on the trypsin equilibrium and the small effect of temperature on the hemoglobin equilibrium. If salicylate or any other denaturing agent causes reversible denaturation, then salicylate must combine more with the denatured than with the native form of the protein. The heat of denaturation as calculated from the effect of temperature on the equilibrium includes the heat of the change in combination with salicylate and, indeed, the heats of all the changes between the original and the final forms of the protein. In the case of heat denaturation of trypsin, energy for the breaking of bonds comes from heat. In the case of the salicylate denaturation of the hemoglobin energy comes from the change in combination with salicylate, and so less thermal energy is required.

Both the trypsin and the hemoglobin equilibria are independent of the protein concentration. This shows that the denaturation in these cases involves neither dissociation nor aggregation. Osmotic pressure measurements have likewise shown no change in molecular weight on denaturation

except in those proteins like myosin which are dissociated by denaturing agents (Huang and Wu, 1930; Burk, 1932; Wu and Yang, 1932; Edsall and Mehl, 1940; Neurath, Cooper, and Erickson, 1942).

Although the hemoglobin equilibrium is independent of the protein concentration, it is extremely sensitive to the salicylate concentration (Anson and Mirsky, 1934b). The peculiar shape of the curve relating the percentage denaturation to the salicylate concentration was explained in terms of a complex equilibrium, involving the reaction of a single molecule of hemoglobin with a considerable number of molecules of salicylate. Steinhardt (1937) later interpreted the great effect of alkali on the rate of denaturation of pepsin to mean that activation of the pepsin molecule involves a change in the ionization of many groups. By plotting the pH against the accurately measured velocity constant of the reaction, he was able to calculate from the slope of the line that the number of groups involved was five. (This does not mean that the activation of pepsin under other denaturation conditions also involves the ionization of five groups). Herriott (1938) by plotting the equilibrium constant of the equilibrium between native and denatured pepsinogen against pH showed that denaturation of pepsinogen involves a change in ionization of two groups.

It was pointed out in the discussion of the salicylate equilibrium (Anson and Mirsky, 1934b) that if denaturation takes place biologically it is probably by a reaction similar to the salicylate denaturation in which the denaturation is extremely sensitive to the concentration of some substance which may be produced and removed under biological conditions.

One is practically forced to conclude from a priori considerations that a high heat of reaction for a reaction which takes place readily at ordinary temperatures means the breaking of many bonds of moderate strength. A few very strong bonds could not be broken thermally at ordinary temperatures at any appreciable rate. It is only with large complex molecules that one has the possibility of breaking a large number of bonds in a single molecule. Ionization of a protein molecule involves many changes in a single large molecule. The heat of complete ionization of a protein is very high if one calculates it per mole of protein rather than per mole of acid as is usually done. All the intermediate ionic forms are stable, and there is no method for estimating the concentrations of the completely ionized and the completely unionized forms. Thus, one does not detect the great sensitivity to temperature of the equilibrium between the completely ionized and the completely unionized forms which must theoretically exist. To detect the great temperature coefficient of a change involving the breaking of many bonds in a single molecule either the intermediate forms must be relatively unstable or the two extreme forms must have some special properties by which they can be estimated.

Non-identity of reversed and native proteins. Roche and his coworkers found that in some cases although after reversal of denaturation the essential properties of the native protein are restored, still by some measurements the reversed protein can be distinguished from the original native protein. For instance, "reversed" methemoglobin as prepared by Roche and Combette (1937) was more easily split into globin and heme by alkali than the original native hemoglobin. "Reversed" serum albumin as prepared by Roche and Chouaiech (1940) had the same molecular weight as the original native serum albumin but differed in solubility.

Neurath and his coworkers (reviewed in Neurath, Greenstein, and coworkers, 1944) have confirmed with different techniques the observation that reversed protein can in some cases be distinguished from the original native protein. They found, for instance, that reversed serum albumin prepared from denatured serum albumin has a different electrophoretic mobility and is digested more rapidly by trypsin than the original albumin. Most of the experiments of Neurath and his coworkers were done with protein denatured by long standing in concentrated urea solution.

In general, the proteins and the denaturing procedures used by Roche and Neurath were not ideal for producing a reversed protein most likely to be identical with the original native protein. In order to obtain the best possible conditions for reversing denaturation completely the following conditions should be observed. The protein chosen should be a one component protein like chymotrypsinogen, and the reversal of its denaturation should be complete by the solubility test, as is also the case with chymotrypsinogen. The denaturation should be rapid, the denaturation procedure should be carried out for the minimum time and with the least concentration of denaturing agent and at the lowest temperature necessary to obtain the changes characteristic of denaturation. The reversal procedure should be begun just as soon as the changes characteristic of denaturation have been obtained. Aggregation should be avoided and reagents such as alkali, which may cause secondary changes, should not be used. These conditions in general were not fulfilled in the experiments of Neurath and his associates who intentionally sought the maximum changes from the long exposure of the protein to urea.

In summary, it may be said that the basic changes involved in the denaturation of some proteins can be reversed but it has been proven in some cases that the reversed protein is not identical with the original native protein. These latter experiments, however, have not been done under conditions which one might suppose to be optimum for obtaining completeness of reversal. The subject, therefore, needs further experimental study.

Reversal of denaturation in the living cell. The luminescence of bacteria is catalyzed by an enzyme, luciferase. If bacteria are heated to a high

enough temperature, this luminescence is irreversibly quenched. If, however, the temperature is just high enough to cause quenching and is maintained for only a short time, then on cooling, all the luminescence is restored. The temperature change required for quenching and restoration of luminescence is very small. Johnson and his associates have concluded from their observations that the great effect of temperature is due to the reversible denaturation of luciferase, just as the great effect of temperature on the heat inactivation of trypsin is due to the reversible denaturation of trypsin (Johnson, Brown, and Marsland, 1942; Johnson, Eyring, and Williams, 1942; Johnson et al., 1945). The observed lowering of the temperature of quenching by alcohol or urethane is interpreted as due to a shift of the luciferase denaturation equilibrium to the denatured side. The observed effect of pressure in restoring luminescence partially quenched by heat or urethane is interpreted as due to a shift of the denaturation equilibrium to the native side. This increasing of luminescence by pressure supposedly takes place only when an appreciable fraction of the luciferase is in the denatured form and is used as a test for the partial denaturation of luciferase by heat or inhibitors. Similar considerations are believed to be applicable to the effects of temperature, pressure and narcotics on many other biological processes.

That denaturing agents such as alcohol and salicylate shift the equilibrium between the native and denatured forms of isolated protein has already been shown. Similar experiments with urethane and with pressure (not the extremely high pressure which causes denaturation) remain to be done.

The reversible heat quenching of luminescence, if it is due to the reversible denaturation of luciferase, must mean either that luciferase gets denatured at a lower temperature than other proteins which accompany it in the living cell, which would seem improbable, or that the denaturation of many other proteins in the cell whose denaturation temperature is lower than that of luciferase is also readily reversible, which again seems improbable. Thus, a priori, the reversible heat quenching of luminescence seems to be a remarkable phenomenon.

The fact that the denaturation of luciferase or any other enzyme can be brought about by heat in vivo and reversed by cooling does not by itself mean that the reversible denaturation of these enzymes ever takes place to any appreciable extent in real life. The evidence is strong, however, that reversible denaturation is at least compatible with the complex structure of the living cell. It is to be hoped that many more attempts will be made to discover reversible heat effects, with the precautions taken of using the minimum temperature needed to produce the effect and of cooling promptly. In particular, it would be extremely interesting to know whether normal enzymatic activity is ever regulated by reversible denaturation.

VI. The All-or-None Character of Denaturation

Definition of all-or-none. By the statement that denaturation is an all-or-none reaction is meant merely the experimental observation that, in the cases so far studied, when a protein is changed so that it is denatured by one test it is denatured by other tests and that when the protein is half denatured by one test it is half denatured by other tests. This result indicates that as denaturation proceeds in a solution of constant composition and temperature or when there is an equilibrium between native and denatured protein, the solution contains, for practical purposes, only two kinds of protein molecules. One kind has been altered from its original properties in that particular solution by all of the tests used. The other kind has been altered by none of the tests used.

There is no intention of implying by the statement that denaturation is an all-or-none reaction that the properties of either the still native or of the already denatured form of a protein are independent of the composition and temperature of the solution any more than that they are independent of the specific structure of the protein. On the contrary, we have seen that the reactivity of the SH groups of denatured egg albumin depends on what organic solutes are present in the solution, that the reactivity of the SH groups of native hemoglobin depends on the pH, and that different native and denatured proteins have quite different properties.

Neurath, Greenstein, *et al.* (1944) define denaturation as "any non-proteolytic modification of the unique structure of a native protein" which, as they state, is not the usual meaning of the word. They describe changes brought about in denatured proteins by changing the composition of the solution as stages of denaturation. They therefore properly conclude that denaturation in their sense of the word is not an all-or-none reaction. It is clear that they are not using the expressions "denaturation" and "all-or-none reaction" in the way those expressions are used in this review. Since there is not agreement about the meaning of these expressions, emphasis should be placed on the experimental observations themselves rather than on the words by which they are described, and expressions such as "all-or-none reaction" should be defined in terms of experimental observations.

Experimental evidence for the all-or-none character of denaturation. In the ideal experiment for testing the all-or-none character of denaturation, several different properties of native, half denatured, and all denatured protein are measured in the same solution. The properties of the native solution are taken as a base line and the percentage denaturation is calculated from the *change* in properties. For practical reasons, the ideal conditions have not been completely realized in the experiments which will now be summarized.

When the enzymes pepsin (Northrop, 1930), trypsin (Northrop and Kunitz, 1932), chymotrypsin (Kunitz and Northrop, 1935)and carboxypeptidase (Anson, 1937) are completely denatured as shown by the solubility test, they are completely inactivated. When they are half denatured as shown by the solubility test they are half inactivated.

When salicylate is added to methemoglobin under certain conditions, no change in spectrum takes place until the salicylate concentration is 0.15 M. Further small increase in the salicylate concentration causes changes in the spectrum and color in the direction of the spectrum and color of denatured hemoglobin. At just the peculiar concentration of salicylate which begins to cause the change in color some of the methemoglobin is changed from a form which is not digestible by trypsin to a form which is digestible by trypsin and some of the methemoglobin is changed from a form which is not precipitated by a particular concentration of ammonium sulfate to a form which is precipitated. Thus, the change in the structure of the methemoglobin molecule which is responsible for the change in spectrum seems to be also responsible for the changes in digestibility and solubility (Anson and Mirsky, 1934b).

When egg albumin is completely denatured by urea in the presence of ferricyanide, as shown by the complete insolubility of the protein on removal of the urea, then all the SH groups of the protein are oxidized by ferricyanide. When half the protein is denatured, as shown by the solubility test, then half the SH groups are oxidized (Mirsky, 1941a).

Experiments of Neurath and Saum. Neurath and Saum (1939) measured the diffusion constants of serum albumin in buffered solutions of increasing concentrations of urea and also measured the relative viscosities of these solutions. They found that the relative viscosity was slightly increased and the diffusion constant slightly decreased by 3 M urea. Increasing concentrations of urea up to 6.6 M caused further marked changes in the relative viscosity and in the diffusion constant. The protein in each solution was monodisperse so far as one could tell from the diffusion measurements. The diffusion test for homogeneity is sensitive enough to show that the solutions did not contain any considerable percentage of molecules which differed extremely in shape from the other molecules present. The test is not sensitive enough to exclude mixtures of molecules of less extreme differences. No measurements were made of the extent of denaturation as shown by the solubility test.

The experiments of Neurath and Saum indicate that the shape of the molecule of denatured serum albumin, or at least the size and shape of the albumin-urea compound, depends on the concentration of urea. The experiments by themselves, however, do not permit any conclusions about

the all-or-none character of the urea denaturation as the all-or-none phrase has been defined here.

In order to test the so-called all-or-none character of denaturation by viscosity measurements of urea solutions, both the viscosity and the amount of protein of changed solubility should be measured as denaturation proceeds slowly. The solubility test should be carried out by dilution with concentrated salt solution so that the denatured protein is precipitated before denaturation can be reversed. Finally, the viscosity of the solution when the protein is half denatured by the solubility test should be compared with the viscosity of a mixture of equal parts of a solution of protein which has been exposed to urea for a very short time and has no denatured protein and a solution of protein which has been exposed to urea for a long time and is all denatured. If the solution during denaturation contains only two kinds of molecules, one like the original unaltered protein, the other like the protein denatured by urea, and if there is no slow reaction between urea and the protein already denatured as shown by the solubility test, then the viscosity of the solution of the protein when it has become denatured by the solubility test should be the same as the mixture of the two solutions of native and denatured protein. Experiments of this sort have not yet been done.

In general, there is as yet only limited knowledge of the extent to which viscosity and other properties of both native and denatured protein depend on the composition and temperature of the solution. By keeping the composition and temperature of the solution constant, however, one can nevertheless study the separate and more restricted experimental question of whether or not under any given conditions, when half the protein has been changed as shown by the solubility test, half has also been changed as shown by the viscosity or any other test.

REFERENCES

Agatov, P. (1941). *Biochemia (U.S.S.R.)* **6**, 269.
Anson, M. L. (1937). *J. Gen. Physiol.* **20**, 663.
Anson, M. L. (1939a). *Science* **90**, 142.
Anson, M. L. (1939b). *J. Gen. Physiol.* **23**, 247.
Anson, M. L. (1940). *J. Gen. Physiol.* **23**, 321.
Anson, M. L. (1941). *J. Gen. Physiol.* **24**, 399.
Anson, M. L. (1942). *J. Gen. Physiol.* **25**, 355.
Anson, M. L. (1944) *in* The Chemistry of the Amino Acids and Proteins, 2d ed., p. 407.
    p. 1115 (Addendum). Ed. by C. L. A. Schmidt.
Anson, M. L., and Mirsky, A. E. (1931). *J. Gen. Physiol.* **14**, 725.
Anson, M. L., and Mirsky, A. E. (1932). *J. Gen. Physiol.* **15**, 341.
Anson, M. L., and Mirsky, A. E. (1934a). *J. Gen. Physiol.* **17**, 393.
Anson, M. L., and Mirsky, A. E. (1934b). *J. Gen. Physiol.* **17**, 399.
Anson, M. L., and Stanley, W. M. (1941). *J. Gen. Physiol.* **24**, 679.

Arnold, V. (1911). *Z. physiol. Chem.* **70**, 300, 314.

Arnow, L. E. (1936). *Physiol. Revs.* **16**, 2671.

Balls, A. K., and Lineweaver, H. (1939a). *Nature* **144**, 513.

Balls, A. K., and Lineweaver, H. (193 b). *J. Biol. Chem.* **130**, 669.

Barron, E. S. G., and Singer, T. P. (1943). *Science* **97**, 356.

Brand, E., and Kassell, B. (1940). *J. Biol. Chem.* **133**, 437.

Brand, E., and Kassell, B. (1942). *J. Biol. Chem.* **145**, 365.

Burk, N. F. (1932). *J. Biol. Chem.* **98**, 353.

Burk, N. F. (1940). *J. Biol. Chem* **133**, 511.

Cannon, R. K., Kibrick, A., and Palmer, A. H. (1941). *Ann. N. Y. Acad. Sci.* **41**, 241.

Crammer, J. L., and Neuberger, A. (1943). *Biochem. J.* **37**, 302.

Edsall, J. T. and Mehl, J. W. (1940). *J. Biol. Chem.* **133**, 409.

Erickson, J. O., and Neurath, H. (1943). *J. Exptl. Med.* **78**, 1.

Fankuchen, I. (1945). *Advances in Protein Chemistry.* See this volume, page 387.

Greenstein, J. P. (1938). *J. Biol. Chem.* **125**, 501.

Greenstein, J. P. (1940). *J. Biol. Chem.* **136**, 795.

Greenstein, J. P., and Edsall, J. T. (1940). *J. Biol. Chem.* **133**, 397.

Greenstein, J. P., and Jenrette, W. V. (1940). *J. Natl. Cancer Inst.* **1**, 91.

Greenstein, J. P., Thompson, J. W., and Jenrette, W. V. (1940). *J. Natl. Cancer Inst.* **1**, 367.

Greenstein, J. P., Jenrette, W. V., and White, J. (1941). *J. Natl. Cancer Inst.* **2**, 305.

Greenstein, J. P., and Jenrette, W. V. (1942). *J. Biol. Chem.* **142**, 175.

Heffter, A. (1907). *Chem. Ztg.* **11**, 822.

Hellerman, L., Chinard, F. P., and Ramsdell, P. A. (1941). *J. Am. Chem. Soc.* **63**, 2551.

Hellerman, L., Chinard, F. P., and Deitz, V. R. (1943). *J. Biol. Chem* **147**, 443.

Herriott, R. M. (1935). *J. Gen. Physiol.* **19**, 283.

Herriott, R. M. (1938). *J. Gen. Physiol.* **21**, 501.

Hess, W. C., and Sullivan, M. X. (1943). *J. Biol. Chem.* **151**, 635.

Huang, T., and Wu, H. (1930). *Chinese J. Physiol.* **4**, 221.

Johnson, F. H., Brown, D., and Marsland, D. (1942). *Science* **95**, 200.

Johnson, F. H., Eyring, H., and Williams, R. W. (1942). *J. Cellular Comp. Physiol.* **20**, 247.

Johnson, F. H., Eyring, H., Steblay, R., Chaplin, H., Huber, C., and Gerhardi, G. (1945). *J. Gen. Physiol.* **28**, 463.

Jordan-Lloyd, D., and Shore, A. (1938) *in* Chemistry of the Proteins. 2d ed., J. and A. Churchill, London.

Knight, C. A., and Stanley, W. M. (1941). *J. Biol. Chem.* **141**, 39.

Kuhn, R., and Desnuelle, P. (1938). *Z. physiol. Chem.* **251**, 14.

Kunitz, M., and Northrop, J. H. (1935). *J. Gen. Physiol.* **18**, 433.

Linderstrom-Lang, K., and Jacobsen, C. F. (1940). *Compt. rend. trav. lab. Carlsberg* **23**, 289.

Lineweaver, H., and Hoover, S. R. (1941). *J. Biol. Chem.* **137**, 325.

Longsworth, L. G. (1941). *Ann. N. Y. Acad. Sci.* **41**, 267.

Matthews, J. E., Jr., Dow, R. B., and Anderson, A. K. (1940). *J. Biol. Chem.* **135**, 697.

MacPherson, C. F. C., Moore, Dan H., and Longsworth, L. G. (1944). *J. Biol. Chem.* **156**, 381.

Miller, B. F. (1933). *J. Exptl. Med.* **58**, 625.

Miller, G. L., and Stanley, W. M. (1941). *J. Biol. Chem.* **141**, 905.

Miller, G. L., and Stanley, W. M. (1942). *J. Biol. Chem.* **146**, 331.

Mirsky, A. E. (1936). *J. Gen. Physiol.* **19**, 559.

Mirsky, A. E. (1941a). *J. Gen. Physiol.* **24,** 709.
Mirsky, A. E. (1941b). *J. Gen. Physiol.* **24,** 725.
Mirsky, A. E., and Anson, M. L. (1930). *J. Gen. Physiol.* **13,** 477.
Mirsky, A. E., and Anson, M. L. (1935). *J. Gen. Physiol.* **18,** 307.
Mirsky, A. E., and Anson, M. L. (1936). *J. Gen. Physiol.* **19,** 439.
Mirsky, A. E., and Pauling, L. (1936). *Proc. Natl. Acad. Sci.* (U. S.) **22,** 439.
Neurath, H., and Saum, A. M. (1939). *J. Biol. Chem.* **128,** 347.
Neurath, H., Cooper, G. R., and Erickson, J. O. (1942). *J. Biol. Chem.* **142,** 249.
Neurath, H., Greenstein, J. P., Putnam, F. W., and Erickson, J. O. (1944). *Chem. Revs.*
    34, 157.
Northrop, J. H. (1930). *J. Gen. Physiol.* **13,** 739.
Northrop, J. H. (1932). *J. Gen. Physiol.* **16,** 333.
Northrop, J. H. (1939). Crystalline Enzymes. Columbia Univ. Press.
Northrop, J. H., and Kunitz, M. (1932). *J. Gen. Physiol.* **16,** 323.
Roche, J., and Combette, R. (1937). *Bull. soc. chim. biol.* **19,** 627.
Roche, J., and Chouaiech, M. (1940). *Compt. rend. soc. biol.* **133,** 474.
Ross, A. F. (1940). *J. Biol. Chem.* **136,** 119.
Rothen, A. (1942). *Ann. N. Y. Acad. Sci.* **43,** 229.
Schubert, M. P. (1936). *J. Biol. Chem.* **114,** 341.
Shramm, G., and Muller, H. (1940). *Z. physiol. Chem.* **266,** 43.
Singer, T. P., and Barron, E. S. G. (1944). *Proc. Soc. Exptl. Biol. Med.* **56,** 120.
Spiegel-Adolph, M. (1936). *Biochem. Z.* **170,** 126.
Stanley, W. M., and Lauffer, M. A. (1939). *Science* **89,** 345.
Steinhardt, J. (1937). *Kgl. Danske Videnskab. Selskab, Math.-fys. Medd.* **14,** 11.
Walker, E. (1925). *Biochem. J.* **19,** 1082.
Winnick, T., Davis, A. R., and Greenberg, D. (1940). *J. Gen. Physiol.* **23,** 275, 283.
Wu, H. (1920). *J. Biol. Chem.* **43,** 189.
Wu, H., and Yang, E. F. (1932). *Chinese J. Physiol.* **6,** 51.

X-Ray Diffraction and Protein Structure

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CONTENTS

I. INTRODUCTION

X-ray diffraction is now more than thirty years old. For many years after the first work by Friedrich, Knipping, and Laue (1) in 1912, most of the efforts of X-ray crystallographers were devoted to the study of comparatively simple structures, structures which could be determined in detail. However, some of the early workers endeavored to apply this technique to more complex structures; a few even studied biological materials. Herzog and Jancke (2) in 1920 obtained fiber diagrams from silk, collagen, and other protein fibers.

Protein crystals were also studied at an early date. The first attempts either gave no diagrams of the sort one would expect from a crystalline material (3) or were completely incorrect from an experimental point of view (4). Only when Bernal and his associates used *single* protein crystals immersed in their mother liquor (5, 6) were reasonable X-ray diagrams obtained.

Enough X-ray studies of proteins have by now been made to result in an impressive collection of data. Undoubtedly there is much valuable information concealed in these data, but, to date, the analyses of these data have not generally been carried as far as had been hoped. Where attempts have been made to carry the analyses to more advanced stages, controversies have usually arisen as to the correctness of the conclusions. There are serious technical difficulties which arise in the course of the analyses of X-ray diffraction data of complex structures. In addition to surveying the more recent work with X-rays on proteins, this paper will attempt to present these difficulties and the possibilities for their solution.

In all X-ray diffraction experiments on proteins, a well defined essentially monochromatic parallel beam of X-rays impinges on a specimen, and the scattered X-rays are recorded on photographic film. These photographic records of the scattered radiation constitute the experimental data. The kind of data one obtains will depend essentially on the specimen used and on the geometry of the X-ray apparatus, and of course the sort of conclusions one can draw will in turn depend on the character of the data (Fig. 1).

All types of specimens have been used for X-ray protein studies, and it has been generally true that the higher the state of orientation — i.e., the closer the specimen approaches in character a single crystal — the more useful the data have proved. Completely unoriented specimens have been studied, both non-crystalline and polycrystalline. One obtains a few halos in the non-crystalline case and a set of comparatively sharp rings when polycrystalline specimens are used. There is generally not much that can be done with such diagrams in the case of proteins. The halos are due to scattering at angles which when translated into Bragg spacings (the justification for the use of Bragg's law for all such cases is not clear) correspond to approximately 4.5 and 10 A. and have been interpreted by Astbury as corresponding to the dimension of the backbone of a polypeptide chain and to an average side chain dimension, respectively. Even when clearly crystalline protein material is used, the diagram (Fig. 1c) is surprisingly simple. Thus the writer has obtained powder diagrams from crystalline horse serum albumin (7) immersed in its mother liquor. While single crystals of the same preparation gave diagrams with hundreds of clearly resolvable spots (reflections), the powder diagram only showed a few distinct lines. Apparently only the very intense reflections are visible in protein powder diagrams; the others blend into a diffuse background. Indeed, in the case of horse serum albumin, while the dimensions of the hexagonal unit cell could be definitely determined from single crystal X-ray data, it was impossible to index the powder diagram unequivocally despite the fact that the unit cell dimensions were known. This failure indicates vividly the shortcomings of the powder method for crystalline proteins. The data *may* be adequate for purposes of identification, but they certainly cannot be used to arrive at any conclusions regarding structure.

An interesting fact which has been commented upon by Bernal is that in single crystal diagrams there seems to be an enhancement of some of the individual reflections at spacings of about 4.5 and especially 10 A. Apparently, these distances correspond to important dimensions in the native protein molecule. As a result powder diagrams of polycrystalline protein specimens show distinct features at these spacings. It should be
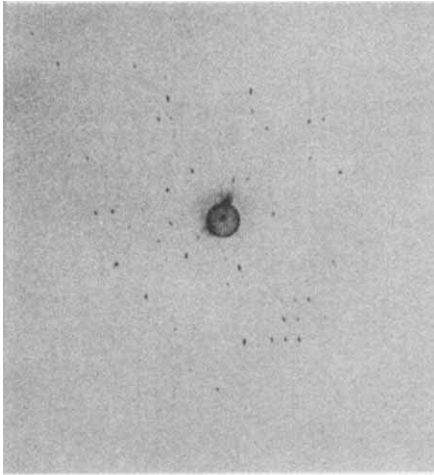
(a)

Wet single protein crystal — Ribonuclease
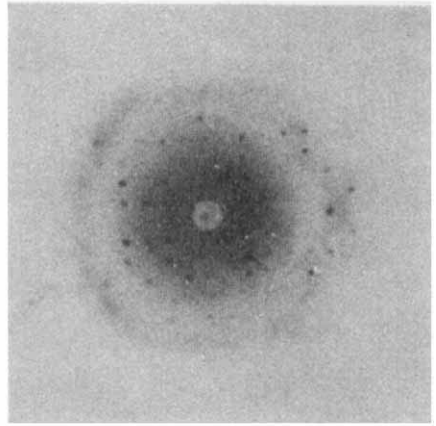
(b)

Dry single protein crystal — Ribonuclease
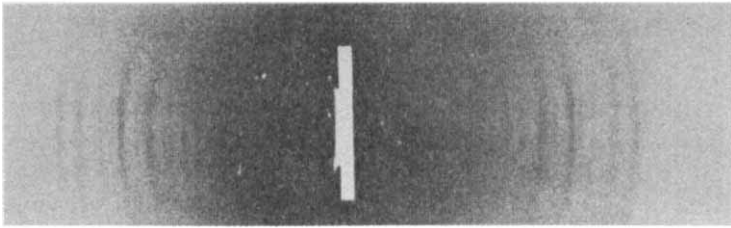
(c)

Powder diagram — Dry Ribonuclease
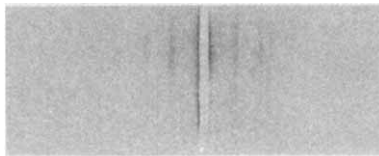
(d)

Equatorial low angle diffraction —
Tobacco Mosaic Virus

Fig. 1

realized, however, that these rings are composite and therefore never as sharp as would be expected from individual lines of a clearly crystalline material, for each line may be due to a large number of individual reflections of about the same spacing. The appearance of broad rings or halos at these spacings is then characteristic of proteins and may be considered as circumstantial evidence of the protein character of a specimen which produces them.

Heat, chemical agents, and radiant energy (ultraviolet light, X-rays, etc.) cause changes in proteins, and apparently these changes sometimes cause changes in the characteristic protein pattern observed at wide angles (10 A. — 2 A.). Undoubtedly a change in the X-ray diagram indicates a transformation within the specimen — but the fact that there is no change in diagram *need not* mean that there is no change in the specimen. This latter statement may seem surprising to those who have heard the X-ray powder diagram described as a sort of unique fingerprint of the specimen in which it has its origin — but fingerprints would not have the unique value in identification which they do have if they consisted only of one or two rather badly defined lines! Two or three broad diffraction lines cannot be considered as characterizing a specific protein — identity or near identity of pattern does not indicate identity or near identity of specimen.

Sometimes — but rarely — powder diagrams can be used to determine the dimensions of a unit cell of a crystalline protein. This possibility occurs when the material crystallizes in the cubic system. Thus the unit cell for ferritin (8) was determined on the basis of powder diagrams alone. Bushy stunt virus was also studied by this method (9) but because only two reflections were observed there seems to be some doubt as to the correctness of the unit cell chosen.

When some orientation is present or is introduced in the specimen, the prospect of obtaining useful information from the data increases greatly. Such orientation is present in natural and in some synthetic fibers and can be produced in solutions of anisotropic molecules by flow. When the character of the orientation is uni-axial, fiber diagrams are obtained. It is then always possible to determine uniquely the period in the direction of the fiber axis. Always for proteins the period has turned out to be intramolecular in origin — small for the fibrous proteins ($\backsim$5–10 A.) and large (68 A.) in the case of tobacco mosaic virus (10).

When only orientation parallel to a given direction is present, it is usually impossible to be sure of details of the structure in directions other than the fiber axis. In almost every case (protein and non-protein) in which the attempt has been made to derive such information from fiber diagrams, the interpretations have been questioned by other workers in the field. When the controversies which arose in these instances were resolved, the

resolution always occurred because some experimenter obtained better data by impressing secondary orientation upon the specimen. Thus Astbury and coworkers have produced secondary orientation in keratin (11) and in myosin (12) and proved that the side chain (10 A.) and the backbone (4.5 A.) spacings are perpendicular to one another. A similar secondary orientation has been observed for dry gels of tobacco mosaic virus (10).

The single crystal represents the ultimate in orientation and whenever adequate single protein crystals were available, it has always been possible to determine uniquely the size and shape of the unit cell thus permitting unequivocal indexing of the significant X-ray reflections. So far only one group of X-ray workers has studied single protein crystals (Bernal and his colleagues). The reason for this is probably the nature of protein crystals. They are usually small, soft, and easily deformed or broken and must generally be studied while immersed in their mother liquor. The experimental difficulties are nevertheless not too great; and techniques for handling and mounting such crystals have been developed by Bernal's group.

While there is at present considerable doubt that the interpretation of X-ray data can be carried far enough to give a reasonable picture of the atomic arrangement within native protein structures, there can be no doubt but that only single crystal X-ray data can hope to lead to any useful conclusions in that direction. For this reason, it is to be hoped that single protein crystals will be much more extensively studied than they now are.

The diffraction of X-rays by crystals is essentially an interference phenomenon occurring between the X-rays scattered by the extranuclear electrons of all the atoms in the specimen. It is convenient to consider all of the electrons in each atom as a single scattering unit, and the scattering power of every atom has been determined (theoretically and experimentally) as a function of $\sin \theta / \lambda$ (θ is half the angle of deviation of the ray of scattered radiation; λ is the wave length of the radiation used). The size and shape of the unit cell determines the directions in which constructive interference occurs between atoms in one unit cell and similarly placed atoms in the other unit cells of the specimen. In these directions, strong X-ray beams can be observed. Bragg and Bragg (13) showed that these beams could be thought of as reflections from planes of atoms — hence the term Bragg reflections. To compute the intensity of a given reflection, only the atoms within one unit cell need be considered. The atomic scattering curve of each atom determines the amplitude of its contribution to the reflection, and the location of the atom in the unit cell determines the phase of its contribution. The contributions of the individual atoms are added vectorially to determine the structure factor of the reflection. The amplitude of the wave (reflection) scattered in the chosen direction is the absolute value of the structure factor. The intensity of the reflection will

be proportional (for protein crystals which may be considered as imperfect crystals) to the square of the amplitude of the resultant wave. With reference to the phase of a wave scattered by an atom at the origin of the unit cell taken as zero, each reflection will have associated with it a phase angle and obviously, just as the amplitudes do, the phase angles depend on the atomic arrangement within the unit cell. Thus in general, the structure factors are complex numbers. Nevertheless, the blackness of the spots on the photographic film depends only on the energy of the X-ray reflections, *i.e.*, on the squares of the amplitudes. Thus because we observe energy, we lose during the experiment the phase angles, and it is this loss which makes for the difficulties and ambiguities which one encounters in trying to determine a structure from the X-ray data.

A crystal is a three-dimensional periodic arrangement of atoms and it can be represented by a three-dimensional Fourier series. It can be shown that when the structure factors of the reflections of indices (*hkl*) are made the coefficients of the corresponding terms of a Fourier series (frequency *hkl*), then the resulting three-dimensional Fourier summation represents to scale the electron density within the unit cell. Maxima in electron density will correspond to atoms. If the phases of the structure factors are determined, then it is possible to determine the structure assuming that enough reflections have been observed to provide the coefficients for the necessary number of terms of the Fourier series. All approaches to the problem of structure determination are really attempts to determine the missing phases regardless as to whether or not Fourier methods are used in the analysis.

Most of the methods that have been used in working out simpler structures have been considered for use in protein work. The most widely used approach is to guess at the structure and then to compute the intensities of the reflections to be expected. If there is reasonable agreement between the computed and observed intensities, the assumed, and of course only approximate structure, can be refined to give better agreement. This adjustment can be conveniently done by Fourier methods since the approximate structure can be used to determine the phases of most of the reflections. This approach has not been too useful in protein work because at present not enough is known of the stereochemistry of the protein molecule to permit of an intelligent first approximation.

Even if one were to make a guess at the atomic arrangement within a protein molecule, that would not be enough to permit of the computation of the intensities, for the molecule would still have to be placed in the unit cell in a number of positions and orientations which would depend on the observed symmetry of the crystal and the size of the unit cell. On the basis of model experiments, there would probably be many different ways to

place the molecules and the intensities would have to be computed for each of these possibilities. Obviously the task of summing up the contribution of the thousands of atoms that are in every protein molecule and moreover to do this for many different arrangements of the molecules in the unit cell would be literally unending. There seems possible at least one elegant way out. Ewald (14) and Knott (15) have suggested the use of molecular structure factors for use in working out crystal structures. The idea of molecular structure factor is an extension of the concept of atomic form factor. One computes as a function of $\sin \theta/\lambda$, the scattering curve of a group of atoms which are often found associated — a benzene ring or an aliphatic chain are simple examples. Such groups need not have spherical symmetry, and their structure factors are also then not spherosymmetrical. However, the structure factor for a given assemblage of atoms — once computed — can be used to determine the contribution of the group to a particular set of reflections for any given orientation of the group. Wrinch (16) suggests that the use of such molecular structure factors could possibly be useful in protein work. Thus the structure factor of a cyclol molecule could be computed and used to check with observed X-ray data. The task would still be an arduous one, but the possibilities are exciting.

Another approach is through the use of Fourier series. If, as is generally the case, the phase angles are not known, the Fourier series which express the electron density cannot be directly computed. Patterson (17, 18) showed that a three-dimensional Fourier series in which the coefficients were the intensities of the X-ray reflections could be given a physical meaning. This summation gives a three-dimensional distribution function, *i.e.*, the peaks represent not atoms but rather the ends of interatomic vectors.

Both the collection of the data necessary for and the summation of a three-dimensional series are tedious and time-consuming affairs. Both for the original Fourier series and for the Patterson summation, it has been shown that only a selected fraction of the data need be used to compute two-dimensional summations which represent projections of the corresponding three-dimensional series onto a plane. These projections naturally contain much superposition of peaks since they are projections and their interpretation is correspondingly more difficult. Harker (19) has shown that in the three-dimensional Patterson summation, certain sections contain particularly valuable information which can be obtained by doing two- (or sometimes one-) dimensional summations. The Harker section, however, requires all the data necessary for a complete three-dimensional Patterson series.

It can be shown that *all* of the Fourier type of summations are transformable into two-dimensional summations. Many methods have been

used for accomplishing these summations. Beevers and Lipson (20, 21), Robertson (22), and others use sets of printed strips. With this technique, one average double summation can be completed in a day. Another technique (23) employs punched cards and standard business machines. These techniques all give at the end numbers which must be plotted to permit the drawing of a contour diagram. Bragg (24, 25) has proposed two other methods, both optical, which use photographic film to produce the final summation. The first suggestion has been taken up by Buerger (26), the second by Huggins (27). Both optical methods have been developed to some extent, but neither as yet to the point where they are likely to be useful for protein studies. It is interesting that Bragg, using the first of these optical techniques, has applied it to hemoglobin (28). The resulting diagram while suggestive is nevertheless inferior to that obtained by mathematical summation, and at present it appears to be true that the more tedious mathematical methods must be used if results as good as the data warrant are to be obtained. For more complete analyses of Fourier methods as applied to molecular structures, the reader is referred to papers by Robertson (29) and Huggins (30).

This rather lengthy survey has, it is hoped, indicated that methods are available for collecting adequate X-ray data and for at least partially analyzing them when obtained. The writer has recently given a more detailed discussion of X-ray diffraction technique as applied to organic materials (43). This may perhaps prove useful to the reader. We next give a brief survey of what has been accomplished and will end with an attempt to suggest likely directions for work in the future.

II. FIBROUS PROTEINS

The fibrous proteins were the first to be studied successfully — successfully at least in the sense that X-ray diagrams were obtained. Until recently most of this work was devoted to the study of scattering at comparatively large angles. Astbury has been the leader in this field and the work has been extensively reviewed by both him (31, 32, 33) and others (34, 35, 36). There has been and still is much controversy about the interpretation of the data. All of the earlier workers used specimens that possessed only orientation parallel to the fiber axis. There was no trouble about determining the periodicity along the fiber axis; neither was there any question about the magnitudes of the spacings at right angles to the fiber axis. The difficulty arose when attempts were made to determine uniquely three-dimensional unit cells to fit the data. Combined with the X-ray data was also the knowledge about atomic sizes, bond lengths, and angles derived mainly from X-ray studies of other structures although strict enough attention apparently was sometimes not paid to such information.

Thus the first structure proposed by Astbury for α-keratin was criticized (37, 34) and finally discarded by its author because it did not allow of the packing of atoms with the sizes and bond angles obtained from other X-ray crystal studies. The writer does not feel justified in presenting here any summary of the many papers which have been published in recent years in this field. No very definite scientific results seem to have been obtained. Thus to quote from the conclusion of the paper by Huggins (36) on "Structure of Fibrous Proteins": "No claim can be made that the structures proposed here (by Huggins) for silk fibroin, keratin, and collagen are proven, that they are correct in all their details, or that they are complete. The writer does claim that they are in considerably better agreement with the available experimental data than are the other structures which have been proposed."

The situation is very different when the low-angle scattering of naturally occurring fibrous proteins is considered. Here, considerable new and most interesting data have been secured from collagen, muscle, keratin, and other fibrous protein materials. These new data were obtained by using better defined X-ray beams, longer specimen-to-film distances, and carefully selected oriented specimens.

MacArthur (38) in this way obtained an exceedingly fine diagram from porcupine quill (α-keratin). MacArthur concludes that there is a period of 658 A along the fiber axis, but this value is obtained not from direct X-ray measurements alone but by introducing Astbury's ideas (33) about the number of amino acid residues a respectable protein should have. Bear (39) has carefully investigated the meridian spacings from porcupine quill tip and finds no X-ray evidence for a period other than 198 A.

MacArthur's work has also clearly shown the composite character of Astbury's 10 A side chain spacing and of the 5.1 A meridian spacing. It would appear that fibrous protein giving an α-keratin X-ray diagram in which these spacings are not obviously composite (assuming the apparatus used is of adequate refinement) are more or less rough approximations of more perfect structures of which porcupine quill tip is one example.

Clam muscle (39), which also gives an α-keratin type wide angle diagram, seems to have a fiber period of 735 A and a somewhat different fine structure for the prominent wide angle diffraction spots.

Feather keratin and silk give β-keratin diagrams, and here again the small angle X-ray scattering is different; for feather keratin (39) appears to have a fiber period of 95 A while no discrete low angle scattering has been observed for silk. The prominent wide angle spots of feather keratin exhibit fine structure; silk shows none.

Collagen has a distinct wide angle diagram, and low angle studies (40) show a fiber period of about 650 A.

The relationship between the wide and low angle scattering is an interesting question which Bear (39) discusses at some length. He concludes that the components of the wide angle spots are high orders of the large periods. Because the assignment of such large indices exactly is impossible at present, he considers it advisable to continue to look upon the wide angle (small spacing) spots as being related to small pseudo-unit cells which are only part of the larger true macro cell. He also suggests why silk shows no large spacings while collagen and clam muscle exhibit very long ones.

Bear (40) has also described the effect of moisture, acid, and tanning on the long meridian spacings of collagen. The "tanned" specimen gave the smallest periods, averaging about 620 A, the dry about 640 A, acid treated 660 A, and the wet specimens about 675 A. No good correlation was observed between the behavior of the meridian and equatorial large angle spacings and that of the long low angle meridian reflections.

This new work, particularly by Bear, has truly opened new vistas in the field of fibrous proteins. It is at its beginning, but already it is clear that many generally accepted ideas about the structure of these proteins must be radically revised or discarded. The data now being obtained seem good enough to permit of the use of Fourier methods, and these, combined with the results of electron microscope studies, should lead to a clear and convincing picture of the macro structure of the fibrous proteins.

III. Virus Proteins

Only tobacco mosaic virus and related strains have been studied (10, 41) in any detail by X-rays. The virus "molecules" are anisotropic shaped cylinders 150 A in diameter and from ten to twenty times as long. Because of the anisotropic shape, dilute solutions are spontaneously birefringent, and oriented gels containing from zero to 50% water can be made. The ordinary X-ray diagrams of such oriented preparations show a fiber type diagram (in which the period however is 68 A, much longer than that given by the fibrous proteins) with a pronounced unresolved equatorial streak at low angles. The use of refined slit systems resolved the equatorial scattering into a set of sharp lines (Fig. 1d). The wide angle diagram was practically independent of the state of hydration while the low angle equatorial scattering was a sensitive function of the state of hydration. Obviously the very complex wide angle diagram has its origin in a quite regular internal structure of the virus particles while the low angle lines are caused by a regular packing of identical parallel molecules.

The intermolecular pattern of the dry gel enabled a determination of the diameter, 150 A, of the virus particle. Even at low virus concentrations

(high hydration), the intermolecular forces were strong enough to maintain a regular hexagonal packing of parallel molecules.

A piece of oriented dry gel of virus placed in water disperses completely. However, for salt and buffer solutions, there are ranges of salt concentration and of pH at which the gel swells to an equilibrium size. Such equilibrium gels also give sharp low angle intermolecular patterns from which can be determined the equilibrium distances as functions of salt concentration and pH.

The wide angle intramolecular diagram indicated a rather detailed regular internal structure for the virus particles. In a sense they could be considered as very small crystallites, having only a few (three probably) unit cells in cross section and twenty-five to fifty along the direction of the length. The intensities of the reflections indicated the presence of a series of smaller sub-units, the smallest of which is about equidimensional of side 11 A.

The virus work was interesting for many reasons. The influence of water on the packing of virus molecules was very great and this permitted the direct demonstration of long range interparticle forces. The diagrams although varying slightly from strain to strain were constant enough to use for identification purposes. Thus Holmes' ribgrass virus was by its X-ray diagrams shown to be a variant of tobacco mosaic despite its very different amino acid analysis. In some early X-ray work on unoriented specimens (42), sharp lines were observed which had been considered as demonstrating the crystalline character of the virus aggregates. These lines are now shown to be completely intramolecular in origin. The ordered aggregates of molecules are not three-dimensional crystalline arrays but rather are a form of liquid crystal.

IV. CRYSTALLINE PROTEINS. SINGLE CRYSTAL STUDIES

Single protein crystals can be made to yield exceedingly detailed X-ray diagrams and yet one must admit that to date the results of such single crystal study have been disappointing, disappointing because very beautiful and complete data have so far only yielded comparatively meager results.

It has always been possible with single protein crystals to determine uniquely the size and shape of the unit cell. Often the space group can also be assigned, and this knowledge of cell size, shape, and symmetry usually is sufficient to determine the number of molecules in the unit cell. The number can definitely be determined if in addition the molecular weight is known very approximately. If the density of the crystal can be measured it is possible to determine the X-ray molecular weight — either hydrated if the crystal was immersed in its mother liquor or anhydrous if the crystal was dry. Table I contains the essential data which are derived from an

analysis of the geometry of the X-ray reflections (43). It will be seen that in general the agreement between X-ray and non X-ray molecular weights is quite acceptable.

Nevertheless, already one difficult step (the density determination) has been introduced. When wet crystals are studied, there is considerable doubt as to just what one measures. When the densities are measured by adjusting a solution in which the crystals are immersed until equilibrium is obtained, the solution undoubtedly affects the state of hydration of the crystal. McMeekin and Warner have studied this question in detail for lactoglobulin (44). They conclude among other things "that previous estimates of the water of hydration of protein crystals are entirely too small." The density of anhydrous crystals can usually be determined reliably, but unfortunately in dry crystals the X-ray patterns are always poorer than those obtainable from the wet crystals.

As soon as attempts were made to use the intensities of the X-ray reflections, progress became very slow. So far, insulin, lactoglobulin, and hemoglobin have been so studied, the last most extensively.

Insulin has been studied both air dry (45) and wet (46). For the dry crystal, Patterson projections and Harker sections were computed. The high symmetry of the insulin unit cell permitted the determination of the principal gross features of the three-dimensional Patterson synthesis. Because no reflections were observed of spacing smaller than 7 A, this Patterson map could not be expected to reveal detail on an atomic scale. Crowfoot did not attempt to push the analysis much further. She did, however, point out the clustering of interatomic vectors of 11 A and 22 A length. Similar but not so extensive computations have been made for wet insulin (46), and a correlation appears to exist between the two vector maps. The molecule does not appear to be much affected by hydration; all that happens is a rearrangement of the molecules relative to one another to accommodate the water. Tobacco mosaic virus and hemoglobin (see below) also seem to exhibit this constancy of molecular geometry. Lactoglobulin has been similarly studied although to date only a brief report of this work has appeared in a review paper (47).

Wrinch has suggested her cyclol, C_2, as the structure for insulin and considers the X-ray data from insulin as supporting her proposals (48). The acute discussion which followed has now subsided with nothing settled save the conviction that what is badly needed are better experiments and more useful data.

Perutz and Boyes-Watson have attempted to supply these experiments and data in some studies of hemoglobin. It is to be regretted that so far only three short papers in Nature have appeared about their work. In the first of these papers (49), crystals of horse methemoglobin were studied in

TABLE I — X-Ray Data on Crystalline Proteins

Proteins, Amino Acids and Peptides by Edwin J. Cohn and John T. Edsall, p. 328–329 (Reinhold Publishing Corporation, New York, N. Y.).

	Best Non-x-ray Molecular Weight	Condition Wet or Dry	"a" in Å (10^{-8} cm)	"b"	"c"	β in degrees	$c \times \sin$	Volume in Å³	n	Volume per molecule (V)	Density (ρ)	Molecular Weight = V 1.65n	Molecular Weight Corrected for Residual Water	Space Group	Smallest Observed Spacing
Ribonuclease (orthorhombic)	13,000 to 15,000	wet													2
		dry	36.6	40.5	52.3	90	52.3	77,300	4	19,300	1.341	15,700	13,700	P2₁2₁2₁	
Ribonuclease (monoclinic)	13,000 to 15,000	wet	30.8	38.5	53.5	107	51	60,000	2	30,000				P2₁	2
		dry	28.7	29.3	45.2	100	44.5	37,400	2	18,700				P2₁	3.7
Insulin*	35,100 to 40,900	wet	144	83	34	90	34	404,000	6	67,000	1.28	52,400		R3	2.4
		dry	130	74.8	30.9	90	30.9	298,000	6	50,000	1.315	39,500	37,400	R3	7
Lactoglobulin (tabular form) "See density note"	37,900 to 41,800	wet	67.5	67.5	154	90	154	702,000	8	88,000	1.257	67,000		P2₁2₁2₁	2.4
		dry	60	63	110	90	110	416,000	8	52,000	1.27	40,000		P2₁2₁2₁	20
Lactoglobulin (needle form)	37,900 to 41,800	wet	67.5	67.5	133.5	90	133.5	608,000	8	76,000				P4₂2₁	
		dry	56	56	130	90	130	408,000	8	51,000	1.30	40,100			20
γ-Chymotrypsin	27,000	wet	69.5	69.5	97.5	90	97.5	471,000	8	58,900				P4₂2₁	2.5
		dry	63.0	63.0	74.5	90	74.5	298,000	8	37,200	1.33	30,100		P4₂2₁	10
Chymotrypsin	41,000	wet	49.6	67.8	66.5	102	65	219,000	2	109,000	1.277	84,500		P2₁	2
		dry	45	62.5	57.5	112	53.5	151,000	2	75,500	1.31	60,000	54,000	P2₁	5

Pepsin	35,500 to 39,200	wet	116	67	461	90	461	3,580,000	54	66,500	1.32	53,000			
		dry													
Horse methemo-globin	66,700	wet	110	63.8	54.2	112	50.2	352,000	2	176,000	1.242	132,000		C2	2
		dry	102	51	47	130	36	188,000	2	94,000	1.27	72,000	66,700	C2	13
Horse serum albumin	70,000 to 73,000	wet	96.7		145	120		1,170,000	6	195,000	1.27	150,000		H	4
		dry	74.5		130	120		610,000	6	102,000	1.34	82,800		H	20
Tobacco seed globulin	(300,000)	wet													
		dry	123	123	123	90	123	1,860,000	4	465,000	1.287	362,000	322,000	F	
Excelsin	294,000	wet													
		dry	149	86	208	90	208	2,670,000	6	445,000	1.31?	350,000	305,800	R3	
Apoferritin	500,000	wet	186	186	186	90	186	6,435,000	8	804,000	1.27	620,000	460,000	F	36
Ferritin	variable	wet	186	186	186	90	186	6,435,000	8	804,000	1,45	706,000	545,000	F	36
Bushy stunt virus	7,600,000 to 10,600,000	wet	394	394	394	90	394	61,000,000	2	30,500,000	1.286	24,000,000		I	
		dry	318	318	318	90	318	32,000,000	2	16,000,000	1.35	13,000,000		I	

* The values given here for insulin are referred to an hexagonal unit cell. The unit cell described on the basis of a rhombohedral lattice gives $a = 44.4$, $\alpha = 114° 28'$, $n = 1$, for dry insulin; and $a = 49.4$, $\alpha = 114° 16'$, and $n = 1$, for wet insulin.

(1) Fankuchen, I., Unpublished.
(2) Fankuchen, I., *J. Gen. Physiol.*, **24**, 315 (1940–41).
(3) Crowfoot, D., *Proc. Roy. Soc. London*, **A 164**, 580 (1938).
(4) Crowfoot, D., *Nature*, **135**, 591 (1935).
(5) Crowfoot, D., and Riley, D., *Nature*, **144**, 1011 (1939).
(6) Crowfoot, D., and Riley, D., *Nature*, **141**, 521 (1938).
(7) Crowfoot, D., and Riley, D., Unpublished data (1940).
(8) Bernal, J. D., Fankuchen, I., and Perutz, M., *Nature*, **141**, 523 (1938).
(9) Perutz, M. F., Ph.D., Thesis, University of Cambridge (1939).
(10) Bernal, J. D., and Crowfoot, D., *Nature*, **133**, 794 (1934).

(11) Crowfoot, D., and Fankuchen, I., *Nature*, **141**, 522 (1938).
(12) Astbury, W. T., Dickinson, S., and Bailey, K., *Biochem. J.*, **29**, 2351 (1935).
(13) Astbury, W. T., and Bell, F. O., Unpublished.
(14) Astbury, W. T., and Bell, F. O., *Tab. Biol.*, **17**, (1), 90 (1939).
(15) Bernal, J. D., Fankuchen, I., and Riley, D., *Nature*, **142**, 1075 (1938).
(16) Rothen, A., *J. Gen. Physiol.*, **24**, 203 (1940–41).
(17) Svedberg, T., *Proc. Roy. Soc. London*, **B 127**, 1 (1939).
(18) Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **18**, 433 (1935).
(19) McFarlane, A. S., and Kekwick, R. A., *Biochem. J.*, **32**, 1607 (1938).
(20) Laufer, M. A., and Stanley, W. M., *J. Biol. Chem.*, **135**, 463, (1940).
(21) Crowfoot, D., *Chem. Revs.*, **28**, 215 (1941).
(22) McMeekin, T. L., and Warner, R. C., *J. Am. Chem. Soc.*, **64**, 2393 (1942).

X-RAY DATA

Ribonuclease (orthorhombic). I. Fankuchen (1).

Ribonuclease (monoclinic). I. Fankuchen (2).

Insulin. Crowfoot (3, 4) (dry), and Crowfoot and Riley (5) (wet).

Lactoglobulin. Crowfoot and Riley (6). Further work is being carried on by these workers (private communication) including Fourier studies and x-ray studies of crystals in various states of hydration (7). See Crowfoot (21).

γ-Chymotrypsin: prepared by Kunitz. X-ray studies by I. Fankuchen (1).

Chymotrypsin. Bernal, Fankuchen and Perutz (8), Perutz (9). (In the original communication (8) *n* was taken as 4. It is now suggested by Perutz that *n* = 2. This gives a molecular weight in better agreement with the data.) Crystals prepared by Northrop.

Hemoglobin. Bernal, Fankuchen and Perutz (8). Further intensive study is being done by Perutz (9). See also Crowfoot (21). Perutz, *Nature* **149**, 491 (1942).

Pepsin. Bernal and Crowfoot (10). These measurements were the first on single protein crystals and repetition of this work would be desirable.

Horse Serum Albumin: prepared by T. L. McMeekin; x-ray studies by I. Fankuchen (1).

Tobacco Seed Globulin. Crowfoot and Fankuchen (11). Fairly old, dry crystals were used prepared by Vickery. Only three extremely faint powder lines were observed. It would be desirable to have these measurements repeated and an effort to obtain data on wet crystals would be worthwhile.

Excelsin. Astbury, Dickinson and Bailey (12) and Astbury and Bell (13). Astbury and Bell refer to this unpublished work in their paper in Tabulae Biologicae (14).

Bushy Stunt Virus. Bernal, Fankuchen and Riley (15). Material furnished by Bawden and Pirie, and x-ray studies of Bernal, D., and Fankuchen, I., *J. Gen. Physiol.*, **25**, 111, 147 (1941).

NON-X-RAY MOLECULAR WEIGHT DATA

Ribonuclease, 12,700, A. Rothen (16).

Insulin, lactogloblulin, pepsin and *excelsin* are taken from Svedberg (17) and are centrifuge measurements.

Chymotrypsin, 41,000, Kunitz and Northrop (18), measured by osmotic pressure.

Hemoglobin: 66,700 is found by chemical methods. The ultracentrifuge value for horse hemoglobin (17) is 63,000–69,000.

Horse Serum Albumin. From Mehl and Oncley, unpublished measurements of sedimentation and diffusion constants yielding about 70,000.

Tobacco Seed Globulin: only the sedimentation constant 12.7×10^{-13} was known (Philpot, unpublished data). This is similar to the values for the other seed globulins whose molecular weights are about 300,000.

Bushy Stunt Virus: molecular weights 7,600,000 and 8,800,000, McFarlane and Kekwick (19); molecular weight 10,600,000, Lauffer and Stanley (20). These molecular weights were used in determining "n" the number of molecules per unit cell.

DENSITIES

Ribonuclease: I. Fankuchen (1).

Insulin: both needle-shaped and flat rhombohedra were studied by Crowfoot. These were shown to possess identical crystal structure. The needles were imperfect and gave density values a trifle lower than the rhombohedra. The value chosen was the highest observed and was obtained from the largest rhombohedral crystals. Residual water 5.35% in air-dried crystals was determined by drying under reduced pressure at 104°.

Lactoglobulin: Wet Tabular, 1.257 in sugar solution (Crowfoot, 6). Wet Needles, dissolved too rapidly to permit of any density determinations. Dry Crystals found to be 1.27 by immersion in *o*-dichlorobenzene and toluene (6). Crowfoot believes this value too low, due to occlusion of air and uses an assumed density of 1.31 (insulin) in computations of molecular weights. McMeekin and Warner (22) suggest that the density of wet tabular crystals is 1.146 and of the dry 1.26. The wet X-ray molecular weight then becomes 61,100. The wet crystals contain 46% water, and therefore the anhydrous molecular weight is 33,000. For the air dry crystals, the X-ray molecular weight is 39,700. Corrected for 10% residual water, this gives an anhydrous molecular weight of 35,800.

Chymotrypsin: Wet. Determined by Perutz (8). Dry, value of 1.31 assumed (8).

Hemoglobin: Wet. Determined by Perutz (8). Dry, value of 1.26 assumed to correspond with value for serum albumin as given by Chick and Martin.

Tobacco Seed Globulin: measured in sodium phosphate buffer solution at pH 5.0. Residual water 10.4%. Determined by drying in vacuum at 100°.

Excelsin: Value of 1.31 assumed by I. Fankuchen to permit computation of molecular weight. Table in paper by Astbury and Bell gives only unit cell data and dry molecular weight. Assumption is made that this value is corrected for residual water.

Bushy Stunt Virus: Wet crystals, 1.286, measured by immersion. Dry crystals, 1.35, from computations of McFarlane and Kekwick (19).

various states of hydration. At all hydrations, the unit cell was monoclinic. The "*a*" axis changed slightly with changing water content, and the "*b*" axis remained constant. The largest change was in the magnitude of the "*c*" spacing and in the β angle. Patterson projections onto the *b* plane were computed for the different states of hydration and compared. It is concluded "that the dry hemoglobin molecule is seen to be a platelet 36 A thick; it is 64 A long in the direction of *b* and probably somewhat shorter along "*a*." It may consist of one layer in the 36 A direction or at most of two 18 A thick layers which in the hydrated molecule are separated by water and salt.

In the second paper (50), oxyhemoglobin is studied. This crystallizes in an orthorhombic unit cell. Patterson projections were made and compared with those of methemoglobin. There are some similarities which enable reasonable guesses to be made as to the orientation of the molecules in the crystal — providing one assumes that essentially the geometry of both molecules is the same.

In the third paper (51), two different ways were used to determine the phase angles of a limited set (00 *l*) of reflections of methemoglobin. First, the change of intensity of the 00 *l* reflections with change of water content was used to limit the choice of phase angles of these reflections. This narrowed the choice to eight possibilities, all of which, it is claimed, ruled out the possibility of the hemoglobin molecule consisting of a double layer structure separatable by water. By introducing the dimensions and density of the molecules, all but one of the possibilities were eliminated. This

choice was checked by studying the differences in the intensities of the 00 *l* reflections in wet crystals, crystallized in pure water and in ammonium sulfate solution. The resulting Fourier analysis suggests a four layer structure for the molecule — each layer 9 A thick. These layers are tied together, however, and form a rigid molecule.

These three papers make a promising beginning in the detailed study of a native protein structure. It is to be hoped that a fuller publication of this work will be forthcoming soon. Only then can a proper evaluation of its importance be made.

V. MISCELLANEOUS PROTEINS AND RELATED MATERIALS

Schmitt (52) has in a recent review in Vol. 1 of this series carefully reviewed the structural studies of biological tissues, and therefore no further discussion of this group of protein rich materials is necessary here.

Spiegel-Adolf and collaborators have published a series of papers (53, 54, 55, 56) in which X-rays and unoriented specimens have been used to trace the effects of chemical and physical agents on proteins. The customary wide angle diagrams were used. Tuberculin proteins (53) show generally the usual protein halos. The quantitative aspects of this and the other papers of this series are open to criticism. The reviewer cannot see how spacings of halos can be given to one part in a thousand (53, 54) when a 1 mm. diameter pinhole system, a 37.7 mm. specimen to film distance, and unfiltered copper radiation are used. In (53) the line widths are used to compute "crystallite" size. No corrections are apparently made for the instrumental contribution to the line breadth, the radiation is not mono-chromatic, and finally there is every reason to doubt that the computation would be meaningful even if it were properly carried out. The line width computations can only apply when the line is a *single, crystalline* reflection, and almost certainly the halos studied are composite and the material non-crystalline.

Powder diagrams of ferritin and apoferritin (8) can be interpreted in terms of a unit cell since both are cubic. They crystallize in the cubic system with identical unit cells in size, 186 A. The line intensities in the two cases are very different. Apparently the protein decides the packing and the iron somehow fills the interstices.

The polyhedral bodies of silkworm disease (55) give a sharp line diagram (7). The innermost line observed is about 40 A. A cubic unit cell of side 75 A fits the data, but this must be considered as a tentative figure.

Proteins denatured in various ways have been formed into oriented fibers (56, 57) which give the β pattern. This again supports the idea that globular proteins are folded configurations of a polypeptide chain.

VI. SUMMARY

In the not too distant future, it is likely that considerable light will be thrown on the structure of proteins by X-ray diffraction investigations. As the preceding part of this paper has indicated, a beginning has been made. Further results will undoubtedly come from many converging directions. Thus, many approaches will require the making of models — models of polypeptide chains both straight and coiled up in various ways. The recent work by Pauling's group on the crystal structure of diketopiperazine (58), glycine (59), dl-alanine (60), β-glycylglycine (61) will undoubtedly be of considerable value. In these papers, the structures were worked out in detail and thus reliable interatomic bond lengths and angles became available. Most of the results were as expected but the bond, $N-CH_2$, was unexpectedly short in all these structures, 1.41 A. Corey (62) has discussed in detail the interatomic distances found in these amino acid crystals in connection with the work on fibrous proteins. It is not unreasonable to assume that in the globular proteins, interatomic relationships exist similar to those found by Corey and his coworkers in the simpler amino acids.

The fibrous protein field also should soon produce interesting results. Several new and rather attractive pictures of what fibrous protein structures look like have been recently proposed but much more important is the fact that new and better experiments are being performed. It is, moreover, now being realized that specimen preparation is important; that proteins are rather delicate structures and that it is essential that the chemical or physical procedures used in the isolation of a fibrous protein be chosen so as to produce a minimum of change from the native state.

The developments that have been and are being made in the use of Fourier series will certainly play a role in protein investigations, not only in the crystalline proteins where they have already been partly applied but in the fibrous proteins as well. The data which are being obtained particularly from the low angle studies seem eminently suitable for such an analysis.

In the crystalline protein field, the situation is perhaps in one sense analogous to that which existed in steroid structures (63, 64). Here an extensive survey (63) of about 100 steroids indicated which steroid crystals might repay an intensive study (64). Only about ten different crystalline proteins have been studied with X-rays; three: insulin, lactoglobulin, and hemoglobin, in some detail. The choice of these three was probably dictated not by their individual promise, but rather by the fact that suitable crystals of these materials were available. Before much more time is spent on any further intensive studies, it might be wise to attempt a more complete preliminary survey of all available crystalline proteins. Such a survey

might well yield several much more promising proteins than those already studied. Ribonuclease may be one of these. The molecule is very small for a protein, two different crystal forms have been found, the crystals of both forms yield excellent X-ray diagrams, both wet and dry (Fig. 1a, 1b).

The actual mechanics of obtaining the X-ray data may be improved also. Heretofore, with single crystals, the oscillation method has been used. Buerger (65) has devised a new technique which permits the direct photography of the reciprocal lattice, one layer at a time, and he believes that it should be useful for crystals with large unit cells — such as proteins.

Another new approach may be in the study of the low angle X-ray scattering of protein solutions. Chymotrypsin has been so studied (66). The method does not give information concerning the internal structure of the molecule but does permit some computation of the molecular size and shape (67).

In 1939 in a lecture on proteins at the Royal Institution, Bernal said: "The problem of the protein structure is now a definite and not unattainable goal, but for success it requires a degree of collaboration between research workers which has not yet been reached." Six years later, this quotation is still a good description of the writer's feelings.

REFERENCES

1. Friedrich, W., Knipping, P., and Laue, M. (1912). *Sitzber. Math.-Physik. Klasse.* Bayer. Akad. Wiss. München **303**, 1912.
2. Herzog, R. O., and Jancke, W. (1920). *Ber.* **53**, 2162.
3. Freudenberg, K. (1927). *Z. Physiol. Chem.* **204**, 233.
4. Clark, G. L., and Corrigan, K. E. (1932). *Physical Rev.* **40**, 639.
5. Bernal, J. D., and Crowfoot, D. (1934). *Nature* **133**, 794.
6. Crowfoot, D. (1935). *Nature* **135**, 591.
7. Fankuchen, I. Unpublished work. Summary of data. See this paper, Table I.
8. Fankuchen, I. (1943). *J. Biol. Chem.* **150**, 57.
9. Bernal, J. D., Fankuchen, I., and Riley, D. P. (1938). *Nature* **142**, 1075.
10. Bernal, J. D., and Fankuchen, I. (1941). *J. Gen. Physiol.* **25**, 111.
11. Astbury, W. T., and Sisson, W. A. (1935). *Proc. Roy. Soc. (London)* A, **150**, 533.
12. Astbury, W. T., and Dickinson, Sylvia (1940). *Proc. Roy. Soc. (London)* B, **129**, 307.
13. Bragg, W. L. (1933). The Crystalline State. Macmillan, New York, N. Y.
14. Ewald, P. P. (1940). *Proc. Phys. Soc. (London)* **52**, 167.
15. Knott, G. (1940). *Proc. Phys. Soc. (London)* **52**, 229.
16. Wrinch, D. Unpublished Work.
17. Patterson, A. L. (1935). *Z. Krist.* A, **90**, 517.
18. Patterson, A. L. (1935). *Z. Krist.* A, **90**, 543.
19. Harker, D. (1936). *J. Chem. Phys.* **4**, 381.
20. Beevers, C. A., and Lipson, H. (1936). *Nature* **137**, 825.
21. Lipson, H., and Beevers, C. A. (1936). *Proc. Phys. Soc. (London)* A, **48**, 772.
22. Robertson, J. M. (1936). *Phil. Mag.* **21**, 176.
23. Shaffer, P. A., Jr., and Pauling, L. Unpublished work.
24. Bragg, W. L. (1939). *Nature* **143**, 678. (1942). *Nature* 149, 470.

25. Bragg, W. L. (1929). *Z. Krist.* **A, 70,** 475.
26. Buerger, M. J. (1939). *Proc. Natl. Acad. Sci.* **25,** 383. (1941). *Proc. Natl. Acad. Sci.* **27,** 117.
27. Huggins, M. L. (1941). *J. Am. Chem. Soc.* **63,** 66.
28. Bragg, W. L. (1942). *Nature* **149,** 470.
29. Robertson, J. M. (1938). *Reports on Progress in Physics* **IV,** p. 332.
30. Huggins, M. L. (1944). *Colloid Chemistry* (*ed.* Alexander), Vol. V, p. 131.
31. Astbury, W. T. (1934). *Cold Spring Harbor Symposia. Quant. Biol.* **2,** 15.
32. Astbury, W. T. (1939). *Ann. Rev. Biochem.* **8,** 113.
33. Astbury, W. T. (1942). *J. Chem. Soc.,* May, p. 337.
34. Neurath, H. (1940). *J. Phys. Chem.* **44,** 296.
35. Bull, H. B. (1941). *Advances Enzymology,* p. 1. (Interscience).
36. Huggins, M. L. (1943). *Chem. Revs.* **32,** 195.
37. Pauling, L., and Niemann, C. (1939). *J. Am. Chem. Soc.* **61,** 1860.
38. MacArthur, I. (1943). *Nature* **152,** 38.
39. Bear, R. S. (1944). *J. Am. Chem. Soc.* **66,** 2043.
40. Bear, R. S. (1944). *J. Am. Chem. Soc.* **66,** 1297.
41. Fankuchen, I. (1941). *Cold Spring Harbor Symposia* **9,** 198.
42. Wyckoff, R. W. G., and Corey, R. B. (1936). *J. Biol. Chem.* **116,** 51.
43. Fankuchen, I. (1945). Chap. 14 in Weissberger, A., Physical Methods of Organic Chemistry, Vol. 1. Interscience, New York, N. Y.
44. McMeekin, T. L., and Warner, R. C. (1942). *J. Am. Chem. Soc.* **64,** 2393.
45. Crowfoot, D. (1938). *Proc. Roy. Soc. (London)* **A, 164,** 580.
46. Crowfoot, D., and Riley, D. (1939). *Nature* **144,** 1011.
47. Crowfoot, D. (1941). *Chem. Revs.* **28,** 215.
48. Wrinch, D. (1937). *Trans. Faraday Soc.* **43,** 1368.
49. Perutz, M. F. (1942). *Nature* **149,** 491.
50. Perutz, M. F. (1942). *Nature* **150,** 324.
51. Boyes-Watson, J., and Perutz, M. F. (1943). *Nature* **151,** 714.
52. Schmitt, F. O. (1944). *Advances in Protein Chemistry* **1,** 25.
53. Spiegel-Adolf, M., Seibert, F. B., and Henny, G. C. (1941). *J. Biol. Chem.* **137,** 503.
54. Spiegel-Adolf, M., Hamilton, R. H., Jr., and Henny, G. C. (1942). *Biochem. J.* **36,** 825.
55. Glaser, O., and Child, G. P. (1937). *Biol. Bull.* **73,** 205.
56. Palmer, K. J., and Galvin, J. A. (1943). *J. Am. Chem. Soc.* **65,** 2187.
57. Senti, F. R., Eddy, C. R., and Nutling, G. C. (1943). *J. Am. Chem. Soc.* **65,** 2473.
58. Corey, R. B. (1938). *J. Am. Chem. Soc.* **60,** 1598.
59. Albrecht, G., and Corey, R. B. (1939). *J. Am. Chem. Soc.* **61,** 1087.
60. Levy, H. A., and Corey, R. B. (1941). *J. Am. Chem. Soc.* **63,** 2095.
61. Hughes, E. W., and Moore, W. J. (1942). *J. Am. Chem. Soc.* **64,** 2236.
62. Corey, R. B. (1940). *Chem. Revs.* **26,** 227.
63. Bernal, J. D., Crowfoot, D., and Fankuchen, I. (1940). *Trans. Roy. Soc. (London)* **A, 802, 135.**
64. Crowfoot, D. (1944). *Vitamins and Hormones* **2,** 409 (Academic Press, New York, N. Y.).
65. Buerger, M. J. (1944). Am. Soc. X-Ray Elect. Diff., Monograph No. 1.
66. Kratky, O., and Sekora, A. (1943). *Naturwissenschaften* **31,** 46.
67. Fankuchen, I., and Mark, H. (1944). *J. App. Phys.* **15,** 364.

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* Numbers in parenthesis are reference numbers. They are included to assist in locating references in which the authors' names are not mentioned in the text. Italic numbers refer to bibliographies of the different papers.

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