The physical chemistry of the proteins / by T. Brailsford Robertson.

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Physiology

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OF
THE PROTEINS

"From the point of view of the physicist, a theory of matter is a policy rather than a creed; its object is to connect or co-ordinate apparently diverse phenomena, and above all to suggest, stimulate and direct experiment. It ought to furnish a compass which, if followed, will lead the observer further and further into previously unexplored regions. Whether these regions will be barren or fertile experience alone will decide; but, at any rate, one who is guided in this way will travel onward in a definite direction, and will not wander aimlessly to and fro." J. J. Thomson, "The Corpuscular Theory of Matter."

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BY

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LONGMANS, GREEN AND CO.
FOURTH AVENUE & 30TH STREET, NEW YORK
39 PATERNOSTER ROW, LONDON
BOMBAY, CALCUTTA, AND MADRAS
1918

DEDICATED TO MY MOTHER

IN AFFECTIONATE ACKNOWLEDGMENT
OF ENCOURAGEMENT AND
INSPIRATION

PREFACE

THE Proteins, with which, as its title indicates, this work has most particularly to deal, have, ever since the publication of the classic researches of Graham, been generally recognized as typical examples of that class of substances which Graham designated "Colloids." This work, therefore, although primarily concerned with the physical chemistry of a limited section of the class, may also, in some measure, be regarded as contributing to an analysis of the properties and behavior of colloids in general, in so far as these permit of illustration by the properties and behavior of the various members of the protein group.

The leading problems in every field of chemical investigation are, to a large extent, determined by the salient properties of the substances which form the subject of inquiry, and for this reason a thorough acquaintance with what may be termed the "descriptive chemistry" of any group of substances is prerequisite to a successful attempt to interpret their behavior. If we were to endeavor to interpret the behavior of the proteins solely in the light of preconceptions derived from the study of the chemistry of the metals, for example, or the simpler organic compounds, we would find that the behavior of the proteins displays merely a bewildering variety of inconsistencies. Their "amphoteric" character, their multiple basicity and acidity, their instability in aqueous solution and, above all, the enormous mass and catenary configuration of their molecules, confer upon them properties which are comparatively unfamiliar in other fields of chemistry or else exaggerate properties which are normally displayed by the simpler chemical bodies only to a comparatively negligible degree.

The proteins in this respect are not exceptional. Thus the mode of investigation and the interpretation of the behavior of the various lipoids must primarily be conditioned by their very general insolubility in water and instability in the presence of oxygen. The behavior of the complex polysaccharides is conditioned by their relative stability and by the extraordinary tendency to op-

tical isomerism which is displayed by the simpler carbohydrate radicals out of which they are built up, while, on the other hand, the behavior of rubber and its congeners is primarily conditioned by the enormous internal molecular friction which leads them to display, to an exaggerated extent, phenomena analogous to hysteresis which, although not wholly negligible in other colloids, for example in the protein group, nevertheless seldom present themselves as salient characteristics of their behavior.

The colloids are therefore an exceedingly heterogeneous group, the only common distinguishing characteristic being the relatively enormous mass and volume of the molecules of the most "typical" representatives of the class, and of course as many gradations of behavior exist as there are gradations in the mass and volume of molecules. Nothing is to be gained, therefore, by endeavoring to force the various members of the colloid group into artificial conformity with definitions which are designed to separate them, as if they were a homogeneous group, from other classes of chemical substances. To describe a particular property or mode of behavior as a "colloidal phenomenon" neither defines nor interprets it and furthermore fails even to describe it, since there are no phenomena which are distinctively "colloidal" and displayed by every member of the colloid group, saving only those phenomena which depend primarily upon the simple factor of the mass or volume of the molecules, and which are therefore predictable from and implied in the properties or behavior of the smaller molecules of the non-colloidal substances.

Similarly, the use of the term "adsorption" to describe the union between colloids and other substances implies a uniformity where no uniformity exists and is, moreover, devoid of utility or meaning unless we attach to the definition some distinct idea of the nature of the underlying forces which condition the union, whether these forces be regarded as consisting of chemical (*i.e.*, atomic) attractions or of capillary (*i.e.*, molecular) attractions. But in forming such concrete ideas we are simply returning to conceptions which are already familiar to us in the "crystalloid" field of chemistry and which call for no definitions which we do not already possess as the result of our general acquaintance with the physical and chemical phenomena which are displayed by simpler and hitherto more familiar substances.

The investigations of recent years, not only upon the behavior

of the proteins, but upon that of the colloids in general, have resulted in the development of two rather sharply differentiated schools of opinion. The one school endeavors, so far as technical difficulties permit, to directly apply, with modifications suggested by the properties and structure of the particular colloid under investigation, the known laws of what may be termed "molecular" physical chemistry to protein and other colloidal systems, while the other school hesitates to do so. The essential question at issue between these two schools, in so far as the proteins are concerned, is, I think, simply this: Are we justified in assuming that the rule of Avogadro is applicable to protein solutions or are we not? In other words, are protein solutions molecularly dispersed systems or are they, rather, suspensions or emulsions? The latter of the two schools to which I have referred avers from *à priori* considerations, implicitly or explicitly, that Avogadro's rule may not be applied to protein solutions, or, at least, that its validity for these solutions should be established before we venture to apply it. The former school prefers to assume that Avogadro's rule does apply to these systems until actual inapplicability demonstrates that it does not. Now the "proof" of the applicability of Avogadro's rule to systems which are admittedly molecularly dispersed has never consisted in anything but the applicability, to these systems, of laws and deductions founded upon Avogadro's rule. The procedure of the former school would appear, therefore, to be sound and well justified by scientific precedent. Following this procedure we will be enabled to correlate and interpret the phenomena which are exhibited by such protein systems as may chance to be molecularly dispersed, while, on the other hand, we shall be enabled to accurately delimit the conditions under which molecularly dispersed protein systems exist and those under which they do not.

In this work I have endeavored to interpret the physico-chemical behavior of the proteins in the light of the laws of Boyle and of Gay-Lussac, as they have been applied to solutions by van't Hoff, and of the Guldberg and Waage mass-law which, as Larmor has shown, is a direct consequence of Avogadro's rule and Boyle's law.* I have also assumed the validity, in protein systems, of the first and second laws of heat, albeit the applicability of the

* Larmor, J., Phil. Trans. Roy. Soc. London, 190 A (1887), p. 276. T. Brailsford Robertson, Journ. Physical Chem., 10 (1906), p. 521.

second law of heat to protein systems has, in some quarters,* been questioned. In considering the *electrochemical* behavior of the proteins I have assumed the applicability of Arrhenius' hypothesis of electrolytic dissociation, of Kolrausch's law of the independent motion of ions, of the Nernst theory of concentration-cells, and further, although this has of recent years been very strongly questioned,† the applicability of the Guldberg and Waage mass-law to reactions between ions. I believe that the utility of these hypotheses justifies us in applying them until still more useful hypotheses shall have been elaborated to amplify or replace them.

A previous edition of this work appeared, in German, six years ago.‡ Since that time our knowledge of the physico-chemical behavior of the proteins has very considerably expanded and increased in exactitude. Among the particularly important investigations which have appeared during this period may be mentioned the invention by Van Slyke of an accurate and simple method of determining free amino-groups (Cf. Chapter I), the work of Pauli upon the combining-capacity of deaminized proteins (Cf. Chapters I, VIII and IX), the work of Schmidt and of af Ugglas upon compound proteins (Cf. Chapter VII), the work of Procter upon the swelling of protein jellies (Cf. Chapter XII), the work of Reichert and Brown upon the crystallography of hæmoglobin (Cf. Chapter XII) and that of Dabrovsky upon the molecular volumes of proteins dissolved in water and in solutions of coagulating salts (Cf. Chapter XIII). The present edition has been almost entirely rewritten and the literature has been brought down to the middle of 1917.

In conclusion I wish to express my very great indebtedness to my wife, for her assistance in preparing the manuscript for the press, to Dr. Hardolph Wasteneys for his assistance in proof-reading, and to Dr. C. L. A. Schmidt for his assistance in proof-reading and in the verification of many references and formulæ.

T. BRAILSFORD ROBERTSON.

BERKELEY, CALIFORNIA,
Nov. 1, 1917.

* von Schroeder, P., Zeit. f. physik. Chem., 45 (1903), p. 75. Dietz, W., Zeit. f. physiol. Chem., 52 (1907), p. 279. Cf., however, Chapter XVII.

† Cf. for example, W. Sutherland, Phil. Mag. Series 6, 14 (1907), p. 1.

‡ "Die physikalische Chemie der Proteine," Dresden, 1912. Theodor Steinkopff.

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PART I

CHEMICAL STATICS IN PROTEIN SYSTEMS

CHAPTER I

THE CHEMICAL CONSTITUTION OF THE PROTEINS

1. The Chemical Homogeneity of the Protein Group. — The chemical similarity between the properties and behavior of the various protein bodies, and the evidence thus afforded of their close chemical relationship, early led protein chemists to ascribe to the proteins a common and characteristic chemical structure, and to endeavor to anticipate the nature of this structure.

The difficulty of securing a quantitative separation of protein bodies is eloquent of their chemical similarity; one by one the standard methods of separation brought forward by the physiological chemist, such as fractional heat-coagulation, fractional precipitation by salts, etc., have been shown to possess but a qualitative value; the separation is seldom or never complete, and the critical temperatures, concentrations, etc., at which precipitations occur are seldom well-defined points, but appear, rather, to represent portions of a continuous curve of solubility. The close relationship between the various protein bodies is furthermore evinced by the similarity of their physical properties, and by the almost universal applicability, within the protein group, of such typical color or other reactions as the proteins display. The general physico-chemical characteristics of the various proteins resemble one another, as we shall see in subsequent chapters, quite as closely as their purely physical or purely chemical characteristics. One of the most striking of these physico-chemical characteristics is their digestibility by the proteolytic enzymes. All true proteins and a large number of the poly-amino-acids are hydrolysable by trypsin, while the overwhelming majority of the proteins are also hydrolysable by pepsin. The chemical relationship between the various proteins would therefore appear to be even more intimate than that between the various disaccharides, since the enzymes which accelerate the hydrolysis of these are, as a rule, specific, the hydrolysis of each

sugar being accelerated to a high degree by one enzyme and by that alone.

2. The Products of the Decomposition of the Proteins. — The proteins are notoriously liable to decomposition, and a complete breaking down of the sparingly diffusible, difficultly crystallizable proteins, into substances which readily diffuse and crystallize can be brought about by a variety of agencies, such as the following:

I. Fusion with alkali.
II. Oxidation with permanganate, chromic acid, etc.
III. The action of halogens.
IV. Hydrolysis, through one or more of the following agencies:

a. Heating in acid solution,
b. Heating in alkaline solution,
c. Treatment with superheated steam.
d. Treatment with enzymes.

Of all these methods that of hydrolysis is the most satisfactory and yields the most uniform and readily interpretable results. It appears that whatever be the method of hydrolysis employed, the end-result, provided the hydrolysis has been complete, is the same, namely, the production of a mixture of amino-acids.

Incomplete hydrolysis, however, results in the production of a number of intermediate substances, variously designated, in the order of decreasing complexity, proteoses (albumoses), peptones and polypeptids. The hydrolysis of the proteins, therefore, occurs in stages, just as, in the hydrolysis of starch, intermediary stages (the dextrins and maltose) are passed through, before the attainment of the last stage of hydrolysis and the complete conversion of the starch into glucose.

It was early recognized that a predominating proportion of the products of the complete hydrolysis of proteins consists of amino-acids, and the most readily detected amino-acids, leucin and tyrosin, were discovered, respectively by Proust in 1818 (54), and by Liebig in 1846 (44). The chemical constitution of these substances, however, was a discovery of much later date, that of leucin having been established in 1868 by Hüfner (31) and of tyrosin in 1869 by Barth (3).

The older methods of isolating individual amino-acids from the mixture which the complete hydrolysis of a protein yields, de-

pended upon the fractional crystallization either of the free amino-acids or of their salts. They were not even approximately quantitative and the isolation and identification of a given amino-acid could only be effected with certainty when that acid was present in relatively large amounts. Until 1890 only mono-amino-acids were known, with certainty, to occur among the products of protein hydrolysis. Then Drechsel (13) discovered lysin and lysatinin, Hedin (29) isolated arginin and Kossel (34) isolated histidin from among the dissociation products of proteins.

The attainment of our present relatively extensive knowledge of the nature and yield of the products of the hydrolysis of proteins is an achievement of the past twenty years, and we owe it in the first place to the labors of Emil Fischer and of Kossel and their pupils.

In 1900 Kossel and Kutscher (39) (40) succeeded in working out a method for the quantitative separation and estimation of the diamino-acids lysin, arginin and histidin which Kossel (35) calls the hexone bases.* The method depends in principle upon the precipitation of arginin and histidin in the form of their silver salts, and of lysin first by phosphotungstic acid and then by picric acid. A partial but not quantitative separation of the diamino-acids from the monoamino-acids in a protein digest, can be procured by precipitation of the former with phosphotungstic acid.

In 1901 Emil Fischer (15) introduced a new method of separating and estimating the monoamino-acids, which, with modifications suggested by experience, is the one to which we owe the greater part of our present knowledge of these products of protein hydrolysis. The hydrolysis is carried out, as a rule, by boiling with hydrochloric acid.† The amino-acids which result are then converted into their esters by dissolving them in alcohol and esterifying by saturation of the solution with dry hydrochloric acid gas. The mixed esters thus obtained are separated by

* Owing to the fact that they each contain six atoms of carbon. An especial interest attaches to these substances, since, unlike the monoamino acids, they are predominantly basic in character, and their relation to the hexoses suggests their possible importance in carbohydrate metabolism.

† N. Zelinsky (74) has suggested the employment of formic acid as a hydrolysing agent. According to this investigator the hydrolysis by formic acid is much more rapid than that which is brought about by boiling the proteins with hydrochloric acid.

fractional distillation *in vacuo*.* The different fractions which are thus obtained each contain the esters of only a few amino-acids. The esters in each fraction are now reconverted into the free amino-acids, and the individual acids are separated, identified and estimated by appropriate methods which differ somewhat for the different acids.

The method, except so far as glutamic acid is concerned, is by no means quantitative. The results which are obtained are minimal yields. The extremely insoluble amino-acids, tyrosin, cystin, and diaminotrioxydodecanic acid are separated from the digest before esterification, and, so far as tyrosin is concerned, our estimate is tolerably quantitative.

By a combination of these methods it has been shown that the protein molecule is built up of a series of amino-acids, and the following amino-acids have been isolated from amongst the products of the hydrolysis of various proteins.†

A. Monoaminomonocarboxylic Acids

1. Glycin: $C_2H_5NO_2$, or amino-acetic acid:
   $CH_2.NH_2.COOH$.
2. Alanin: $C_3H_7NO_2$, or α-aminopropionic acid:
   $CH_3.CH(NH_2).COOH$.
3. Valin: $C_5H_{11}NO_2$, or α-aminoisovalerianic acid·
   $\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!\diagdown\!\!\diagup CH.CH(NH_2)COOH$.
4. Leucin: $C_6H_{13}NO_2$, or α-aminoisocaproic acid:
   $\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!\diagdown\!\!\diagup CH.CH_2.CH(NH_2)COOH$.
5. Isoleucin: $C_6H_{13}NO_2$, or α-amino-β-methyl-β-ethylpropionic acid:
   $\begin{matrix} CH_3 \\ C_2H_5 \end{matrix}\!\!\diagdown\!\!\diagup CH.CH(NH_2).COOH$.
6. Phenylalanin: $C_9H_{11}NO_2$, or β-phenyl-α-aminopropionic acid:
   $C_6H_5.CH_2.CH(NH_2).COOH$.
7. Tyrosin: $C_9H_{11}NO_3$, or β-parahydroxyphenyl-α-aminopropionic acid:
   $HO.C_6H_4.CH_2.CH(NH_2)COOH$.

* N. Zelinsky, A. Annenkov, and J. Kulikov (75) accomplish the separation of the individual amino-acids by first neutralizing the excess of hydrochloric acid with lead hydroxide and then subjecting the mixed hydrochlorides of the ethyl esters to fractional distillation.

† Cited from Aders Plimmer (53).

8. Serin: $C_3H_7NO_3$, or β-hydroxy-α-aminopropionic acid:
$CH_2(OH).CH(NH_2)COOH$.

9. Cystin: $C_6H_{12}N_2O_4S_2$ or dicystein, or di-(β-thio-α-aminopropionic acid):
$HOOC.CH(NH_2).CH_2.S - S.CH_2.CH(NH_2).COOH$

B. Monoaminodicarboxylic Acids

10. Aspartic acid, $C_4H_7NO_4$, or aminosuccinic acid.
$HOOC.CH_2.CH(NH_2).COOH$.

11. Glutamic acid, $C_5H_9NO_4$, or α-aminoglutaric acid:
$HOOC.CH_2.CH_2.CH(NH_2)COOH$.

C. Diaminomonocarboxylic Acids

12. Arginin, $C_6H_{14}N_4O_2$, or α-amino-δ-guanidinvalerianic acid:

$$HN = C\begin{matrix}\diagup NH_2 \\ \diagdown NH.CH_2.CH_2.CH_2.CH(NH_2)COOH.\end{matrix}$$

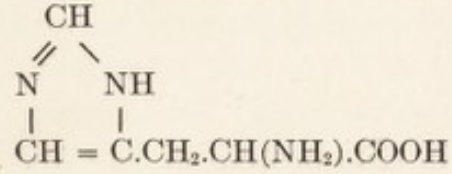
13. Lysin: $C_6H_{14}N_2O_2$ or α-ϵ-diaminocaproic acid:
$H_2N.CH_2.CH_2.CH_2.CH_2.CH(NH_2).COOH$.

D. Diamino-oxy-monocarboxylic Acids

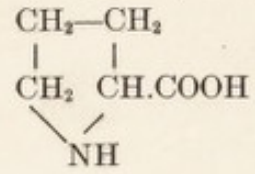
14. Caseinic acid, or diaminotrioxydodecanic acid:
$C_{12}H_{26}N_2O_5$.

E. Heterocyclic Compounds

15. Histidin, $C_6H_9N_3O_2$, or β-iminazolyl-α-aminopropionic acid:

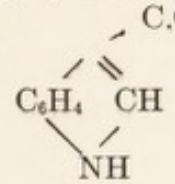


16. Prolin, $C_5H_9NO_2$, or α-pyrrolidin carboxylic acid:



17. Oxyprolin, or oxypyrrolidin carboxylic acid:
$C_5H_9NO_3$.

18. Tryptophane, $C_{11}H_{12}N_2O_2$, or β-indole-α-aminopropionic acid:



3. The Connection between the Amino-Acid Content and the Properties of the Proteins. — The various proteins are found to be built up of the several units enumerated above together, possibly, with units which we have not yet succeeded in isolating. Some of the proteins are built up out of all of these units in varying proportions, others out of a lesser number, so that in some cases amino-acids which are predominant among the products of the hydrolysis of one protein are absent from among the products of the hydrolysis of another. Nevertheless the general relationship of the various units and their occurrence in different proportions in the various proteins accounts not only for the differences, but also for the similarity between them.

In many instances a definite parallelism can be traced between the chemical and physical behavior of the proteins and their amino-acid content. Thus the albumins, which are soluble in distilled water and are not coagulated by half-saturation of their solutions with ammonium sulphate, contain no glycin, while the globulins which are (when uncombined with bases or acids) insoluble in distilled water, and are coagulated by half-saturation of their solutions with ammonium sulphate, do contain this amino-acid. The alcohol-soluble vegetable proteins contain only a trace (probably attributable to associated impurities) of glycin and some of them contain no lysin, their content of diamino-acids is very small, while their content of glutamic acid and of prolin is very high. The phosphoproteins (casein, vitellin) are also rather high in glutamic acid. Gelatin is characterized by its high glycin content and keratin (the protein of hair and feathers) by its high cystin content. The histones, which are predominantly basic substances, contain about 30 per cent of diamino-acids, while the protamins, which are still more predominantly basic, contain only small amounts of monoamino-acids, salmin containing over 80 per cent of arginin, while sturin contains 67 per cent of its nitrogen as arginin, 10 per cent in the form of histidin and from 6 to 7 per cent in the form of lysin (41).

Kossel (35) has expressed the view that all proteins are built up around a protamin nucleus and that this accounts for the fact that the majority of the proteins yield, on hydrolysis, a certain proportion of diamino-acids.

4. The Synthesis of Proteins. — The marked predominance of amino-acids in the products of protein hydrolysis, long ago led protein chemists to surmise that the amino-acid structure, or some derivative of that structure, must be represented in a high degree in the protein molecule, and it was in following this clue that Schützenberger (64) carried out one of the earliest and most successful attempts to synthesize bodies of a protein character. Recognizing that the decomposition of proteins into amino-acids is essentially a phenomenon of hydrolysis, he regarded dehydration as an essential feature of any attempt at protein synthesis, while the abundance of amino-acids among the products of protein hydrolysis and the presence therein of bodies related to urea, led him to believe that protein synthesis must consist in the linkage of amino-acids with molecules of urea and the elimination of water. Accordingly amino-acids were mixed with urea and phosphorus pentoxide and heated to 125° C. The product was a pasty solid, soluble in water and readily precipitated by alcohol. It was, furthermore, precipitated from aqueous solution by the usual protein precipitants and gave the biuret and xanthoproteic reactions.

This experiment of Schützenberger's left us, however, very much where we were, so far as real knowledge of the structure of the protein molecule is concerned. The knowledge of the fact that a mixture of amino-acids and urea yields, under certain treatment, a body or bodies more or less closely resembling the proteins, furnished us little or no information regarding the structure of the protein molecule which we did not already possess in the fact that the disintegration products of the proteins are predominantly amino-acids. Prior to Schützenberger, Grimaux (28) had shown that condensation-products of amino-benzoic acid and of aspartic acid (probably comparable with the octaspartic acid of Schiff (62)) resemble the proteins in many of their properties; but these experiments also threw no light upon the structure of the protein molecule beyond emphasizing the already sufficiently evident probability that the amino-acid grouping plays an important part in the building up of the protein molecule.

The clue which led, through a series of remarkable researches to our present comparatively extensive knowledge of the groupings within the protein molecule, was obtained by Curtius (9) who,

in 1883, observed that ethyl glycocollate, in watery solution, tended to form glycocoll anhydride:

(In the absence of water)

$$\underset{\text{(Glycocoll)}}{NH_2.CH_2.COOH} + C_2H_5OH = \underset{\text{(Ethyl glycocollate)}}{NH_2.CH_2.COOC_2H_5} + H_2O.$$

(In the presence of water)

$$\underset{\text{(Ethyl glycocollate)}}{NH_2.CH_2.COOC_2H_5} + \underset{\text{(Ethyl glycocollate)}}{NH_2.CH_2COOC_2H_5} =$$

$$O=C\begin{matrix}\diagup CH_2-NH \diagdown \\ \diagdown NH.CH_2 \diagup\end{matrix}C=O + 2\,C_2H_5OH.$$

(Glycocoll anhydride)

Obviously, if the closed ring representing the glycocoll anhydride molecule could be opened up without destroying the stability of the molecule, a new amino-acid would be formed, one degree more complex than the original amino-acid (glycocoll). This possibility occurred to Emil Fischer, and he found, in fact, that if the glycocoll anhydride which is thus prepared be boiled for a short time with concentrated hydrochloric acid, the following change occurs:

$$\underset{\text{(Glycocoll anhydride)}}{O=C\begin{matrix}\diagup CH_2.NH \diagdown \\ \diagdown NH.CH_2 \diagup\end{matrix}C=O} + HCl + H_2O = \underset{\text{(Glycyl-glycin chloride)}}{O=C\begin{matrix}\diagup CH_2.NH_2.HCl \\ \diagdown NH.CH_2.COOH\end{matrix}}$$

On now treating the glycyl-glycin chloride with silver oxide, silver chloride is precipitated and free glycyl-glycin is obtained. (23). If, however, the glycocoll anhydride be originally treated with *alcoholic* instead of with watery HCl, the ethyl ester of glycyl-glycin is obtained:

$$\underset{\text{(Glycocoll anhydride)}}{O=C\begin{matrix}\diagup CH_2.NH \diagdown \\ \diagdown NH.CH_2 \diagup\end{matrix}C=O} + C_2H_5OH = \underset{\text{(Glycyl-glycin ester)}}{O=C\begin{matrix}\diagup CH_2NH_2 \\ \diagdown NH.CH_2.COOC_2H_5\end{matrix}}$$

It would almost appear, therefore, as if we had only to repeat this cycle of operations indefinitely in order to secure the most complex poly-amino-acids; but this is not so easy as it might appear at first sight; the instability of amino-acids consequent upon the high reactivity of the NH_2 group, and the consequent difficulty of obtaining simple anhydrides renders this procedure

impossible. Moreover the anhydride-ring is in many cases (e.g., leucin anhydride) very difficult to break up when it has once been formed.

In the search for methods of overcoming these difficulties, Fischer found that the instability of the amino-acids could be eliminated by the introduction of radicals into the NH_2 group, and he and Fourneau synthesized phenyl-cyanate-glycyl-glycin and carboxyethyl-glycyl-glycin ester ($C_2H_5O.OC.NH.CH_2.CO.NH.CH_2.CO.OC_2H_5$) which are both chemically stable bodies. In subsequent investigations Fischer found that by gentle heating, combination between the esters of the carboxyethyl amino-acids and other amino-acid esters could be directly brought about (25); in this way carboxyethyl-diglycyl-leucin ester was formed ($C_2H_5O.OC.NH.CH_2CO.NH.CH_2.CO.NH.CH.C_4H_9.CO.OC_2H_5$).

The difficulty was here encountered, however, that the carboxyethyl group, once introduced, cannot be eliminated again.

The method which Fischer devised to overcome this difficulty (25) was extremely ingenious. The introduction of a radical into the NH_2 group appeared to be a necessity, forced upon us by the impossibility of otherwise securing simple anhydrides of the acids. It occurred to Fischer, however, that the radical thus introduced into the NH_2 group might itself be made a carrier of amino-acid groups into the molecule. This anticipation proved to be correct. The radical which Fischer first utilized was the chloracetyl group ($ClCH_2.CO-$); when chloracetyl is allowed to act upon glycyl-glycin ester (obtained by the methods described above) chloracetyl-glycyl-glycin ester is obtained:

$$\underset{\text{(Chloracetyl chloride)}}{Cl.CH_2.COCl} + \underset{\text{(Glycyl-glycin ester)}}{NH_2.CH_2.CO.NH.CH_2.COOC_2H_5} = \underset{\text{(Chloracetyl glycyl-glycin ester)}}{ClCH_2CO.NH.CH_2.CO.NH.CH_2.COOC_2H_5} + HCl.$$

By saponification of this ester, free chloracetyl-glycyl-glycin is obtained; on now treating this with a concentrated aqueous solution of ammonia, the Cl atom in the chloracetyl group becomes replaced by an $-NH_2$ group and diglycyl-glycin is obtained:

$$\underset{\text{(Chloracetyl-glycyl-glycin ester)}}{ClCH_2.CO.NH.CH_2.CO.NH.CH_2.COOC_2H_5} + 2NH_3 = \underset{\text{(Diglycyl-glycin ester)}}{NH_2.CH_2.CO.NH.CH_2CO.NH.CH_2.COOC_2H_5} + NH_4Cl.$$

In other words, the chloracetyl group, introduced to protect the NH_2 group of the amino-acid is, after it has performed its protective function, itself transformed into an amino-acid group, through the replacement of the halogen atom by NH_2. Obviously, other halogen-containing acid groups may be used in place of chloracetyl, and in this way a great variety of amino-acid groups can be introduced into the NH_2 group. Thus Fischer employs:

Chloracetyl-chloride for the introduction of glycyl.
α-Bromopropionyl-chloride for the introduction of alanyl.
l-α-Bromopropionyl-chloride for the introduction of d-alanyl.
α-Bromobutyryl-chloride for the introduction of α-aminobutyryl.
α-Bromisocapronyl-chloride for the introduction of leucyl.
α-Bromophenylacetyl-chloride for the introduction of phenylglycyl.
α-Bromohydrocinnamyl-chloride for the introduction of phenylalanyl.
α-Phenylbromopropionyl-chloride for the introduction of phenylalanyl.
α-δ-Dibromovaleryl-chloride for the introduction of prolyl.
Fumaryl-chloride for the introduction of asparagyl.

By this method the chain of amino-acids is lengthened at the amino-group end. Theoretically, it appeared possible to also lengthen the chain at the carboxyl end of the molecule, by acting upon the esters of the amino-acids with the acid chlorides of other amino-acids. Until 1904, however, the acid chlorides of amino-acids were unknown and all attempts to prepare them had failed, owing to the same reason which limits the use of the first method of synthesizing poly-amino-acids, described above, namely the reactivity of the NH_2 group. It will be recollected that Fischer found that the NH_2 group could be protected by the introduction of radicals, and, utilizing this fact, in 1904 Fischer succeeded in devising a method of preparing the acid chlorides of the amino-acids (17). The acid chlorides thus prepared react with the esters of other amino- or poly-amino-acids to form poly-amino-acid chains of greater length. Thus:

$$\underset{\text{(Bromisocapronylglycyl chloride)}}{C_4H_9.CHBr.CO.NH.CH_2COCl} + 2\,\underset{\text{(Glycin ester)}}{NH_2.CH_2.COOC_2H_5}$$

$$= \underset{\text{(Glycin ester hydrochloride)}}{HClNH_2.CH_2.COOC_2H_5} + \underset{\text{(Bromisocapronylglycyl-glycin ester)}}{C_4H_9.CHBr.CO.NH.CH_2CO.NH.CH_2COOC_2H_5}.$$

Subsequent saponification of the bromisocapronyl-glycyl-glycin ester and treatment with ammonia yields the poly-amino-acid (tripeptid) leucyl-glycyl-glycin:

$$C_4H_9.CH(NH_2).CO.NH.CH_2.CO.NH.CH_2.COOH.$$

If the bromisocapronylglycylchloride be made to act upon glycyl-glycin ester, and the product be treated in the same way, the *tetrapeptid*, leucyl-diglycyl glycin results:

$$C_4H_9.CH(NH_2).CO.NH.CH_2CO.NH.CH_2CO.NH.CH_2.COOH.$$

These methods of synthesis proved inadequate where hydroxy-amino-acids are concerned, because the phosphorus pentachloride, used in the formation of the acid chloride, attacks the OH group. It was, however, ascertained that the OH could be protected by acting upon the hydroxy amino-acid with methyl chlorcarbonate (20), which converts the OH into $-OCO_2CH_3$, which is not attacked by PCl_5 and is readily removed by hydrolysis. In this way it has proved possible to introduce phenyl-carboxylic acids, such as tyrosin, into poly-amino-acid chains.

By these methods, and modifications of these methods, Fischer has succeeded in building up long chains of amino-acid groups, these chains being collectively termed, by Fischer, *peptids*. Chains consisting of two links, i.e., combinations of two amino-acids, Fischer terms dipeptids; such, for example, are glycyl-glycin, alanyl-alanin and leucyl-leucin; chains consisting of three links he terms tripeptids, such being, for example, diglycyl-glycin, and leucyl-glycyl-glycin; chains consisting of four links are termed tetrapeptids, and so on, the higher members of the series being collectively termed *polypeptids*.

The surpassing interest of these investigations lies in the fact that Fischer considers many of his polypeptids to be, in all probability, identical with some of the natural peptones and sub-peptones; while others probably merit inclusion among the proteins themselves. Thus the octadecapeptid l-leucyl-triglycyl-l-leucyl-triglycyl-l-leucyl-octaglycyl-glycin, and the tetradeca-peptid l-leucyl-triglycyl-l-leucyl-octaglycyl-glycin so closely resemble, in general properties, the ordinary proteins, that, as Fischer puts it, they would have been classed as proteins had they been first met with in nature (18) (19). Thus they give the biuret reaction, form opalescent watery solutions, and the tetradecapeptid is precipitated by ammonium sulphate, by tannic acid and by phosphotungstic acid. As they do not contain tyrosin, tryptophane or cystin they fail to give such protein color reactions as depend upon the presence of these groups. The molecular weight of the octadecapeptid is 1213, and the

substitution of phenylalanin, tyrosin and cystin in the place of glycin groups would increase this weight two or three times, giving a value which is of the same order as that of the more modern estimations of the (minimal) molecular weights of many of the natural proteins.*

A whole series of the polypeptids give the typical peptone biuret reaction, and such as contain tyrosin give also Millon's reaction. The biuret reaction is, with the glycin compounds, first encountered in the tetrapeptid, but it is given by other tripeptids. It is more intense the greater the length of the polypeptid chain, and it is also intensified by the carboxyl group or by conversion of the carboxyl group into an acid amide group. The majority of the polypeptids are readily soluble in water, and such as are with difficulty soluble in water are readily soluble in dilute mineral acids and alkalies with which they combine; they are less soluble in solutions of acetic acid. As a rule they are insoluble in absolute alcohol, but in alcohol containing a little watery ammonia they are generally soluble; on boiling off the ammonia they are precipitated again. Under conditions involving dehydration, e.g., heating or treatment of the esters with alcoholic ammonia, the dipeptids are converted into diketopiperazines which are ring-compounds. Under similar conditions the polypeptids are modified in an analogous manner, with the formation of ring-compounds.

Upon hydrolysis the peptids break down into their constituent amino-acids, the imino groups in the polypeptid molecule being converted, by the taking up of water, into amino groups. A

* Emil Fischer strongly inclines to the opinion that the higher estimates, such as 12,000 and 15,000 for the minimal weight of the protein molecule, which are freely cited in the older literature upon proteins, are in error, since the admixture of a small quantity of another protein might easily raise the calculated value to this magnitude, and we have no proof of the chemical individuality of the majority of our protein preparations, even when, as sometimes happens, they are crystallizable. An exception appears to be afforded by hæmoglobin, the minimal weight of which is placed by many observers, employing adequate chemical and physico-chemical technique, at or in the neighborhood of 16,000. But then hæmoglobin is probably to be regarded as a salt-like compound of, possibly, two or more molecules of a basic, histone-like protein (globin) with a non-protein acid, namely hæmatin. In a similar way, we shall see (Chaps. V and IX) that casein, which is possessed of a minimal molecular weight of 4000–4400, forms, under certain conditions, salts of which the molecular weight is double or four times this.

very large number of them are hydrolysed by the proteolytic enzymes, pepsin, trypsin, etc., and in some cases, at all events, it is certain that the hydrolysis takes place in stages, as it does with the proteins and peptones (1). Further discussion of the mode of hydrolysis of the polypeptids by enzymes will be found in the chapters dealing with the chemical dynamics of protein systems.

5. The Occurrence of Peptids among the Products of Protein Hydrolysis. — It is highly probable that many of the bodies which are known to the biochemist as "proteoses" "peptones," etc., will turn out to be identical with some of the polypeptids already synthesized; this identity has, however, not yet been proven. But several of the simpler peptids and one tetrapeptid have already been isolated from protein digests. These are to be regarded as products of incomplete hydrolysis, intermediate between the higher complexes and the simple amino-acids which result from their complete decomposition.

The presence of a dipeptid amongst the products of the hydrolysis of a protein of silk, silk-fibroin, was detected by Fischer and Bergell in 1902 (16). Later, Fischer and Abderhalden (9) showed that its anhydride, which they isolated, was the diketopiperazine of glycyl-alanin, and that it could not possibly have arisen, by synthesis from glycin and alanin, during the process of its isolation. At the same time they isolated another dipeptid from among the products of the incomplete hydrolysis of silk-fibroin, namely glycyl-l-tyrosin, while from among the products of the incomplete hydrolysis of elastin glycyl-l-leucin was obtained. Later, Fischer and Abderhalden (22), by partial hydrolysis of the silk-fibroin, obtained a peptone-like substance, precipitable by phosphotungstic acid, easily soluble in water, insoluble in alcohol, precipitable from its aqueous solution by saturation with ammonium sulphate or sodium chloride, which proved to be a tetrapeptid, yielding, on hydrolysis, two molecules of glycin, one of alanin and one of tyrosin. Its molecular weight, determined by the cryoscopic method, was 350. The synthetic pentapeptid, l-leucyl-triglycyl-l-tyrosin, possesses very similar properties, so that the peptones are not necessarily exceedingly complex substances, nor is excessive complexity necessary in order that substances of this type may be precipitable from their aqueous solutions by saturation with ammonium sulphate.

A number of other peptids have been isolated by various observers (42) (48) from among the products of the incomplete hydrolysis of proteins.

6. The Analysis and Characterization of Proteins by the Determination of the Chemical Groups Characteristic of the Different Amino-acids. — The hydrolysis of the proteins is accompanied by a very marked increase in the total number of free amino groups. This is due to the fact, now incontestibly established, that the various amino-acid radicals of the protein molecule are attached to one another in an end to end or catenary linkage, through the union of the amino group of one amino-acid with the carboxyl group of the adjacent acid, in accordance with the general equation:

$$H_2NR_1COOH + H.HNR_2COOH = H_2NR_1COHNR_2COOH + H_2O.$$

The reversion of this reaction, in hydrolysis, leads, of course, to the transformation of an imino or potentially imino group into a free amino group and the series of such transformations which constitutes the process of the hydrolysis of a protein leads to the appearance of a large number of free amino groups which were not present as such in the unhydrolysed protein molecule.

Free amino groups in the aliphatic series have the well-known property of reacting with nitrous acid with the liberation of nitrogen, in accordance with the equation:

$$RNH_2 + HNO_2 = ROH + N_2 + H_2O.$$

Very ingenious advantage has been taken of this fact by D. D. Van Slyke (69) (70) (2) in the method which he has devised and which is now very widely employed for the determination of the distribution and partition of nitrogen within the protein molecule.

This method consists essentially in the following process. The protein having been in the first place subjected to complete hydrolysis, the ammonia in the mixture of products (derived from "amid" nitrogen in the protein molecule) is first removed by vacuum distillation and separately determined. The residual mixture of products is then treated with phosphotungstic acid, which results in the precipitation of the diamino-acids, namely cystin, arginin, lysin and histidin. A determination of sulphur yields a measure of the cystin-content. Arginin has the property of yielding one-half of its nitrogen in the form of ammonia on

boiling with alkali. The quantity of ammonia developed on boiling the precipitate with alkali therefore affords a measure of the content of arginin. The total nitrogen in the precipitate is now determined and from it is subtracted the proportion of nitrogen which is contributed by the cystin and arginin content. The residual nitrogen is derived from lysin ($= x$) and histidin ($= y$). On treatment with nitrous acid lysin yields a volume of free nitrogen corresponding to the whole of its nitrogen content ($= x$), while histidin yields a volume of free nitrogen which corresponds to two-thirds of its nitrogen content ($= \frac{2}{3} y$). The amino-nitrogen content of the precipitate is therefore determined by the nitrogen yield on treatment with nitrous acid and after subtraction of the amino-nitrogen contents of arginin (= one-fourth of its total nitrogen) and of cystin (= the whole of its nitrogen content), the residual amino nitrogen evidently represents the whole of the lysin nitrogen plus three-fourths of the histidin nitrogen. But the determination of the total nitrogen in the precipitate and the subtraction therefrom of the cystin and arginin nitrogen has already given us a measure of the total nitrogen yielded by the lysin and histidin. Subtracting, therefore, the amino-nitrogen yield of these amino-acids the difference evidently corresponds to one-third of the histidin nitrogen, from which the contents of histidin and lysin may readily be computed.

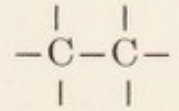
In the filtrate, after the separation of the diamino-acid precipitate, the total nitrogen and the amino-nitrogen are separately determined. The difference yields a measure of the nitrogen contained in pyrrollidine (prolin and oxyprolin) or indol (tryptophane) rings.

During the hydrolysis by hydrochloric acid a small amount of a very deeply colored precipitate separates out. The nitrogen content of this precipitate is the so-called "melanin" or "humin" nitrogen. According to Gortner and Blish (27) this is derived from a portion of the tryptophane and, in the presence of a sufficiency of carbohydrate, the yield of melanin nitrogen is a quantitative measure of the tryptophane content of the protein.

7. Types of Union in the Protein Molecule. — Following the recognition of the fact that the proteins are complexes built up by the union of amino-acids, the question of the mode of union

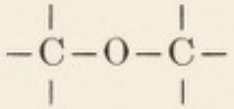
between them became one of paramount importance. Hofmeister (30) has pointed out that it is possible to conceive of several ways in which the acids may be linked together such as:

A. Direct union of the carbon atoms, as:



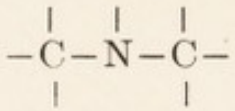
under which condition the molecule would be an immense chain of carbon atoms, and would not be readily hydrolysable, as the proteins are, into its constituent amino-acids.

B. Ether-like unions, as:

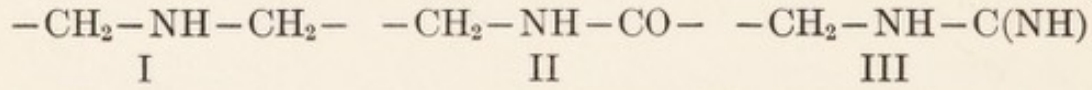


Such unions, however, would only be possible when one of the two amino-acids contained a hydroxyl group. Only tyrosin, serin and oxyprolin, among the amino-acids which occur in the proteins contain such groups, however, therefore this mode of union cannot be of general occurrence in the proteins.

C. The carbon atoms may be united by a nitrogen atom, as:

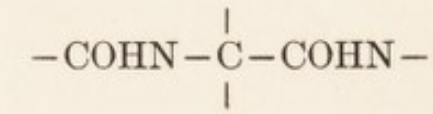


Several varieties of this mode of union are possible, such as:



I II III

The syntheses accomplished by Emil Fischer, which I have described above, the occurrence of peptids among the products of the partial hydrolysis of proteins, and their digestibility by proteolytic enzymes, demonstrate that type II is the most general mode of union among the amino-acids which constitute the protein molecule. The fact that the proteins give the biuret reaction also supports this view. It has been shown by Schiff (61) that only those substances which contain two $-CO-NH-$ groups, or two $-CS-NH-$, or two $C(NH)-NH$ groups, or, under certain conditions, two $-CH-NH-$ groups yield the biuret test. That such groups as:



occur in the protein molecules, as they do in the molecules of the polypeptids, is therefore highly probable and is furthermore confirmed by the extreme paucity of free amino groups in the native protein molecule.

The content of free amino groups in the native protein molecule may be determined either by the yield of nitrogen on treatment with nitrous acid (43) (69) (38) (71) or by the method of titration with formaldehyde, originally proposed by Sörensen (66) (67) (46) (38) which latter method also enables us to indirectly estimate the free carboxyl groups. An examination of the various proteins by either of these methods reveals the fact that the content of free amino groups in the unhydrolysed protein molecule is very small indeed. Thus Van Slyke and Birchard have obtained the following results (71):

PERCENTAGE OF TOTAL NITROGEN PRESENT IN FREE AMINO GROUPS

Hæmoglobin	6.0
Casein	5.5
Hæmocyanin	4.3
Gelatin	3.1
Edestin	1.8
Gliadin	1.1
Zein	0.0
Heteroalbumose	8.1
Protoalbumose	9.9

On the other hand, edestin, after complete hydrolysis by hydrochloric acid, yields a volume of free nitrogen, on treatment with nitrous acid, corresponding to no less than 79 per cent of its total nitrogen content (69). The investigations of Kossel and Gavrilov and of Van Slyke and Birchard have in fact shown that the free amino nitrogen in the unaltered protein molecule exactly corresponds in quantity with one-half the lysin nitrogen. Hence zein, which contains no lysin (49) yields no free nitrogen on treatment with nitrous acid. The period required for complete interaction of proteins with nitrous acid (30 minutes) is longer than that required by the α-amino groups (3 to 4 minutes), but corresponds to that found for the ω-amino group of lysin. From these facts Van Slyke and Birchard infer that one of the two amino groups of lysin, the ω group, exists free in the protein molecule, and that this group represents, within at most a fraction of a per cent of the total protein nitrogen, the entire amount

of the free amino nitrogen determinable in the native proteins by the nitrous acid method. The α-amino groups which constitute the remaining and greater part of the free amino nitrogen found after complete hydrolysis are, in the intact protein molecule, practically all condensed into peptid linkings.

In the primary albumoses or first split-products of protein hydrolysis, the relations are different. The free amino groups in hetero- and protoalbumose exceed one-half the content of lysin nitrogen by 3.0 and 4.8 per cent of the total nitrogen respectively, indicating that an appreciable proportion of the α-amino groups are uncovered in even the first steps of hydrolysis.

8. Some Consequences of the Polypeptid Structure of the Protein Molecule. — The polypeptids are as essentially amino-acids as the amino-acids out of which they are built up. Thus glycyl-glycin is as typically an amino-acid as glycocoll itself, since it possesses an $-NH_2$ group as well as a $-COOH$ group and for this reason is presumably capable of forming compounds, not only with acids and bases, but also with neutral salts (52). On undergoing electrolytic dissociation it may be supposed to yield either hydrogen (H^+) ions, or hydroxyl (OH') ions, owing to the occurrence of a reaction with water of the type:

$$R\begin{matrix}\diagup NH_2 \\ \diagdown COOH\end{matrix} + H.OH = R\begin{matrix}\diagup NH_3OH \\ \diagdown COOH\end{matrix}$$

just as ammonia, in aqueous solution, partially reacts with water to form NH_4OH.

It is usually conceded that these elements in the structure of the proteins afford an explanation of the power which they possess of neutralizing both acids and bases, in other words the "amphoteric" character of the proteins. To this opinion I have also formerly inclined, but an accumulation of data irreconcilable with this view induced me some years ago (57) to abandon it. Since that time evidence of a perfectly conclusive character has been obtained and we may now regard it as an established fact that some elements in the protein molecule other than terminal $-NH_2$ or $-COOH$ groups are responsible for the acid- and base-neutralizing power which is possessed in such a marked degree by many proteins (*vide* Chapters IV and V).

In the first place, the investigations of Levites and D. D. Van Slyke, referred to above, have shown that only a very small

proportion of the nitrogen in proteins is present within their molecules in the form of $-NH_2$ groups. Thus in the case of edestin, as we have seen, only 1.8 per cent of the total nitrogen is present in the form of free $-NH_2$ groups.

Now edestin, as Osborne (47) has shown, is insoluble, when in the free condition, in water. It forms an insoluble hydrochloride containing 14×10^{-5} equivalents of HCl per gram and, on further addition of acid, passes into solution. Its combining-capacity for acids does not remain constant, however, for at neutrality to tropæolin, which corresponds to a free acidity of from 0.01 to 0.001 H^+ (60) it neutralizes 127×10^{-5} equivalents of acid per gram. Hence, if the acid is neutralized by $-NH_2$ groups of edestin, the number of these groups in each molecule of edestin must be at least $\frac{1}{1}\frac{2}{4}\frac{7}{7} = 9$.* From the former determination it would appear that the molecular weight of edestin is 7000, and this corresponds with the molecular weight indicated by its tyrosin and glutamic acid content (40) (1 mol. tyrosin + 3 mols. glutamic acid + · · ·). Nine $-NH_2$ groups in this molecule would correspond to over ten per cent of the total nitrogen, or almost exactly to the whole $-NH_2$ content of the arginin, calculated as the free diamino-acid, which the edestin molecule contains. Since only 1.8 per cent of the total nitrogen of the edestin molecule is present therein in the form of $-NH_2$ groups no less than eighty per cent of the neutralizing power of the edestin for acids must be accounted for in some other fashion than by the assumption of a union of the acid with free $-NH_2$ groups.

From the investigations of Erb (14), although the exact interpretation of his results is in some respects open to question (Chap. IV), it would appear that the combining-weight of egg albumin for acids may be as low as 152, while its molecular weight is, according to Hofmeister, 5400 or some multiple of this. Hence upon the assumption that terminal $-NH_2$ groups bind these acids, there must be at least 35 of them in egg albumin (45), which would correspond to no less than 69 per cent of the total nitrogen in this protein.

* Osborne believes that an insoluble "monochlorhydrate" is also formed, containing 7×10^{-5} equivalents per gram which would raise the number of $-NH_2$ groups to 18, but would, at the same time, double the estimate of the molecular weight.

It has been pointed out by Kossel and Cameron (37) that the acid-combining capacity of the protamin, salmin, is equal to the combining capacity of the guanidin groups of the arginin radicals which it contains, yet salmin yields no nitrogen on treatment with nitrous acid. Sturin (41) contains 67 per cent of its nitrogen in the form of arginin, 10 per cent in the form of histidin and 6 to 7 per cent in the form of lysin. It yields nitrogen on treatment with nitrous acid corresponding to the ω-amino group of the lysin. Only about three out of every hundred nitrogen atoms in sturin are therefore present in the form of free $-NH_2$ groups. Yet one hundred nitrogen atoms in sturin will neutralize no less than 24 equivalents of acid. All of these protamins, moreover, possess a certain, although relatively inferior power of neutralizing bases.

The number of terminal $-COOH$ groups in any protein cannot be much in excess of the terminal $-NH_2$ groups, as the formol-titration and behavior towards cupric hydroxide (32) (33) (66) (67) show, and also because the protein would otherwise be overwhelmingly acid in character (30) and the majority of the proteins possess a distinct capacity for neutralizing acids, even when they are themselves predominantly acid. Now free casein * is insoluble in water, but when combined with acids or with bases it is soluble. To carry one gram of casein into solution 11.4×10^{-5} equivalents of base just suffice, indicating a combining-weight for casein of about 8800. The tyrosin, glutamic acid and sulphur contents of casein indicate a minimal molecular weight of from 4000 to 4400.

In the presence of *excess* of base, however, casein attains a maximal combining capacity (measured by the gas-chain) of 180×10^{-5} equivalents per gram, so that it behaves like a 16-basic acid, and if $-COOH$ groups bind the base there must be 16 of them in the molecule, corresponding to 25 per cent of the total oxygen, or, almost exactly, *to the percentage of the total oxygen which is contained in the $-COOH$ groups of the glutamic acid in the casein molecule, calculated as the free acid.* In order to provide so many free carboxyls, the form of the casein molecule would necessarily be that of a branched chain, or the radiating spokes of a wheel, at the centre of which must exist unions of the type: $-C-C-$

* For references to the sources of these data Cf. Chap. IV.

and the regular decomposition of the proteins into their constituent amino-acids, upon hydrolysis, would be unintelligible (30). Moreover, in the synthetical polypeptids, which closely resemble the natural peptones in their behavior, the linkage of the amino-acids is not radial but catenary in character * and the peptids which have been isolated from the mixed products of partial protein hydrolysis are likewise catenary in structure.

In the second place, as Vernon has pointed out (72), although the power of the sum of the decomposition-products of a protein to neutralize bases is greater, yet it is only very slightly greater than that of the unhydrolysed protein. Now in the process of hydrolysis the $-COHN-$ groups of the protein are split into $-NH_2$ and $-COOH$ groups; yet this results in no pronounced gain of combining-capacity for bases. The obvious conclusion is that the $-COHN-$ group within the protein molecule must be nearly as efficient in accomplishing the neutralization of bases as the $-COOH$ group of the constituent amino-acids out of which the protein is built up.

Direct proof, however, that terminal $-NH_2$ groups are not responsible for any appreciable proportion of the acid-combining capacity of proteins has been furnished by the recent experiments of Blasel and Matula (5) and Pauli and Hirschfeld (51). These investigators prepared deaminized gelatin by acting upon gelatin with nitrous acid, thus destroying all the free $-NH_2$ groups in the molecule. They then compared, with the aid of the gas-chain, the acid-combining capacity of the deaminized gelatin with that of normal gelatin. They found that the combining-capacity of deaminized gelatin for acids is but slightly inferior to that of ordinary gelatin, indicating, beyond any question, that the combining-capacity of gelatin for acids is, in very large proportion, attributable to elements of the molecule other than free $-NH_2$ groups. The inference is unavoidable that the elements of the molecule which actually participate in the union with acids are, in very large proportion, the $-COHN-$ groups within the body of the protein molecule.

Very strong evidence that the same structural elements of the protein molecule are responsible for the neutralization of bases by proteins has also been recently afforded by the investigations

* Even when dicarboxylic acids enter into the compounds, Cf. Fischer and Koenigs (24), and Fischer and Schmidlin (26).

of Osborne and Leavenworth (50) who have shown that edestin combines with, or holds in solution, 34.67 per cent of its weight of copper in the form of cupric hydroxide. This, if we assume that each copper atom unites with one nitrogen atom, involves the union of cupric hydroxide with ten out of every sixteen atoms of nitrogen in the edestin molecule. Now this is exactly the proportion of nitrogen which edestin yields in the form of amino-nitrogen after complete hydrolysis. In other words it is exactly equal to the proportion of $-COHN-$ groups which the unhydrolysed molecule contains. Precisely similar results were obtained with gliadin.

To account for the high acid- and base-combining capacity of the proteins we must therefore look, not to the terminal $-NH_2$ or $-COOH$ groups, but to the $-COHN-$ groups within the body of the protein molecule. Now two varieties of this union can be conceived, between which it has not proved possible as yet to decide by any direct method of analysis. Thus glycyl-glycin may conceivably be either:

Keto-form

$$H_2N.CH_2.CO-HN.CH_2.COOH$$

or

Enol-form

$$H_2N.CH_2.C(OH)=N.CH_2.COOH$$

and our analytical data, and the modes of decomposition and synthesis of the proteins and peptids do not enable us with any degree of certainty to distinguish between them. Neither form is therefore inconsistent with our present knowledge of the synthesis and hydrolysis of proteins and polypeptids, but while the keto-form of the $-COHN-$ group would conceivably possess the power of neutralizing acids it offers no probable point of union for bases. The enol-form, on the contrary, would provide a point of union for either acids or bases.

According to Werner's theory of valencies the nitrogen in either of these unions contains two latent valencies, positive and negative, which, while the nitrogen is trivalent, neutralize one another, but which, when the nitrogen becomes pentavalent are capable, respectively, of neutralizing a negative and a positive radical. The enol type of union carries with it the possibility of the following types of reaction:

$$-COH=N- + Na^+ + OH' = -CONa^{++} + \begin{matrix} H \\ | \\ \diagdown N- \\ | \\ OH \end{matrix} \qquad \text{(i)}$$

and

$$-COH=N- + H^+ + Cl' = -COH^{++} + \begin{matrix} H \\ | \\ \diagdown N- \\ | \\ Cl \end{matrix} \qquad \text{(ii)}$$

yielding, in each case, *only protein ions.*

I have already incidentally dwelt upon the fact, in connection with Kossel's theory of a protamin nucleus of proteins and in connection with the neutralizing powers of edestin for acids and of casein for bases, that there is reason to suspect that diamino and dicarboxylic radicals in the protein molecule play a predominant part in accomplishing the neutralization of acids and bases, and electrochemical data, as we shall see, lend further support to this view. Accordingly, the above formulæ, which represent the reaction when only a single $-COHN-$ bond is involved should probably, at least in many instances, be doubled, and in the following possibilities would then exist:

In combination with bases,

$$R\begin{matrix} COH.N- \\ COH.N- \end{matrix} + KOH + H_2O = R\begin{matrix} COK^{++} \\ COH^{++} \end{matrix} + \begin{matrix} H\ OH \\ \diagdown\ \diagup \\ \diagdown N- \\ \diagdown N- \\ \diagup\ \diagdown \\ H\ \ OH \end{matrix} \qquad \text{(iii)}$$

$$R\begin{matrix} COH.N- \\ COH.N- \end{matrix} + 2\ KOH = R\begin{matrix} COK^{++} \\ COK^{++} \end{matrix} + \begin{matrix} H\ OH \\ \diagdown\ \diagup \\ \diagdown N- \\ \diagdown N- \\ \diagup\ \diagdown \\ H\ \ OH \end{matrix} \qquad \text{(iv)}$$

In combination with acids,

$$\begin{matrix} -COH.N \\ -COH.N \end{matrix}\diagdown\diagup R + HCl + H_2O = \begin{matrix} -COH^{++} \\ -COH^{++} \end{matrix} + \begin{matrix} H\ \ Cl \\ \diagdown\ \diagup \\ \diagdown N \\ \diagdown N \\ \diagup\ \diagdown \\ H\ \ OH \end{matrix}\diagdown\diagup R \qquad \text{(v)}$$

$$\begin{matrix} -COH.N \\ -COH.N \end{matrix}\!\!\diagdown\!\!\diagup R + 2\,HCl = \begin{matrix} -COH^{++} \\ -COH^{++} \end{matrix} + \begin{matrix} H\ \ Cl \\ \diagdown\ \ \diagup \\ \diagup\!\!\diagdown N \\ \diagdown\!\!\diagup N \\ \diagup\ \ \diagdown \\ H\ \ Cl \end{matrix}\!\!\diagdown R \qquad \text{(vi)}$$

It is obvious that in reactions (iii) and (v) the molecules of water may or may not participate in the reaction; also that the anionic groups in reactions (iii) and (iv) may or may not be united to form a single quadrivalent cation (derived from a dicarboxylic acid group). As we shall see (Chapter X), no evidence has been found (at least among the compounds of casein or of serum globulin with bases) of the occurrence of reactions of the type represented by equation (iii). Equation (iv) faithfully represents, so far as the electrochemical data are concerned, the mode of combination of bases with these proteins, and the anionic groups are probably united to form one quadrivalent union. The union of serum globulin and ovomucoid with acids follows equation (v) (with or without the molecule of water) when the concentration of acid is low; but at higher acidities ovomucoid, at least, combines with hydrochloric acid in the manner indicated by equation (vi).

In the succeeding chapters of this work we shall see that this hypothesis regarding the mode of union between the proteins and acids and bases is supported by the following facts:

(i) The compounds which the proteins form with acids and bases, when dissolved in water, are excellent conductors of electricity, and true electrolytes (65) (55) (4), yet they do not, for example, yield chlorine ions, when the compound in question is a hydrochloric acid compound (8) (59), nor does the potassium hydrate compound yield potassium ions, or the calcium hydrate compound calcium ions (56). The equivalent conductivities of the compounds at infinite dilution are such as would indicate the presence *only of bulky organic ions*, travelling under a given fall of potential at the constant minimal equivalent velocity of about 20×10^{-5} cm. per sec. per volt per cm. fall in potential at 30° C. which is characteristic for such ions (6).

(ii) Edestin will displace NaOH from its combination with hydrochloric acid (T. B. Osborne, Cf. Chapter V) and casein,

although insoluble in water, will displace carbonic acid from its combination with calcium hydrate (W. A. Osborne, Cf. Chap. V). Solutions of caseinates of the bases may be obtained which are pronouncedly acid in reaction (Robertson, Cf. Chap. V). Since in all these cases the *molecular* concentration of the protein is very low, and the compounds which are formed are quite highly electrolytically dissociated (Cf. above), were the formation of these compounds due to the replacement of OH′ groups of $-NH_3OH$ groups by acid radicals or of H^+ groups in $-COOH$ groups by bases, then the "strength," i.e., the degree of dissociation of edestin as a base must be equal to or greater than that of NaOH, while that of casein as an acid must be much greater than that of H_2CO_3 and comparable with the degree of dissociation of NaOH at very high dilutions (since Na caseinate may be prepared which is acid in reaction). Such conclusions, applied to bodies which are amphoteric, are, of course, absurd. Were the formation of potassium caseinate due to the formation of a salt such as

$$RCOO' + K^+$$

an acid solution of this compound could no more exist than an acid solution of potassium aluminate. As in similar cases which occur in the domain of inorganic chemistry, we can interpret these phenomena only by assuming that the basic radical in the casein compounds, and the acid radical in the edestin compound are bound up in a non-dissociable form. Since the casein compounds, at least, when in solution in water, are notable conductors of electricity, they must dissociate at some other point in the molecule than that of the union between the base and the protein.

(iii) Each equivalent* of a monobasic acid or monacid base neutralized by serum globulin or casein yields *two equivalents* of the protein compound (Cf. Chap. X). This obviously corresponds with the mode of dissociation depicted above, while, if terminal $-NH_2$ or $-COOH$ groups accomplished the union, each equivalent of neutralized acid or base would produce only *one equivalent* of salt.

(iv) On successive additions of 1, 2, 3, etc., equivalents of

* That is, gram-molecule divided by the valency of the combining radical, in this instance unity.

monacid bases to a solution of an organic polybasic acid of the type $R(COOH)_m$, and the formation of salts by the replacement of H atoms in the $-COOH$ groups, the osmotic pressure of the solution would increase (provided the salts were highly dissociated) in the same proportion, namely 2, 3, 4, etc. The experimental fact, for casein (58), is that the osmotic pressure (= depression of the freezing-point) increases in the proportion 2, 4, 6, etc., i.e., each successive equivalent of neutralized base gives rise to an equal number of ions. This obviously corresponds with what would be expected were the union and its mode of dissociation of the type outlined above.

In addition to these, a host of minor details in the behavior of the protein salts, which would be very hard to explain upon any other basis, admit, as we shall see, of a simple explanation on the basis of the hypothesis outlined above.

The poly-amino-acid structure of the proteins, however, carries with it other possibilities which are of importance in interpreting their behavior. Some measure of the dissociations

$$R\begin{cases}NH_2\\COO'\end{cases} + H^+$$

and

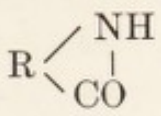
$$R\begin{cases}NH_2\\COOH\end{cases} + H_2O = R\begin{cases}NH_3^+\\COOH\end{cases} + OH'$$

must undoubtedly occur, although, in the majority of cases (a notable exception being afforded by the protamins) these modes of dissociation must play a very subordinate part. Then the terminal $-NH_2$ and $-COOH$ groups (and at least one of these must exist at either end of a chain of amino-acids) may neutralize themselves internally, thus:

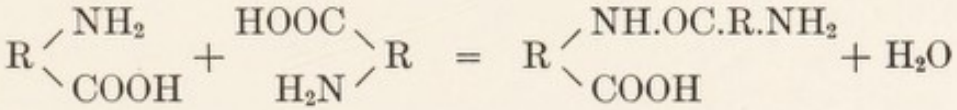
$$\begin{array}{c}NH_3\\ \diagup\quad\diagdown\\ R-COO\end{array}$$

forming what Winkelblech terms an "internal salt" (73). Such a molecule, whatever the mode of union, would of course form compounds with acids or bases with much more difficulty than the protein which had not undergone such internal neutralization, since, before dissociation could occur the ring-formation would

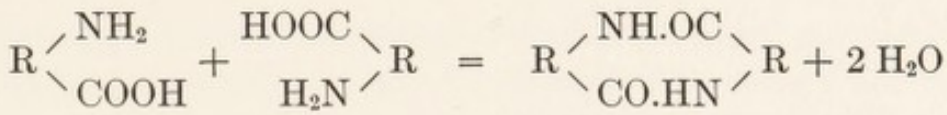
have to be opened up. The internal-salt formation may go a step further, with the formation of anhydrides such as



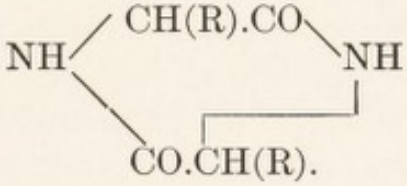
such anhydride formation being frequently observed in the polypeptids. Then two molecules of an amino-acid, and therefore, of a poly-amino-acid such as protein, may unite with one another in either of two ways, thus:



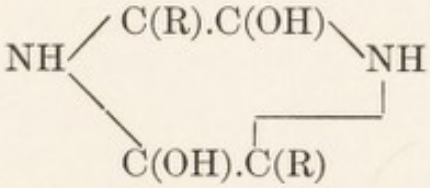
or



the product being, in the first instance, a poly-amino-acid of a higher order and greater molecular weight, and in the second an "internal salt" or anhydride.* Another type of anhydride which may be formed is that of a diketopiperazin, of the general formula:



and these anhydrides may exist in two isomeric forms, the keto and enol forms, the former being represented by the above formula, and the latter by the general formula:



The enol form carrying with it the possibility (in the presence of water) of the transient formation of salts with bases before the ring breaks up.†

* Such as leucyl-glycin anhydride.

† Cf. R. H. Aders Plimmer (53), Part 2, p. 36.

9. "Racemized" Proteins. — It has recently been shown by Kossel (36) and by Dakin (11) (12) that treatment of proteins with rather concentrated alkali leads to a progressive diminution of the optical rotary power of the solutions. This Dakin attributes to an internal racemization or, more correctly, enolization of the protein molecule analogous to that which occurs in the hydantoins (10). He depicts the reaction as follows:

$$\mathrm{R-}\overset{\displaystyle \mathrm{NH-CO-} \atop |}{\mathrm{CH}}\mathrm{-CO-NH-CHR-COOH}$$

$$\rightleftarrows \mathrm{R-}\overset{\displaystyle \mathrm{NH-CO-} \atop |}{\mathrm{C}}\mathrm{= C(OH)-NH-CHR-COOH}$$

the alkali tending to shift the equilibrium towards the right. The central carbon atom instead of being attached by its valencies to four different groups is now attached to three groups, to one of them by a double bond, and any optical activity which it may have possessed owing to assymetry must have been lost.

The "racemized" proteins thus prepared are totally resistant to hydrolysis by pepsin, trypsin or erepsin. Putrefactive bacteria do not attack "racemized" casein although they slowly decompose the "racemized" caseose (proteose) which is simultaneously formed through partial hydrolysis of the casein and subsequent "racemization" of the higher products of the hydrolysis. "Racemized" egg albumin (7) and casein (63) are non-antigenic. The amino-acids resulting from the complete hydrolysis of "racemized" protein by acid are for the most part optically inactive (12).

A number of objections have been urged by Kober (32) against Dakin's interpretation of the progressive loss of optical activity of proteins in alkaline solutions. In the first place he points out that the presence of an oscillating hydrogen atom within the molecule might be anticipated to lead to the development of bands in the absorption spectrum and no absorption-bands are observable in protein solutions excepting those in the ultraviolet spectrum which are attributable to the phenylalanin and tyrosin radicals. This objection is deprived of force, however, by the fact that in the hydantoins, in which analogous "enolization" does demonstrably occur, no absorption-band is developed.

The objection, also raised by Kober, that racemized polypetids are hydrolysable by trypsin is not valid because the reaction depicted by Dakin is not, correctly speaking, racemization but "enolization" and the structures of polypeptids formed from racemic amino-acids and the so-called "racemized proteins" are probably not in the least analogous. Finally, the fact, also cited by Kober as an objection to Dakin's hypothesis, that not all of the amino-acids resulting from the complete hydrolysis of enolized proteins are optically inactive, merely tends to show that not all $-COHN-$ groups are equally affected by alkali.

According to Underhill and Hendrix (68) crude "racemized" proteins produce toxic symptoms when introduced into the circulation of animals. Purified "racemized" proteins exert no toxic action. A portion, but not all of the toxic substance can be removed from "racemized" proteins by extraction with alcohol. Evidently the main reaction is complicated by the occurrence of side-reactions chief among which must be hydrolysis which is rather rapid in solutions of the alkalinity employed.

In terms of the hypothesis of protein ionization outlined above the "racemization" of proteins by alkali must consist in an exchange of hydrogen atoms in the enol group in accordance with an equation such as:

$$\begin{array}{l} \quad\quad NH-CO- \\ \quad\quad\quad | \\ R-CH-C(OH) = N-CH-RCOOH \\ \quad NH-CO- \\ \quad\quad | \\ R-C = C(OH)-NH-CH-R-COOH \end{array}$$

with consequent transformation of the double bond connecting carbon and nitrogen in the $-COHN-$ group into a single bond.

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CHAPTER II

THE PREPARATION OF PURE PROTEINS

1. The Proteins as Chemical Individuals. — With the exception of casein and the protamins and those proteins such as hæmoglobin, fibrin, egg albumin and certain of the vegetable proteins which may be prepared in crystalline condition, it cannot be positively affirmed that any of the proteins which have hitherto been isolated in a "pure" condition are, in reality, chemical individuals and not mixtures, tolerably constant in composition, of two or more proteins or of one protein with the products of its partial hydrolysis or with other colloidal substances. Our methods of isolation and purification are admittedly inadequate, and the most diverse opinions exist regarding the appropriate criteria of the purity of any given protein. The reason for this latter fact is probably to be sought in the imperfection of our knowledge of the properties, chemical, physical, and physico-chemical, of the proteins as a class; lack of knowledge implying, of course, the lack of a basis for comparison and of a standard for calibration.

The proteins are, as a rule, non-crystallizable or only crystallizable with difficulty and under such conditions as to involve contamination with a variable and usually indeterminate proportion of impurities, while repeated re-crystallization usually leads to a more or less sensible alteration in the properties of the protein, which may be attributable, in the majority of cases, to a partial hydrolysis. The salts which the proteins form with the inorganic bases and acids are, as a rule, either soluble or else, if insoluble, of such a nature (for example, the protein salts of the heavy metals) that the inorganic constituent cannot be removed again from the molecule without altering its properties.

A limited number of the proteins are insoluble in distilled water when uncombined with bases or acids, and these, of course, afford exceptionally favorable opportunities for isolation and purification. Precipitation of these proteins in the free condition can

usually be brought about by inducing a certain reaction (i.e., hydrogen or hydroxyl ion concentration) in their solution, through the addition of acids or of bases, the hydrogen- or hydroxyl-ion concentration being sufficient to set the protein free from its combination with bases or acids respectively and insufficient to lead to the formation of a new salt with the acid or base employed for its precipitation. Thus, for example, casein is precipitated from milk by the addition of acetic acid, "insoluble" serum globulin from serum by dilution and the passage of CO_2, histones from cell-extracts by the addition of ammonia.

The isolation of proteins of this class is usually most conveniently carried out by employing for the precipitation an acid or base which is insufficiently dissociated to transform the protein into a base, if an acid is employed, or into an acid if a base is employed for the precipitation, so that the protein which is at first precipitated does not, on adding an excess of the acid or base, form a salt and pass into solution again. For instance, if a "strong" acid such as hydrochloric acid be added to milk, or to any solution of a caseinate of an alkali or an alkaline earth, free casein is at first precipitated. But if the addition of the hydrochloric acid be continued the casein passes into solution again and it is now found that the casein is behaving as a base and that a certain proportion of the acid is neutralized by it. If a "weak" acid such as acetic be employed, however, the acidity of the mixture is insufficient to transform the casein into a base, and a considerable excess of acetic acid may be added to the mixture without danger of loss of material owing to resolution of the protein.

The acid or base which is employed in setting free the insoluble uncombined proteins of this class must, however, be sufficiently strong to set free the protein from its salts; for example, CO_2 cannot be employed to precipitate casein because casein is a stronger acid than CO_2 and displaces it from carbonates. But CO_2 can be and is successfully employed to precipitate "insoluble" serum-globulin because this protein is a weaker acid than casein and can be displaced from its salts by so feeble an acid as carbonic acid. Undoubtedly correlated with this is the fact that serum globulin is more readily transformed into a base than casein, so that acetic acid redissolves and is partially neutralized by serum globulin, although it is insufficiently strong except in very high concentrations to redissolve casein.

It is clear, therefore, that proteins of this class must be precipitable only within a very narrow range of hydrogen- and hydroxyl-ion concentrations and that the probability of simultaneous precipitation of more than one protein is very slight unless the proteins chance to be acids or bases of very nearly equal strength. In very few cases, however, do we possess, at present, any very reliable guarantee that this does not occur, and in a few instances, at all events, we are positive that it does occur. For example, the various members of the paranuclein series, which are derived from casein by its partial hydrolysis, are all precipitable by acetic acid, and, some of them at least, are not redissolved by the addition of moderate excess of acetic acid. True, there are recognizable differences between the basic functions of the various paranucleins and casein. The paranucleins are precipitated in solutions of lower acidity than casein, and doubtless an acid could be found the solution of which, at a concentration sufficiently acid to precipitate paranuclein, would not be sufficiently acid to precipitate casein. But this acid has not, as yet, been discovered and so a reliable method for separating casein from paranuclein in a solution containing a mixture of these proteins is yet to seek; while the separation of the different members of the paranuclein group from one another is a task replete with difficulties and uncertainties.*

It is not intended to imply, by the above remarks, that the isolation of free casein is as a rule attended with danger of contamination with paranuclein, because, fortunately, the presence of paranuclein in solutions of casein is not to be feared unless the casein has undergone appreciable hydrolysis in an acid medium. I am merely pointing out the imaginary difficulties which would be encountered in endeavoring to separate casein from paranuclein, were they found in nature in the same fluid, as an illustration of the very real difficulties which unquestionably attend the separation and isolation of other proteins which are insoluble in distilled water when uncombined with acids or bases.

All that can be said, therefore, when a protein isolated by means of a certain procedure, is found repeatedly to yield the same physical, physico-chemical and chemical constants, is that it is a protein or mixture of proteins of *constant composition provided that particular method of isolation be employed and no other.*

* *Vide* T. Brailsford Robertson (31).

Only when a protein has been prepared in crystalline form or by many different observers in many different ways and has always been found to possess the same physical, physico-chemical and chemical constants, can we pronounce it, with any degree of safety, a definite chemical individual. So far this has only been accomplished for a very limited number of proteins.

It cannot with propriety be assumed, therefore, that the following methods for the isolation of "pure" proteins yield *definite chemical individuals*, save those for the isolation of the crystalline proteins and of casein and of the various members of the protamin group. But the methods which follow are those which, to the knowledge of the author, have been shown to yield, at least, *mixtures of appreciably uniform composition, appreciably free from non-protein contamination.*

Very many well-known methods for the isolation and purification of various proteins are, of course, omitted, not because they do not in all probability yield products conforming to the above restrictions, but because either evidence of this fact is lacking, or they have never been employed in research of a distinctly physico-chemical character.*

2. Casein. — The following is the method of preparation employed by Hammarsten (8):

"Milk is diluted with four volumes of water and the mixture treated with acetic acid to 0.75 to 1 per mille. Casein thus obtained is purified by repeatedly dissolving in water with the aid of the smallest quantity of alkali possible, by filtering and reprecipitating with acetic acid and thoroughly washing with water. Most of the milk-fat is retained by the filter on the first filtration, and the casein contaminated with traces of fat is purified by treating with alcohol and ether."

According to Bosworth and Van Slyke (3) casein prepared in this manner is always contaminated by a small admixture of dicalcium phosphate and contains about 0.14 per cent of phosphorus attributable to this source. The method of preparation which these investigators employ to secure a product free from this contamination is as follows:

Separator skim-milk is diluted with seven or eight times its volume of distilled water, and dilute acetic acid (6 cc. of glacial

* For a number of methods for the separation of various animal proteins *vide* Hammarsten (8).

acetic acid diluted to 1 litre) is carefully added until the casein separates completely, after which the clear solution is removed by syphon as soon as the precipitate settles. Distilled water is then added, the mixture stirred vigorously and the precipitate allowed to settle, after which the wash water is syphoned off. Water is again added, and the casein is dissolved by adding, for each litre of milk used, 1 litre of dilute NH_4OH (6 cc. of strong reagent diluted to 1 litre). When the solution is complete the whole is filtered through a thick layer of absorbent cotton. The casein is then precipitated again with dilute acetic acid; the precipitate is allowed to settle, and is then washed, redissolved in dilute NH_4OH and filtered, the process of precipitation, washing, dissolving, etc., being repeated not less than four times. Finally an excess of strong NH_4OH (10 cc.) is added and then 20 cc. of a saturated solution of ammonium oxalate. The mixture is allowed to stand twelve hours or more. Calcium is precipitated as oxalate in very finely divided condition, too fine to permit its satisfactory removal by ordinary methods of filtration. Better aggregation of the precipitate can, however, be effected by means of centrifugal force. The centrifuged mixture is then filtered through double thickness of filter paper. The filtered solution is next treated with dilute HCl (10 cc. HCl, sp. gr. 1.20 diluted to 1 litre) until the casein is precipitated. The precipitate is washed with distilled water until free from chloride and is then placed on a hardened filter paper in a Buchner funnel, as much water as possible being now removed by suction. The mass is next transferred to a large mortar and thoroughly triturated with 95 per cent alcohol. The alcohol is then removed by suction on a Buchner funnel and the casein is placed in a mortar and triturated with absolute alcohol. Most of the alcohol is removed and the casein treated twice with ether in a mortar by trituration, the ether being removed each time by means of suction on a Buchner funnel. The material is then placed in a large evaporating dish and spread out in a layer as thin as possible, allowed to stand twelve hours or more in a warm place, and then finely ground in a mortar until the particles pass through a 40-mesh sieve and dried for two days over H_2SO_4 in a desiccator under diminished pressure.

Casein prepared in this manner contains only 0.71 per cent of phosphorus; corresponding almost exactly to the theoretical per-

centage calculated on the supposition that casein contains two atoms each of phosphorus and sulphur. Previous estimates, derived from analyses of casein prepared by Hammarsten's method, indicated a phosphorus content of about 0.85 per cent.

Casein "nach Hammarsten" is obtainable upon the market in a high degree of purity. For the investigations described in this work I have always employed the C. P. casein "nach Hammarsten" which is manufactured in Germany for Messrs. Eimer and Amend of New York. This substance is free from appreciable fat and would appear to be contaminated only by a small amount of an acid, water-soluble substance. This I remove by further purification in the following manner (30) (35):

Half a pound of casein is triturated with about 12 litres of distilled water, the water being added in six successive portions. On each addition of water the casein is well stirred up in a porcelain mortar and allowed to settle, the supernatant water is then poured off and fresh water is added. It is next washed in a similar manner in 5 kilos of absolute alcohol, and then in 5 kilos of ether "distilled over sodium." The mortar, containing the casein drained as free from superfluous ether as possible,* is then placed in an incubator over sulphuric acid at 40°–50° C., the flame is turned out under the incubator, and it is allowed to cool for about 24 hours. The casein is now found, if these operations have been conducted carefully, to be in the form of a dry, pure white powder, still containing, however, a considerable quantity of ether. The presence of a color in the powder indicates contamination, either with moisture or with some other impurity.

The casein is now spread out, within the incubator, in a layer not over 1 cm. deep, the flame under the incubator is lighted and it is allowed to stand for 24 hours at 40°–50° C. The casein is then found to be free from appreciable water or ether.

The casein which is thus prepared gives every indication of being a pure product and a definite chemical individual. It is insoluble in distilled water, save in traces which adhere to the casein par-

* At this point it is necessary to avoid exposing the mortar to the moist air of the room for a moment longer than is necessary, otherwise the evaporating ether causes the condensation of sufficient moisture to spoil the product unless it is again treated with alcohol and ether.

ticles,* and it neutralizes, to phenolphthalein and to litmus, exactly the quantities of alkali determined by Söldner (41), Lacquer and Sackur (15) and Van Slyke and Hart (45).

Casein which has been carefully prepared in the manner outlined above, floats upon the top of and is not readily wetted by water or watery solutions of bases; if, however, it contains a mere trace of moisture, it is readily wetted by all save the most alkaline solutions. In order to successfully and completely dissolve perfectly anhydrous casein it is necessary to first add to it a very little of the solution in which it is to be dissolved, rub it up into a paste, and then add, while stirring, the remainder of the solution.

If casein which is readily wetted by water be desired (34) it is necessary to omit the desiccation over H_2SO_4. For this purpose $CaCl_2$ should be employed as the desiccating agent, and desiccation should be continued for 12 hours at about 30° C. Casein prepared in this way readily sinks in and is wetted by water and aqueous solutions; it contains, however, not very significant traces of moisture and a considerable amount of adherent ether.

3. "Insoluble" Serum Globulin. — There appear to be at least two globulins in serum, the one insoluble in distilled water, which is precipitated on dialysis of the serum, or dilution and the subsequent addition of a weak acid; the other soluble in water, precipitable by saturation with magnesium sulphate after the removal of the insoluble fraction (16) (5) (27).

According to Hardy these globulins differ markedly in their phosphorus content, the insoluble globulin containing from 0.07 to 0.08 per cent phosphorus; the soluble globulin only a trace (about 0.009 per cent) of phosphorus (9).

According to Hammarsten (7) the individuality of these proteins, and the freedom of any given preparation of the one from an admixture of the other is open to serious doubt. Moreover, as Taylor has shown (44), the "insoluble" globulin is readily converted, by hydrolysis, into the "soluble" form. The relationship between the various serum globulins would therefore appear to be somewhat analogous to that between the various members of the paranuclein group.

* So that when litmus paper is dipped into a suspension of casein particles in distilled water the paper is reddened where it is touched by the undissolved particles, while the fluid which bathes them remains clear and neutral (30).

The following method of preparing serum globulin is based upon the observation of Alexander Schmidt (38) and Kühne (14) that "insoluble" serum globulin can be precipitated from serum, previously diluted with ten volumes of water, by the passage of CO_2. It appears to be difficult or impossible to bring about this precipitation in the total absence of salts of stronger acids, e.g., NaCl. I have repeatedly observed that if serum globulin, precipitated by CO_2 and carefully washed, be dissolved in a minimal quantity of NaOH or KOH it cannot be reprecipitated by dilution and the passage of CO_2, although a distinctly acid and very opaque solution results.

Three litres of ox-serum are diluted with ten volumes of distilled water, and CO_2 is bubbled through it at a good rate for about half an hour. The globulin which is thus precipitated is allowed to settle in tall glass cylinders,* the supernatant fluid being syphoned off after settling. The precipitate is then washed with about 60 litres of distilled water, in two portions. The globulin is then dissolved in a minimal quantity of $N/10$ HCl † and immediately reprecipitated by cautious neutralization with $N/10$ KOH. This precipitate, after settling and decantation of the supernatant fluid, is washed in 60 litres of distilled

* For the settling and washing of protein precipitates I employ wide-mouthed glass cylinders from 50 to 60 cm. high and about 10 cm. in diameter, closed by ground glass stoppers. The syphon is provided with a side tube through which it can be filled and can then be closed by a rubber tube provided with a pinch-cock. When the protein is being washed with alcohol or with ether, the syphon is filled with alcohol so as to avoid accidental contamination of the contents of the cylinder with water. The syphon is supported on a stand which is so arranged that the height of the opening of the syphon above the precipitate can be adjusted by turning a screw. The greater part of the liquid is first rapidly run off with the opening of the syphon a good way above the precipitate. The syphon is then lowered and the remainder of the liquid run off more slowly.

† This can be calculated from the fact that 1 litre of serum yields about 5 grams of globulin and that about 20×10^{-5} gram equivalents of the strong monobasic acids just suffice to dissolve one gram. If acetic acid be preferred, about 100×10^{-5} equivalents are required to dissolve 1 gram (9).

It has been shown by Hammarsten (7) that "insoluble" serum globulin is not readily "denatured," i.e., altered in properties, by acids. "A solution of serum globulin containing 0.2–0.3 per cent acetic acid remains unchanged for days at a low temperature, and one can recover the serum globulin unaltered by neutralization." In the presence of stronger acids denaturation is a matter of some hours.

water in 6 successive washings, the precipitate, after each agitation with distilled water, being allowed to settle for 24 hours in the presence of toluol, after which the supernatant fluid is drawn off and the globulin suspended in a fresh quantum of distilled water.* The thick suspension of globulin which is thus obtained after the final washing is kept, in the presence of toluol, in a stoppered bottle and used in this form, since globulin, if washed with alcohol and ether and dried, is redissolved only with difficulty.

The suspension is well shaken before withdrawing a measured sample. The globulin content of the suspension is determined to within ± 0.01 gram per 100 cc. by placing 25 cc. samples in small and accurately weighed beakers, evaporating the fluid to dryness on a water-bath, and then drying the residue over H_2SO_4 at 70° until its weight becomes constant.

4. Fibrin. — The following is the method of preparing fibrin which is employed by Bosworth (2).

Fresh ox-blood is collected in a large bottle and, as soon as possible, transferred to wide-mouthed precipitating jars and allowed to coagulate. The clots are then removed, broken into small pieces, and washed in running water to remove the serum and blood corpuscles. The washed masses of fibrin are passed through a mincing machine, placed in a large (8-litre) bottle, a little toluol added, and the bottle filled with 0.2 per cent sodium hydroxide solution. This solution causes the fibrin to swell and after about 36 hours the whole content of the bottle resembles a thin jelly. This jelly is broken up, one-half transferred to another 8-litre bottle, and after the two bottles are filled by the addition of water they are allowed to stand for an additional 36 hours. The jelly is by then almost completely dissolved and the contents of both bottles are filtered, first through cheese-cloth, then linen and finally paper. The clear filtrate is then diluted with an equal volume of water and placed in tall wide-mouthed precipitating jars. Dilute acetic acid (0.3 per cent) is then added cautiously. At a certain point a flocculent precipitate of fibrin appears which quickly settles to the bottom of the jars.

* This suspension is, of course, equivalent to a prolonged dialysis of the protein, the precipitate acting as a dialyser of enormously extended surface, permitting the passage of associated diffusible impurities into the distilled water and retaining the protein.

The supernatant liquid is syphoned off, the precipitate washed with water, dissolved in dilute sodium hydroxide (0.05 per cent) and again precipitated with acetic acid. This process is repeated three times, the final precipitate being washed with alcohol and ether and dried over sulphuric acid in an evacuated desiccator.

The behavior of fibrin strongly resembles that of casein in all stages of its preparation except in its extreme sensitiveness to a slight excess of acid or alkali, for unlike casein, it is readily soluble in weak acetic acid.

5. Hæmoglobin. — The following is the method of preparation recommended by Preyer (26).

The blood is collected in a vessel and allowed to coagulate and to stand for several hours (or, better, for a day) in a cool place. Then the serum with the white corpuscles and the fat which have collected above the clot is removed and the coagulum is washed with distilled water and then cut into very small pieces, and these pieces in turn are repeatedly washed with cold distilled water. Then the clot is comminuted, best by freezing and reducing the frozen mass to powder. This powder is placed in a filter paper and washed with cold distilled water until the filtrate no longer gives any very bulky precipitate with bichloride of mercury. The coagulum is extracted by water heated to from 30 to 40 degrees and filtered, and the filtrate is collected in a large cylindrical vessel standing in ice. A small measured portion of the red solution thus obtained is gradually mixed during constant agitation with small quantities of alcohol until a slight precipitate forms. This determines how much alcohol may be added to the whole solution without a precipitate appearing. A slightly smaller proportion of alcohol is now added to the remaining filtrate and the mixture is placed in a cooling medium. After a few hours the crystals separate in great abundance. The crystals, owing to the large volume of water used, are very easily filtered off in the cold. They are then washed with cold water containing a little alcohol until the filtrate yields only an insignificant cloudiness upon the addition of acetate of lead or mercuric chloride. The product yielded is a very large one. The crystals may be purified by repeated washing by decantation until the wash-water does not become cloudy with bichloride of mercury, lead acetate or silver nitrate. They are then nearly pure, and the ash is free from phosphoric acid and consists of

pure iron oxide. If this is not the case, then they must be dissolved in warm water and recrystallized. At a temperature of less than 0° C. the crystals can be dried in the air without decomposition.

Preyer states that, of all kinds of blood, that of the horse is best adapted for the production of very large quantities of pure hæmoglobin.

For a variety of rapid methods of preparing small quantities of crystals from the blood of different species the reader is referred to the monograph on the Crystallography of Hæmoglobins by Reichert and Brown (28).

6. Crystallizable Egg-Albumin. — The method of Hopkins and Pinkus (10) is universally employed for the preparation of this protein.

Two hundred cc. of egg-white obtained from newly-laid fowls' eggs are mixed with an equal bulk of saturated ammonium sulphate solution, the latter being very gradually added and the mixture stirred briskly with an egg-beater between each addition. It is then allowed to stand over night. The mixture is then filtered and to the clear filtrate more saturated ammonium sulphate solution is added until a permanent precipitate is obtained. Distilled water is then added, a few drops at a time, until the solution is just clear again. Ten per cent acetic acid solution is then added drop by drop until a slight but definite precipitate has appeared. The bottle is then immediately stoppered and allowed to stand. After 24 hours an abundance of uniformly crystalline precipitate has settled out consisting of rosettes of needles. If a somewhat greater proportion of acetic acid is employed the rosettes are mixed with sheaths and fan-shaped aggregates of crystals. Recrystallization of this first product is carried out by filtering off the crystals from the mother-liquor, redissolving in a moderate amount of water, acidifying with a few drops of dilute acetic acid and adding saturated ammonium sulphate solution until a faint turbidity is produced. In 24 hours a large proportion of the protein will have recrystallized. This process may be repeated 5 or 6 times in the course of a week. The crystals which are finally obtained may be freed from inorganic contamination by prolonged dialysis against running distilled water.

7. Ovovitellin. — The following method of preparation is that employed by Osborne and Campbell (23), modified by Plimmer (25) and further modified by the author (33).

Twenty-five yolks of eggs are carefully washed, without breaking the enveloping membrane, in a stream of water, so as to remove all traces of the whites. To the yolk is then added an equal volume of 10 per cent sodium chloride solution and the solution which is thus obtained is extracted from ten to twelve times with twice its volume of ether in separatory funnels, extracting several times after the ethereal layers fail to yield a precipitate, due to the presence of lecithin, upon the addition of acetone. The complete extraction occupies a period of from two to three weeks. The watery layer which is finally obtained is then poured into twenty volumes of distilled water, and the precipitate of ovovitellin which is thus obtained is allowed to settle in tall glass cylinders. The supernatant fluid is then syphoned off and the precipitate redissolved in 10 per cent sodium chloride and reprecipitated in the same manner. This process is repeated. Finally the vitellin is dissolved in very dilute sodium hydrate and the solution filtered, the filtrate being allowed to drop directly into dilute acetic acid, thus reprecipitating the vitellin. This precipitate is suspended in distilled water and allowed to settle in tall glass cylinders. The supernatant water is then drawn off and the washing with water repeated several times. The precipitate is then washed in 6 litres of 99.8 per cent alcohol. After allowing the precipitate to settle, the supernatant alcohol is syphoned off and the washing in alcohol repeated twice. The vitellin is then washed twice in ether "distilled over sodium," employing 6 litres each time. The thick suspension of vitellin in ether finally obtained is quickly poured into a hardened filter and allowed to filter and dry over sulphuric acid at 40 degrees for 48 hours. The vitellin is thus obtained as a white, somewhat coarse powder.

About a gram of this ovovitellin was placed in about 30 cc. of alcohol and boiled for some five minutes. About 5 cc. of the alcohol, after filtration, was then tested directly for lecithin by the addition of several volumes of acetone. The remainder of the alcohol was evaporated down to dryness and the residue (barely visible) taken up in about 10 cc. of ether. This ether was then tested by the addition of several volumes of acetone. Both tests proved entirely negative, not the slightest opalescence being produced by the acetone; thus the ovovitellin did not contain any lecithin. Hence the older statements to the effect that

vitellin is always associated and probably combined with lecithin are attributable to inadequate extraction of the yolks with ether and the consequent incomplete removal of the lecithin.

It is extremely difficult to prepare homogeneous solutions of ovovitellin. A five per cent solution in alkali ($N/10$ KOH) is a thick jelly, opalescent owing to the presence of a multitude of air bubbles entangled in it while stirring. In endeavoring to prepare more dilute solutions it is found very difficult to avoid the formation of small lumps of jelly within the solutions, and these are exceedingly difficult to break up, and dissolve with extreme slowness. Although vitellin is soluble in dilute solutions of the strong acids, yet when the powder is directly mixed with an acid solution it will not dissolve or does so with extreme slowness; it is soluble in acid solutions only when freshly precipitated and still wet.*

It is possible to obtain clear, homogeneous 1 per cent solutions of ovovitellin (not more concentrated) by gradually dropping the vitellin from above into the solvent while undergoing violent stirring, and maintaining the stirring for about an hour.

8. The Vegetable Proteins. — The following methods of preparing the globulin of flax-seed will serve as an illustration of the general nature of the technique which is employed in preparing the various vegetable proteins. For further information concerning this subject the reader is referred to the monograph by Osborne (22) in which he summarizes the result of his important and extensive researches upon the preparation and properties of the vegetable proteins. According to Osborne (20) flax-seed globulin may be extracted from flax-seed meal in either of three ways, namely by extraction with distilled water, or with sodium chloride solution or with dilute KOH. The following are the methods of preparation employed by him.

(*a*) Extraction with distilled water.

The ground flax-seed is freed from oil by extracting with benzene and from the greater part of the outer coating of the seed by sifting through a fine sieve. One hundred grams of this meal are extracted for about 24 hours with distilled water at temperatures between 20 and 40° C. The filtered extract has a yellow color, is acid in reaction and very slightly turbid.

* Exactly the same phenomenon is encountered with casein. Cf. Van Slyke and Van Slyke (46), T. Brailsford Robertson (32).

This extract is then saturated with pure ammonium sulphate. The copious precipitate is collected on a filter and, after the completion of filtration, dissolved in about 800 cc. of water. The solution is then filtered and placed in a dialyser. After twenty-four hours a considerable precipitate has formed, which is seen under the microscope to consist entirely of perfectly formed octahedral crystals. After dialysing for about five days the solution is found to be free from chlorides, and the precipitate is then filtered off and washed with water, followed by dilute alcohol gradually increased in strength to absolute alcohol; it is finally washed with ether and dried over sulphuric acid.

The preparation, after drying, still retains its crystalline character. The meal yields about 10 per cent of its weight in globulin.

(*b*) Extraction with sodium chloride solution.

One hundred grams of the flax-seed meal are extracted three successive times with 20 per cent sodium chloride solution, and the clear, filtered, bright yellow extracts are united and dissolved in 10 per cent sodium chloride, and this solution, after filtering off an insoluble residue, is submitted to dialysis. After five days the solution, free from chlorides, is filtered and the precipitate washed with water, alcohol and ether and dried. The preparation, after drying, still retains its crystalline character. One hundred grams of meal yield about 11.6 grams of protein. This protein is identical with the globulin obtained by extraction with water.

(*c*) Extraction with dilute KOH solution.

Flax-seed globulin, prepared by either of the above methods, is soluble in dilute KOH and can be precipitated from this solution unchanged in composition or properties, by cautious neutralization with hydrochloric acid.

Flax-seed meal is extracted with 100 cc. of 0.01 KOH per gram. After filtration it is saturated with ammonium sulphate and the precipitate filtered off and dissolved as completely as possible in 10 per cent sodium chloride. This solution is dialysed until the chlorides are removed and the subsequent treatment is the same as that employed in the two previous preparations.

This preparation is imperfectly crystalline, but identical in composition with the other preparations. One hundred grams of meal yield about 17.5 grams of protein.

The plant globulins are, like serum globulin, precipitable from

their solutions in sodium chloride by dilution and the passage of CO_2. It is stated by Osborne, however, that the separation which is secured in this manner is rarely so complete as that which is accomplished by dialysis (22). The plant globulins are, according to Osborne, very readily "denatured" by strong acids, even when diluted.

For the preparation of some of the seed-proteins it is sufficient to extract the crushed seeds with hot sodium chloride solution; on cooling, the protein crystallizes out spontaneously (6) (19) (29).

Osborne inclines to the view that the majority of the vegetable globulins, instead of being predominantly acid substances, as they are generally believed to be, are, in reality, predominantly basic. This question will be commented upon in a later chapter (Chap. V).

9. The Alcohol Soluble Vegetable Proteins (Gliadin, Zein, etc.). — The following method of preparing gliadin, based upon that employed by Osborne and Harris (24), has been communicated to me by Dr. J. E. Greaves:

Gluten is prepared by kneading dough, made from wheat flour, in a stream of cold water until all of the starch has been washed out; it is then partially dried and a moisture determination is made. The moist gluten thus obtained is finely chopped and mixed with twenty times its weight of alcohol of such a strength that with the water in the gluten it forms an alcoholic solution containing 70 per cent of alcohol by volume. The mixture of gluten and 70 per cent alcohol is then allowed to stand, with frequent shaking, for 48 hours. After allowing the mixture to stand for 10 hours the supernatant alcoholic solution of gliadin is syphoned off and filtered through very finely shredded and well-washed and dried asbestos until a perfectly clear filtrate is obtained. The filtrate is then evaporated, under a pressure of about $\frac{1}{2}$ atmosphere until frothing prevents further concentration. It is then cooled and very slowly poured, with constant stirring, into about one hundred times its volume of ice-cold distilled water, containing 10 grams of sodium chloride per litre. The gummy mass, which usually collects on the stirring-rod, is dissolved by the addition of the least possible amount of absolute alcohol, and this solution is evaporated, under reduced pressure, to a thick syrup; the syrup is then cooled and poured, in a very fine stream, with constant stirring, into hot absolute alcohol. The precipitate is dissolved

in 70 per cent alcohol and this solution is evaporated under reduced pressure, with the occasional addition of absolute alcohol, until a thick syrup is again obtained. The gliadin is precipitated from this syrup by the method just described, washed three times with ether (distilled over sodium), partially dried over sulphuric acid, ground up as finely as possible, and then completely dried over sulphuric acid, in partial vacuum, at room temperature. The conditions of the precipitation, of course, involve contamination with NaCl. According to Osborne (21) there is only one alcohol-soluble protein in flour; on standing in alcoholic solution, however, a protein insoluble in alcohol or alcohol-water mixtures is precipitated within a few hours. Kosutany (13) believes that this substance is derived from gliadin by the splitting off of water and is identical with glutenin, the alcohol-insoluble and water-insoluble protein constituent of gluten. According to Osborne (21), however, glutenin differs qualitatively from gliadin in that, upon hydrolysis with hydrochloric acid, glutenin yields glycocoll and lysin, while gliadin yields neither of these amino-acids.

10. Ovomucoid. — The following method of preparing ovomucoid is a modification of that originally employed by Mörner (17) (33) (36).

The whites of eggs are beaten up to a froth and allowed to stand in shallow vessels over night. The subnatant fluid is then poured off, the froth being rejected. This fluid is diluted to five times its volume with distilled water, and to every litre of the diluted fluid is added 130 cc. of approximately $N/10$ acetic acid (made up by diluting 10 cc. of glacial acetic acid to 1750 cc.). This mixture is heated slowly to boiling point, being rapidly and uniformly stirred meanwhile, and after being allowed to boil for about 3 to 5 minutes, is put aside in rather shallow vessels for about 12 hours. At the end of this time most of the coagulum has floated to the top and the clear pale yellow subnatant fluid is filtered through hardened filter paper. Filtration is very rapid, and the filtered fluid, when boiled, either with or without further addition of acetic acid, remains perfectly clear. The fluid which is thus obtained is now slowly evaporated to one-fifth of its volume, the temperature of the fluid never being allowed to rise above 55° C. After allowing this fluid to cool, the protein is precipitated from it by the addition of ten volumes of 99.8 per cent alcohol and is allowed to settle in tall glass cylinders. The supernatant

fluid is then syphoned off and the precipitate is washed in the same volume of alcohol as that employed in the precipitation. This washing is repeated, again employing the same volume of alcohol, and the precipitate is allowed to steep in this alcohol for about 24 hours, in order to remove all adherent or combined acetic acid. The alcohol is then syphoned off and the precipitate is washed in the same volume of ether (distilled over sodium). This washing is repeated. The ether is then syphoned off and the thick suspension of protein in ether thus obtained is rapidly poured into a hardened filter, the filter and the contained suspension of protein in ether being at once transferred to an incubator and the filtration continued over sulphuric acid at 40 degrees (to avoid condensation of atmospheric moisture on the filter).

After the completion of filtration, the ether which has filtered off is removed from the incubator, and the precipitate is allowed to dry for 24 hours. The protein is then obtained in the form of chalky cakes which are very readily broken up into impalpable powder. This powder is passed through a fine sieve and conserved in a glass stoppered bottle.

It is found inadvisable to work with fewer than six dozen eggs at one time as, otherwise, the amount of precipitate which is finally obtained is so small that the danger of excessive caking and partial decomposition, in drying, due to the deposition of traces of moisture upon the filter, is very great.

Twenty-four dozen eggs of average size yield about 40 grams of ovomucoid.

About a gram of ovomucoid, prepared in this manner, was dissolved in about 100 cc. of $N/2$ hydrochloric acid, and this solution was boiled until 30 cc. of fluid had distilled over. This distillate was then tested for acetic acid. It contained a trace of an acid of the fatty series, sufficient to yield a slight coloration with ferric chloride, but insufficient to yield a precipitate of ferric hydrate on boiling, or to yield the ethyl acetate test.*

Ovomucoid is a gluco-protein, and yields, on boiling with strong acid, about 30 per cent of glucosamin (47) (40) (18) (42) which, according to Steudel (42), is contained in the molecule not in the form of glucosamin but in the form of an antecedent which yields glucosamin on treatment with strong acids.

* The solutions of ovomucoid prepared in this manner have themselves a distinct odor of ethyl acetate.

11. Gelatin and Deaminized Gelatin. — The only practicable method of purifying gelatin which has been devised consists in subjecting the best qualities of commercial gelatin to very prolonged dialysis against running distilled water (4). Deaminized gelatin is prepared by Blasel and Matula (1) by the following method:

About 200 grams of the best commercial gelatin is dissolved in one litre of warm water. To this solution is added 200 grams of sodium nitrite dissolved in one litre of water. After cooling, 140 grams of glacial acetic acid is carefully added. The mixture is allowed to stand for twelve hours and then heated on a water-bath for two hours. The deaminized gelatin is then precipitated by the addition of solid ammonium sulphate and purified by prolonged dialysis (2 weeks) against running distilled water.

12. Globin. — Globin may best be prepared by the following modification of the method devised by Schulz (39) (37).

A thick suspension of ox-corpuscles is obtained by centrifugalizing freshly defibrinated ox-blood, the volume of the suspension being about one-third that of the blood from which it is derived.

After pipetting off the supernatant serum the suspension is diluted to the original volume of the blood from which it was derived by the addition of $N/6$ NaCl solution and the centrifugalization is repeated, the supernatant fluid being removed as before. This is repeated six times in order to free the corpuscles from adherent serum. After the last centrifugalization the corpuscle suspension is not again diluted with NaCl.

The thick suspension of corpuscles which is thus obtained is diluted to ten times its volume by the addition of distilled water. The corpuscles are thus "laked" and the contained hæmoglobin is discharged into the water, forming a clear solution which is allowed to stand in tall glass vessels for twenty-four hours in order to permit the leucocytes to settle. The upper portion of the fluid is then decanted and employed, the lower portion of the fluid being rejected.

Two and one-half litre portions of this fluid are placed in six-litre bottles, to the contents of each bottle are added 56 cc. of concentrated HCl (sp. gr. 1.18) and the mixture is thoroughly shaken and allowed to stand at room temperature for one hour.

The addition of the acid causes a flocculent precipitate to appear and the mixture turns dark brown. Two and one-half litres of ether are then added to each bottle and the contents shaken thoroughly until they attain an oily consistency. Rubber stoppers with two perforations are then fitted into the necks of the bottles. Through one perforation is inserted a long glass tube reaching to the bottom of the bottle, to act subsequently as an air inlet, and through the other is inserted a short tube just reaching to the bottom of the stopper and provided with a rubber tube and pinch-cock. The stoppers are then tied down and the bottles quickly inverted and allowed to stand at room temperature for twenty-four hours. By this time the contents of the bottles, if the temperature of the room is not too warm, should have separated into two layers, an upper, jelly-like, very deeply colored ether layer containing the greater part of the hæmatin hydrochloride, and a lower, aqueous layer containing the globin to some extent contaminated by hæmatin hydrochloride. The latter is then drawn off through the shorter of the two tubes inserted through the stopper.*

To this fluid is added four volumes of a mixture of equal parts by volume of alcohol and ether. A light-colored precipitate is obtained, leaving the fluid very deeply colored. This precipitate is collected upon a hardened filter, washed once with a volume of alcohol equal to that of the watery fluid from which it was precipitated, drained and then scraped off the paper and dissolved in a volume of $N/10$ HCl equal to that of the fluid from which the globin was precipitated. This solution is now diluted by the addition of three times its volume of distilled water, and 20 per cent ammonia solution is carefully added until a precipitate just appears. One cubic centimetre of strong ammonia per litre is then added and the dense, flocculent precipitate is collected on a filter. The precipitate is then washed in large volumes of absolute alcohol, alcohol-ether mixture and ether. After draining in a dry atmosphere (preferably within an incubator over H_2SO_4) the precipitate is allowed to dry for 24 hours in an incubator over H_2SO_4, then pulverized and sifted and returned to the incubator to dry over H_2SO_4 for one week.

* The separation of the fluid into two layers is sometimes very slow. In such cases separation may be hastened by centrifugalization.

13. The Protamins. — Kossel, to whose investigations we owe our comparatively extensive knowledge of these, "the simplest proteins," prepares them in the following manner (12).

The minced ripe testicles of herrings (yielding clupein), salmon (yielding salmin) or sturgeons (yielding sturin) are shaken up in water, whereby a suspension of spermatozoa is obtained. This fluid is coagulated with acetic acid and the precipitate washed with alcohol and ether and dried. About 100 grams of the dry mass is then shaken up in 500 cc. of a 1 per cent solution of H_2SO_4 for one-half hour and filtered; this extraction is repeated six times and the extracts are mixed. The filtered extract is precipitated with three times its mass of alcohol, the fluid is syphoned off and the precipitate is dissolved in hot water and again precipitated with alcohol.

This precipitate of protamin sulphate is dissolved in about one and one-half litres of hot water and the solution is allowed to cool, when a small part of the sulphate separates out as a yellow or brownish colored oil. This least soluble portion of the protamin sulphate is rejected. The supernatant fluid is collected and evaporated to a small volume when the greater part of the protamin sulphate separates out as an oil. The mixture is placed in a separatory funnel and the oil drawn off.

This preparation may be freed from the last traces of associated nucleic acid in the following manner: It is dissolved in hot water and then precipitated with sodium picrate. This precipitate is well washed with water and then freed from picric acid by shaking it up with ether in the presence of an excess of H_2SO_4. The protamin sulphate is precipitated out of the sulphuric acid solution by the addition of alcohol. This precipitation should be repeated. The protamin sulphate should now come down as a loose white precipitate. If the precipitate has a glutinous appearance, the solution in water and precipitation by alcohol should be repeated. Finally, the precipitate is dried at 110 to 120° C.

According to Kossel, clupein and salmin are identical, the formula of the sulphate being a multiple of $C_{30}H_{57}N_{17}O_6$, 2 H_2SO_4. The most probable formula for sturin sulphate is, according to the same author, 4 $C_{36}H_{69}N_{19}O_7$ + 11 H_2SO_4.

These substances are strong bases; when one precipitates the H_2SO_4 out of a solution of clupein sulphate by the addition of

the equivalent mass of baryta, the resultant solution of free clupein has a strongly alkaline reaction (12) (43).

Salmin carbonate yields an alkaline solution owing to hydrolytic dissociation (43). Taylor states that the salmin yielded by the Pacific salmon is identical with that which is yielded by the European salmon.

The protamins readily form "basic salts" in which the proportion of H_2SO_4 to protein is less than in the fully neutralized compound; they also tend to combine with water to form various hydrates. Thus salmin sulphate which is insufficiently dried has the formula $C_{30}H_{57}N_{17}O_6$, $2\ H_2SO_4 + H_2O$.

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CHAPTER III

THE QUANTITATIVE ESTIMATION OF THE PROTEINS

1. The General Principles Underlying the Quantitative Estimation of Proteins. — The extraordinary bulk of the protein molecule in comparison with the molecules of the simpler substances which were the first to claim investigation by analytical chemists, places unusual difficulties in the way of the estimation of proteins by the traditional methods of analytical chemistry. Although the proteins act as acids and bases, yet their amphoteric character seriously interferes with direct titration by acidimetric methods, for both the acidic and basic functions of the proteins are weakened and modified by their simultaneous presence in the same molecule, and it is but rarely, in cases such as those afforded by casein and the various members of the protamin group, that the predominance of the one function is sufficiently great to permit of its utilization in the determination of the protein by direct titration (1) (18) (32). Even in these cases we are additionally hampered by the great magnitude of the combining-weight of the protein, leading to a proportionate enhancement of the normal margin of acidimetric inexactitude, and also by the fact that many of the indicators which are customarily employed in alkalimetry or acidimetry form compounds with proteins, either ceasing to function as indicators, or displaying novel or abnormal color-changes or even directly entering into and disturbing the very equilibrium, between protein on the one hand and an acid or base on the other hand, which we desire to measure.

Since the nitrogen-content of the proteins is high and nitrogen is one of the elements which we are able to determine with the greatest precision, nearly all the efforts of analytical chemists in this field have until recently been aimed at reducing the estimation of protein to an estimation of nitrogen. The problem thus resolved itself into one of separating the protein in question from other proteins and from nitrogenous contaminations. In cases in which this preliminary isolation is not difficult of attain-

ment, as in the case of the casein in milk, no more accurate and generally reliable method of procedure has yet been devised. Unfortunately in many, if not in the majority of, instances it is the fulfilment of this preliminary condition of complete separation from nitrogenous contaminations which presents the most serious difficulties. For instance the most valuable reagent for accomplishing the separation of various members of the globulin group from one another and from other proteins is ammonium sulphate, but the coagulum which is produced by this reagent, necessarily employed in high concentration, is heavily contaminated by the ammonium salt and must be freed therefrom by very prolonged dialysis before it can be utilized for a determination of nitrogen. Prolonged dialysis, on the other hand, is not only time-consuming but also involves the possibility of serious errors arising from autohydrolysis or even hydrolysis due to enzymes or to bacterial contamination, due to the protracted period during which the labile protein must be exposed to the action of water. Any alternative method of freeing the protein from contamination by ammonium salts, such as resolution followed by recoagulation with some other reagent, is attended with many difficulties and uncertainties attributable in large proportion to the modifications of the actions of coagulating agents which are brought about by the saline contamination which we desire to remove.

The high combining-weight of the proteins attributable to the mass of their molecules, is, as we have seen, the chief obstacle to the application of chemical methods in their quantitative estimation. In the application of *physical* methods for this purpose, however, the bulk of the protein molecule, far from being a disadvantage, is frequently a positive advantage and may indeed enable us to employ physical methods of estimation not ordinarily applicable to the smaller molecules of the majority of inorganic or the simpler classes of organic substances, for the great majority of the physical properties of a substance are determined by the mass or volume of its molecules and those properties which are magnified by increasing mass of the molecule are of course displayed exceptionally prominently by the proteins.

It appears extremely probable therefore that in the future we will come to rely more and more upon physical methods for the estimation of the characteristically bulky molecules of colloidal substances. Many attempts have been made to estimate

the protein-content of fluids by their optical rotation. This particular physical property of the proteins was, however, a most unfortunate choice, since it is one of the few physical properties which is independent of the absolute mass or volume of the molecules displaying it, and the specific rotatory power of proteins is extremely low in comparison with that of a large proportion of optically active substances of much smaller molecular dimensions.

In recent years two methods of estimating protein, based upon the measurement of physical qualities of their solutions or suspensions, have been suggested and applied to specific problems in very considerable detail. These are the nephelometric method, based upon measurements of opacity, and the refractometric method. Both methods are capable of attaining a high degree of accuracy and may be rendered to a great extent independent of contamination by non-protein substances. It therefore appears probable that they may eventually prove applicable to the estimation of a variety of proteins under conditions which would preclude, or render exceedingly difficult, reduction of the estimation to a determination of nitrogen.

2. The Nephelometric Method of Estimating Proteins. — This method, which we owe to Kober and his collaborators (6) (7) (8) (9) (10), depends upon the measurement of the relative opacities of suspensions of coagulated proteins, the measurement of relative opacity being carried out in a modified form of colorimeter. A full description of this instrument will be found in a recent article by Kober and Graves (9).

The success of the nephelometric method depends upon the discovery of specific coagulating agents which will bring the protein into stable suspension in masses of approximately uniform, or at least reproducible, magnitude, of sufficiently large diameter to scatter incident light-rays and insufficiently large to result in instability of the suspension. The discovery of the most suitable coagulating agent for each protein and under each set of modifying circumstances constitutes, indeed, the chief difficulty of the method, but, once the correct coagulant is found and the circumstances attending its employment have been standardized, the method is susceptible of a very high degree of accuracy, far surpassing indeed that attainable through the estimation of nitrogen.

The coagulating reagent employed by Kober is sulphosalicylic acid. The following are the details of the method as applied by Kober to the estimation of the various proteins in milk (8).

Five cubic centimetres of milk are carefully measured into a 250-cc. graduated flask and after adding 200 cc. of distilled water and 10 cc. of decinormal sodium hydroxide solution, water is added to the mark and the solution is thoroughly shaken. Ten cubic centimetres of this mixture is then placed, together with 2 cc. of ether (which has previously been washed with a 10 per cent aqueous solution of sodium hydroxide) in a centrifuge tube which is then tightly stoppered with a cork and vigorously shaken. After allowing the mixture to stand until the layers have separated, or after centrifuging for one to two minutes, the cork is removed and 5 cc. of the aqueous layer is withdrawn. This is done by closing the top of the pipette with the finger and inserting it quickly into the centrifuge tube. If this is done correctly the ether solution will not contaminate the sample. This 5 cc. of the aqueous layer is then diluted in a volumetric flask to exactly 50 cc.

The milk treated in this way has an almost inappreciable turbidity, not more than 1.6 per cent of the turbidity which is subsequently produced by the coagulating agent. Such residual turbidity as it possesses is almost exactly equal to that of the standard solution of casein with which it is subsequently compared, and is therefore without influence upon the accuracy of the comparison with the standard.

To 10 cc. of this solution is now added 10 cc. of a 3 per cent solution of sulphosalicylic acid and the suspension of coagulated milk protein which is thus obtained is matched in the nephelometer with the following standard: One volume (5 cc.) of a 0.01 per cent casein solution to which is added two volumes (10 cc.) of 3 per cent sulphosalicylic acid.

The volume of solution employed is not a correct aliquot of the original sample of milk, owing to the fact that in the extraction with ether some ether is dissolved by the aqueous layer and some water by the ether layer. A determination of the volume increase of a solution of sodium caseinate shaken up with ether shows that 10 cc. of the clear aqueous layer of defatted diluted milk is equal to 9.1 cc. of the diluted milk before extraction with

ether. Correction must be made for this change of volume in the estimate of protein-content.

In order to obtain the casein-content of the milk, the casein from a fresh portion of milk is precipitated in accordance with the "official" method by the addition of acetic acid, and the opacity of the suspension obtained in an aliquot part of the filtrate, by adding four volumes of 3 per cent sulphosalicylic acid solution, is determined nephelometrically. This determination yields an estimate of the "globulin and albumin" fraction which, subtracted from the "total proteins" estimated above, yields the amount of casein precipitable by the "official" method.

A modification of this method of protein estimation has been utilized by Pfeiffer, Kober and Field (10) in the estimation of the globulins and total proteins in cerebrospinal fluid.

It will be seen that the chief difficulty attaching to the nephelometric method arises from the necessity of achieving constant conditions of coagulation, a difficulty which is especially serious when the coagulating process has to be superadded to the procedures incident to the separation from one another of the various proteins in a mixture in which it is desired to determine each protein separately. This difficulty does not attach to the refractometric method described below, in which, once the separation of the various protein fractions has been achieved, no further procedures other than dilution, etc., are necessitated before a determination can be made. On the other hand, the refractometric method does not permit the extreme accuracy which may be attained with the nephelometric method under favorable conditions.

3. The Refractometric Method of Estimating Proteins. — This method, originally employed by Reiss (11) (12) (13) (14) (15) (16) for the estimation of the total proteins in blood-serum, has been modified by the author so as to render it applicable to the separate determination of the non-proteins, globulins, albumins and total proteins in a little over 1.5 cc. of blood-serum (24). The following are the details of the method:

(*a*) Estimation of the non-proteins.

Glass tubes 25 cm. long, having an inside diameter of 5 mm. and walls about 1 mm. thick, are sealed at one end. It is well to blow gently into the tube while the sealed end is still soft, thus making the contour of the bottom of the tube hemispherical and dimin-

ishing the tendency to crack on cooling. Into one of these tubes, which has been carefully cleaned and dried, is now introduced exactly 1 cc. of serum with the aid of an accurately calibrated pipette with a capillary tip. Such pipettes may be prepared by taking lengths of narrow-bore glass tubing drawn out to a capillary at one end, introducing 1 cc. of mercury, and marking with a diamond the extremities of the mercury column. The mercury is then delivered into another similar tube, which is similarly marked, and the operation repeated until the desired number of pipettes is obtained. Prepared in this way the pipettes all deliver (between the marks) the same volume of fluid, and if this is the case the exact volume employed is immaterial provided it is in the neighborhood of 1 cc.

In delivering the serum, wetting the upper part of the tube and the formation of air-bubbles should be carefully avoided.

The serum having been introduced, with another pipette calibrated against the first, deliver 1 cc. of $N/25$ acetic acid solution, which may be made up with sufficient accuracy by diluting 4 cc. of glacial acetic acid to 1750 cc.

A short length of thick platinum, silver, or nickel wire is now introduced into the tube and the upper end is sealed off in a flame, taking care not to heat the contents. After cooling, the tube is shaken and the short length of wire contained in it brings about a thorough admixture of the contents. The tube is now placed in a beaker of cold water of such a depth as to immerse the top of the contained column of liquid several millimeters below the surface. It is well to rest the bottom of the tube upon a wad of glass wool or a piece of wire gauze to avoid the cracking of the tube by bumping during the subsequent boiling. The water is now slowly heated to boiling and allowed to boil energetically for exactly two minutes. The tube is then removed from the boiling water and allowed to cool to room temperature.

When cool the tube is broken open a little above the surface of its contents and the coagulum is broken up. This is best accomplished with the aid of a platinum wire about 0.6–0.7 mm. in diameter and provided with several slight bends. This is inserted into the tube and the upper end twirled between the thumb and forefinger. The fluid and the coagulum are now separated by centrifugalization, the fluid is withdrawn by the aid of a dry, clean pipette, and the refractive indices of the fluid

and of $N/50$ acetic acid solution (prepared by diluting the $N/25$ acetic acid used above to exactly one-half with distilled water) are determined simultaneously. By determining the refractive indices of the fluid and of $N/50$ acetic acid simultaneously, the necessity for regulation of the temperature at which the readings are made is obviated, for although the absolute values of the refractive indices are affected by temperature, the differences between them are independent of the temperature at which they are determined (19). In carrying out a large number of estimations, however, it is necessary to eliminate the possible error due to progressive changes in the temperature of the dark-room by redetermining the refractive index of the solvent (in this instance $N/50$ acetic acid) at frequent intervals.

The refractive index of a 1 per cent solution of NaCl is 0.00160 greater than that of distilled water. The refractivities of 1 per cent solutions of other inorganic salts and of glucose and urea are of very similar magnitude. To within a sufficient degree of accuracy, therefore, the percentage of non-protein substances in the serum may be estimated by dividing their refractivity by the factor 0.00160. The result must be multiplied by 2, because the serum has been diluted to one-half with $N/25$ acetic acid.

For the determination of the refractive index, I employ a Pulfrich refractometer, which reads the angle of total reflection to within one minute. A sodium flame is used as the source of light. The refractive index may be estimated from the angle of total reflection with the aid of the table which is supplied with the instrument.

When it is desired to make a number of successive determinations, the cup of the refractometer should be carefully dried with absorbent cotton and lens paper before a new sample of fluid is introduced.

(*b*) Estimation of the albumins.

Glass tubes 9–10 cm. long are prepared, having an inside diameter of 5 mm. and one end closed. Tubes which have been employed in the estimation of the non-protein constituents, after having been cleaned and dried, may be cut down to the proper length and utilized for this purpose. Small corks or short pieces of glass tubing sealed at one end, the sealed end being pressed against the open end of the longer tubes and held in position by

short pieces of rubber tubing, are employed as stoppers. Into one of these tubes is introduced, with the aid of a pipette similar to that described above, 0.5 cc. of a saturated solution of ammonium sulphate (prepared by dissolving an excess of ammonium sulphate in hot water, allowing the excess to crystallize out on cooling and then removing the fluid from the subnatant crystals and keeping in a well-stoppered container). With another pipette which has been calibrated against the first, introduce 0.5 cc. of serum, drop in a piece of platinum, silver or nickel wire, affix the stopper and shake thoroughly with as little delay as possible. It is necessary to introduce the ammonium sulphate first, as otherwise, being of greater specific gravity than the serum, it sinks through the serum, portions of which are thus exposed for some time to ammonium sulphate of higher concentration than one-half saturated. This leads to a precipitation of albumins which do not readily redissolve, and the results obtained are erroneous and irregular. If the ammonium sulphate is introduced first the serum floats on the top of it and energetic shaking brings about almost immediate admixture of the serum and the reagent. It is well, while shaking, to hold the thumb against the bottom of the tube, thus diminishing the danger of cracking the tube by the impacts of the heavy piece of wire.

The tube, with the stopper still affixed, is now centrifuged. The precipitate soon settles and sufficient supernatant fluid may be drawn off to fill the tip of a pipette and the space between two marks known to hold about 0.25 cc. (prepared as above). This quantity of the supernatant fluid is delivered into another clean, dry tube of the type employed in the precipitation, and 0.25 cc. of distilled water is added with the aid of a pipette calibrated against the first. A piece of wire is dropped in, a clean stopper affixed, and the contents are shaken. We now determine the refractive index of this fluid and that of one-quarter saturated ammonium sulphate solution prepared (and kept as a stock solution) by mixing equal volumes of saturated ammonium sulphate solution and distilled water and adding to this mixture an equal volume of distilled water. The difference between these refractive indices represents one-fourth of the combined refractivities of the albumins and of the non-protein constituents. Multiplying by 4, therefore, and subtracting the refractivity of the non-protein constituents, we have the refractivity of the

albumins. Dividing this by 0.00177, we obtain the percentage of albumin in the serum. It is necessary to dilute the ammonium sulphate solution of albumins because the refractivity of proteins in half-saturated ammonium sulphate solutions is abnormal (21).

(*c*) Estimation of the globulins.

Determine the refractive index of the serum and that of distilled water. Subtracting from the difference the known refractivity of the non-proteins and the known refractivity of the albumins, we obtain the refractivity of the globulins. Dividing this by 0.00229 we obtain the percentage of globulin in the serum. Adding together the percentages of albumins and globulins we obtain the percentage of total proteins.

The following is an illustrative determination, employing ox-serum obtained by whipping and centrifuging freshly-drawn blood:

Refractive index of the fluid obtained after acidifying with acetic acid and boiling (3 determinations) 1.33024
Refractive index of $N/50$ acetic acid 1.32902
Difference $\times$ 2 ..... 0.00244
Estimated concentration of non-proteins = $\frac{244}{100}$ = 1.5 per cent.
Refractive index of the fluid obtained after precipitation of the globulins and subsequent dilution:
1st determination 1.35189
2nd determination 1.35172
3rd determination 1.35181
4th determination 1.35189
Refractive index of $\frac{1}{4}$ saturated $(NH_4)_2SO_4$ 1.34932
Differences $\times$ 4:
1st determination 0.01028
2nd determination 0.00960
3rd determination 0.00996
4th determination 0.01028
Refractivity of the albumins:

1st determination	0.01028 − 0.00244	= 0.00784
2nd determination	0.00960 − 0.00244	= 0.00716
3rd determination	0.00096 − 0.00244	= 0.00752
4th determination	0.01028 − 0.00244	= 0.00784
Average		0.00759

Concentration of albumins:

1st determination = $\frac{784}{177}$ = 4.4 per cent
2nd determination = $\frac{716}{177}$ = 4.1 per cent
3rd determination = $\frac{752}{177}$ = 4.3 per cent
4th determination = $\frac{784}{177}$ = 4.4 per cent
Average 4.3 per cent

Refractive index of the serum	1.34686
Refractive index of distilled water	1.32887
Difference	0.01799

Refractivity of the proteins $= 0.01799 - 0.00244 = 0.01555$
Refractivity of the globulins $= 0.01555 - 0.00759 = 0.00796$
Concentration of globulins $= \frac{7.96}{2.23} = 3.5$ per cent
Concentration of total proteins $= 3.5 + 4.3 = 7.8$ per cent

Since the percentage of total proteins is dependent upon the dilution of the blood and is therefore highly variable, while the relative proportions of globulin and albumin are much more constant, it is convenient to express these in percentages of the total proteins. Thus in the above result, taking the total proteins as 100, we find that the globulins formed 45 per cent and the albumins 55 per cent of the total proteins.

This method has been applied to the study of the proteins in human blood-serum under normal conditions and a variety of disease-conditions by A. H. Rowe (25) (26) (27) (31). It has also been applied to the study of the changes in the ratio of globulin to albumin in blood-serum during immunization and infection by Righetti (17), Hurwitz and Meyer (4) and Schmidt (28). The globulins, albumins and non-proteins in the blood-sera of a variety of mammals and birds have also been estimated refractometrically and characteristic differences found in the relative proportions of these constituents in widely-separated classes of animals (2) (3) (5) (23) (30) (33) (34).

The refractometric method has also been applied to the estimation of casein in milk (20) and to the estimation of the digestive activity of proteolytic ferments (22) (29).

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CHAPTER IV

THE COMPOUNDS OF THE PROTEINS

1. The Amphoteric Character of the Proteins. — The fact that one and the same protein can combine either with a base or with an acid appears to have been first clearly stated by Platner (33) in 1866. The term "amphoteric" which is now used to designate substances possessing this power is due to Bredig (4).

The source of this dual combining-capacity of the proteins has been discussed in Chap. I. In 1886, Strecker (51) pointed out that amino-acids can, like other substituted ammonias, bind acids by their $-NH_2$ groups, or, as we now know, through the transformation of trivalent into pentavalent nitrogen; while they can also bind bases in virtue of their possession of a $-COOH$ group. It is probable that in most cases a proportion of the amino-acid, when dissolved in water, combines with water to form a compound analogous to ammonium hydrate, which is in chemical equilibrium with the unhydrated form also present in the solution. Thus amino-acetic acid,

$$CH_2\begin{cases} NH_2 \\ COOH \end{cases}$$

when dissolved in water, is believed to partially undergo the reaction

$$CH_2\begin{cases} NH_2 \\ COOH \end{cases} + H.OH = CH_2\begin{cases} NH_3.OH \\ COOH \end{cases}$$

the trivalent nitrogen becoming pentavalent, and the molecule splitting off both hydrogen and hydroxyl ions in definite proportions. As I have stated, however, the transformation is not complete, nor is it by any means an easy matter, as a rule, to determine its extent (25) (39).

Nevertheless, as I have pointed out in Chap. I and as will be more clearly revealed in the chapters dealing with the electrochemistry of the proteins, it is very improbable that terminal $-NH_3OH$ or COOH groups in the proteins molecule are involved

in the union of the majority of proteins with inorganic acids and bases. The active agents in accomplishing these unions are, more probably, $-COH.N-$ groups *within* the protein molecule.

Whatever may be the *method* of union between inorganic substances and the proteins, however, there is now no longer any doubt that it is of very general occurrence. The methods which have been employed by various investigators to demonstrate the existence of compounds of the proteins with inorganic substances are very diverse in nature, but they may be conveniently classed as *direct* and *indirect*. The direct methods consist in the precipitation of the (supposedly) unaltered compound by appropriate reagents (in the case of compounds such as those with the heavy metals, which are insoluble in water, addition of a reagent is of course unnecessary), followed by the elementary analysis of the precipitate, or in the analysis of soluble compounds of proteins with substances which, when uncombined, are insoluble in water. The indirect methods may be variously designated: the indirect method of precipitation, the method of electrical conductivity, the cryoscopic method, the potentiometric method, the method of catalysis, the indicator method and the method of "masking" the physiological effects of inorganic substances by the addition of proteins to their solutions. The results obtained by these methods will be considered separately.

2. The Direct Method of Demonstrating the Existence of Protein Compounds by Precipitation or Coagulation. — This, which is the method most frequently employed, consists, usually, in precipitating or coagulating the protein salt by the addition of suitable reagents, the reagent which is most commonly employed being alcohol.

The very important question immediately suggests itself, whether or not the reagent which is employed for this purpose precipitates the protein compound unaltered, as it exists in the solution, or whether the compound is altered in the process of precipitation. On the whole it may be said that too little attention has been given to this question by investigators who have employed this method and that in consequence we possess very little accurate information on the subject.

It appears, however, that alcohol may be relied upon, at all events within certain limits, to precipitate the salts of some proteins with bases in an unaltered condition, that is, containing

the same relative proportion of base to protein as the pre-existing compound in aqueous solution. Thus Van Slyke and Hart (52) employed alcohol to precipitate calcium caseinate, and they found that the precipitate from solutions neutral to litmus contained exactly the quantity of calcium (uncombined with hydrochloric acid) which corresponds with the quantity of calcium hydrate which casein will neutralize to litmus while in aqueous solution. Similarly the precipitate from solutions neutral to phenolphthalein contained the calculated quantity of calcium. As we shall see in considering the electrochemical phenomena which accompany the coagulation of the caseinates by alcohol, coagulation of these salts through the addition of alcohol to their aqueous solutions is preceded by a profound decrease in their degree of dissociation (43); hence the caseinate is precipitated in combination with the proportion of base which was bound up by it in its aqueous solution, because, immediately preceding precipitation, the combined base is bound up in an undissociated molecule.

It has also been shown that serum-globulin is precipitated from serum by alcohol in the form of a salt with a base, and not in the form of the free protein, since the mixed proteins which may thus be precipitated are completely soluble in distilled water, and this solution yields a precipitate on passing CO_2 through it (42).

It would not be safe to assume, however, without special investigation, that alcohol precipitates all protein salts without alteration of the relative proportion of base or acid to protein which subsists in these compounds as they exist in aqueous solution. So far this can only be positively affirmed for the "neutral" and "basic" caseinates, that is, the caseinates the solutions of which are respectively neutral to litmus and to phenolphthalein.

The influence of other coagulating agents upon the composition of the protein salts which they precipitate is even more uncertain than that of alcohol, and in one case at all events, namely that of the precipitation of sodium caseinate from solutions containing a considerable excess of sodium hydrate by means of ammonium sulphate, we are in possession of definite evidence that the act of coagulation is accompanied by a change in the composition of the protein salts.

Spiro and Pemsel (49) employed ammonium sulphate to precipitate protein salts; their procedure was as follows: A given weight of protein was dissolved in a measured quantity of standardized NaOH solution, and the protein-base compound was then precipitated by the addition of a suitable excess of a saturated solution of ammonium sulphate. The quantity of alkali bound by the protein and precipitated with it was then determined by titration of the filtrate. The following were their results, employing casein:

Solution	Mg of NaOH precipitated with 1 gram of casein	Equivalent grm. mol. of NaOH precipitated with 1 gram casein
1.14 grams casein in 10 cc. $N/5$ NaOH..	26.1	65×10^{-5}
1.41 grams casein in 15 cc. $N/5$ NaOH..	29.2	73×10^{-5}
1.24 grams casein in 20 cc. $N/5$ NaOH..	33.1	82×10^{-5}
1.31 grams casein in 30 cc. $N/5$ NaOH..	34.3	36×10^{-5}

Now I have shown (40) employing the potentiometric method (*vide infra*), which is a static measurement, not involving any change in the dynamical condition or composition of the body of the solution, that in all of the above solutions investigated by Spiro and Pemsel the amount of alkali which was actually bound by one gram of casein *while in solution* must have been at least 160×10^{-5} gram equivalents. Hence it is clear that in the act of coagulation by ammonium sulphate these salts of casein are very materially altered in composition.

The method of coagulation by heat has been comparatively little employed for the investigation of protein compounds with acids. It cannot be employed for the investigation of compounds of proteins with bases because although proteins in alkaline solution are denatured by heat the denatured protein is only flocculated in an acid medium (6). It has been shown by Chick and Martin that in acid media the protein coagulum binds a certain proportion of the acid, but whether or not the protein salt, as it exists in the solution prior to coagulation, is flocculated without alteration, cannot at present be definitely stated.

At the present stage of our knowledge therefore, the direct method of demonstrating the existence of protein compounds cannot be trusted to yield accurate quantitative data, and results and conclusions based upon this method are to be accepted with the greatest caution.

3. The Direct Method of Demonstrating the Existence of Protein Compounds by the Solution of Otherwise Insoluble Substances. — This method has been employed by W. A. Osborne (30) who has shown that casein, when triturated with calcium carbonate, carries a definite proportion of the calcium into solution in the form of the soluble calcium caseinate with the evolution of CO_2. Strychnin is similarly carried into solution by casein.

Osborne and Leavenworth (31) have shown that edestin will hold in solution a quantity of cupric hydroxide corresponding to no less than 34.67 per cent of its weight of copper, which, assuming that each atom of copper is united to one nitrogen atom, implies that no less than ten out of every sixteen nitrogen atoms contained in the edestin molecule participate in forming the compound with cupric hydroxide. This is exactly the proportion of nitrogen atoms which is present in edestin in the form of $-COHN-$ groups. Precisely similar results were obtained with gliadin.

4. The Indirect Method of Precipitation. — Cohnheim and Krieger (9) have elaborated an ingenious method of determining the acid-binding capacity of the proteins which is based upon the fact that phosphotungstic acid forms insoluble salts with proteins. If the protein is combined with an acid, and calcium phosphotungstate is employed, double decomposition takes place as follows:

$$\underset{\text{(soluble)}}{\text{Protein hydrochloride}} + \underset{\text{(soluble)}}{\text{calcium phosphotungstate}} = \underset{\text{(insoluble)}}{\text{protein phosphotungstate}} + CaCl_2$$

hence the quantity of calcium chloride present in the filtrate, after filtering off the precipitate and carefully washing it (34) is taken as a measure of the quantity of hydrochloric acid which was bound by the protein just before precipitation. The procedure is as follows: Weighed amounts of protein are dissolved in measured volumes of acid of known concentration. The protein is then precipitated by the addition of an *excess* of the phosphotungstate and the filtrate, plus the washings of the precipitate, is titrated against standard alkali. An excess of the phosphotungstate is stated to be necessary because the phosphotungstate of protein is believed to undergo extensive hydrolytic

dissociation so that it is stable, and precipitation is complete, only in the presence of an excess of the reagent.

It is obvious that this method is open to all of the objections attaching to the direct method of precipitation considered above. In our present stage of knowledge we are very much in the dark concerning the actual chemical mechanism of the precipitation of proteins by reagents such as phosphotungstic acid and we are by no means certain, indeed it is highly improbable, that only one compound of a given protein with phosphotungstic acid exists or that its composition is independent of the excess of phosphotungstic acid employed. Hence it is not certain that in the substitution of phosphotungstic acid for hydrochloric acid, under the conditions outlined above, one equivalent of phosphotungstic acid replaces one or even a constant number of equivalents of hydrochloric acid. Results obtained by this method are at present, therefore, only of qualitative value.

The experiments of Erb (11), who employed this method, must nevertheless be quoted here in order to point out a misconception which his interpretation of the results involves. One cubic centimetre of a 5 per cent watery solution of vitellin was mixed with 1, 2, 3, etc., cubic centimetres of normal HCl and the volume of each solution was made up to 10 cc. To these were added a constant excess of calcium phosphotungstate, the precipitate was filtered off and washed, and the filtrate titrated. The following is the way in which Erb expresses his results:

Cc. of normal HCl added	Cc. $N/10$ NaOH required to neutralize the filtrate	HCl bound in cc. $N/10$ HCl	Theoretical excess of $N/10$ HCl	Hydrolytic dissociation per cent	1 gram protein binds mg. of HCl
0.1	0.1	0.9	−1.9	69.0	63
0.2	0.8	1.2	−0.9	59.6	87
0.3	1.5	1.5	0.1	48.3	108
0.5	3.0	2.0	2.1	31.0	146
0.7	4.5	2.5	4.1	13.7	183
0.9	6.2	2.8	6.1	3.4	204
1.0	7.2	2.8	7.1	3.4	204
1.5	12.1	2.9	12.1	0.0	212

It will be seen that the quantity of HCl which is bound by one gram of vitellin increases with increasing concentration of HCl in its solution. We shall see that this phenomenon is a general one where proteins are concerned. It appears that the

binding-capacity of the vitellin reaches a maximum in the higher concentrations of acid. Assuming that this is the true binding-capacity of vitellin but that the compound which is formed undergoes hydrolytic dissociation according to the balanced equation:

$$\text{Protein hydrochloride} + \text{water} \rightleftarrows \text{Protein} + \text{HCl}.$$

Erb calculates, in the fourth and fifth columns of the above table, respectively, the "theoretical excess" of HCl (unbound) and the percentage hydrolytic dissociation of the protein salt in each mixture. This assumption is, however, unquestionably incorrect. The acid- or base-combining capacity of the proteins is a function of the concentration of acid or base in their solution and not independent of it, as it is in the case of a strong monobasic acid which is incapable of undergoing polymerization. This is very readily shown in the case of casein, which is insoluble when uncombined and which would therefore be precipitated if its salts underwent appreciable hydrolytic dissociation. If the alkali-binding capacity of casein were independent of the concentration of alkali in its solution, therefore, the addition of any quantity of alkali beyond that necessary to carry the casein into solution should be without effect upon the number of equivalents of alkali neutralized by one gram of casein. As one increases the amount of alkali in its solution, however, the alkali-binding capacity of casein, as estimated by the potentiometric method, increases from 11.4×10^{-5} equivalents per gram to 180×10^{-5} equivalents per gram, or 16 times the amount of alkali necessary to carry it into solution (40). The alkali-binding capacity of casein is therefore not a constant in the presence of varying amounts of alkali. The same considerations apply, of course, to the varying combining-capacity of serum globulin in the presence of a varying excess of acid or alkali (39).

In the case of proteins which are soluble in water when uncombined with acids or with bases the demonstration of the fact that their combining-capacities vary with the quantity of acid or alkali in their solutions is not such a simple matter, but it is not difficult to infer, from data to which reference will be made in succeeding chapters, that such variations in combining-capacity are an essential feature of the behavior of these proteins also.

It is obvious that almost any sufficiently neutral salt of an acid which forms insoluble compounds with the proteins might

be employed in investigations such as those cited above, in the place of calcium phosphotungstate. von Rhorer has employed potassium mercury iodide (K_2HgI_4) and calcium picrate.

5. The Method of Electrical Conductivity. — This method is based upon the fact that the salts of the proteins are, in solution, less highly ionized than the majority of the strong inorganic acids or bases in equivalent concentration. Hence on adding proteins to a solution of one of these latter, the total number of ions per liter of the solution is diminished. Moreover, the protein ions which are formed have a low velocity of migration (16) (37) (38) (40) (41) as might be anticipated, having regard to the magnitude of their mass and volume. Hence, on adding protein to a solution of a strong acid or of a strong base, the conductivity of the solution is diminished and this diminution affords a measure of, although it is not directly proportional to, the quantity of acid or base bound by the protein. This method was first employed in a systematic manner by Sjöqvist (47). The following figures are illustrative of his results:

	Egg albumin dissolved in 0.025 N HCl.									
Grams albumin in 100 cc.....	0	0.53	1.06	1.59	2.13	2.55	3.19	4.25	5.23	6.38
Mol. Cond. $\times 10^7$.	340.5	271	207	151	108	86	71.5	67	67	68

The molecular conductivities were calculated for a 0.025 m. HCl solution. It will be observed that after a certain quantity of albumin has been dissolved in the acid (4.25 per cent) the molecular conductivity of the solution approaches constancy, indicating that all of the acid is bound by the protein; the inference being that the conductivity thereafter measured is that of the protein hydrochloride alone, the addition of further albumin contributing but little to the conductivity of the solution, since free, unneutralized protein is but sparingly ionized.

Details of more recent results obtained by this method and their interpretation will be found in the chapters dealing with the electrochemistry of the proteins. Since this measurement is a static one, the objections which apply to the two methods previously described do not apply to it.

6. The Cryoscopic Method. — This method depends upon the diminution in the total ionic + molecular concentration in solu-

tions of strong acids or bases, upon the addition to them of proteins. As in the previous method, the measurement is a static one, with this difference, however, that the measurement is necessarily conducted at or in the neighborhood of 0° C. and that the dynamical equilibrium of the system under investigation is shifted to the equilibrium pertaining at this temperature. Regarding the magnitude of this shift for any given temperature range we have but meagre data, but in the light of results obtained with the caseinates and with the salts of ovomucoid (Chap. IX) it is probably in these instances and for the temperature-range 30–0° C., not greater than the experimental error of the method.

The cryoscopic method was first employed, for this purpose, by Bugarzsky and Liebermann (5), who found that upon adding 6.4 grams of egg albumin to 100 cc. of 0.05 *N* HCl or NaOH the difference between the freezing-point of the solution and that of water is reduced nearly 50 per cent, indicating a diminution by nearly 50 per cent of the total number of ions *plus* molecules per liter of the solution. Upon adding similar quantities of protein to solutions of sodium chloride, little or no alteration in the freezing-point of the solutions could be detected. From these results it is usually inferred that the proteins do not bind neutral salts. This, however, is not an altogether justifiable inference. Hardy (*loc. cit.*) has detected a slight, but what he considers unmistakable, depression in the *electrical conductivities* of salt solutions upon saturation with serum globulin, and he assumes that a compound with the neutral salt is actually formed, but that it is only stable in the presence of excess of the salt, so that at any given salt-concentration only a small proportion of the salt is held in combination. Mellanby (28) has arrived at similar conclusions. This possibility becomes more plausible when we view it in the light of the Guldberg-Waage mass law. Representing the reaction between protein and NaCl thus:

$$\text{Protein} + \text{NaCl} \rightleftarrows \text{Protein}-\text{NaCl Compound},$$

we may suppose that the station of equilibrium lies far to the right. An excess of NaCl would displace this equilibrium towards the left and a greater proportion of the protein would exist in the solution in the form of the protein-salt compound, i.e., the compound would be more "stable." Turning now, to the above-

quoted results of Bugarzsky and Liebermann, it is evident that, save in the presence of a considerable excess of neutral salts, little or no alteration in the freezing-point of the solution would result from the addition to it of protein, since the depression of conductivity observed by Hardy only amounted to 2 per cent of that of a $N/10$ solution, and the cryoscopic method is not sufficiently sensitive to reveal, with certainty, such slight variations in the freezing-point of solutions. For this purpose the method of electrical conductivity is much to be preferred. An alternative interpretation of these results will be found in Chap. VI.

7. The Potentiometric Method. — This method is also based upon the fact that the addition of protein to a solution of a strong base or acid diminishes the concentrations of the ions of the inorganic constituent of the mixture. As in the previous methods, the measurement is static. But, instead of involving the measurement of the *total* ionic or ionic plus molecular concentration of the mixture this method enables us to directly determine the concentration of a given ion. The results are therefore much more readily interpreted than those obtained by the two methods previously described and, indeed, furnish us with a direct measure of the quantity of a (highly dissociated) base or acid which is actually bound in the solution, by the protein. For a brief description of the principles underlying potentiometric measurements in concentration-chains the reader is referred to the appendix; for fuller details to such works as those of Hamburger (15) and Michaelis (29).*

The potentiometric method was first employed for this purpose by Bugarzsky and Liebermann (5). These investigators employed two different concentration-chains. The one, the mercury-chain, was built up as follows:

Hg	HgCl (solid), HCl	NaCl	NaBr, HgBr (solid)	Hg
1	2	3	4	5

In the first instance the potential of the chain for a certain concentration of HCl in 2 was measured, then a weighed amount

* For the modifications in technique imposed by the presence of proteins in the solutions under investigation *vide* Appendix.

of protein was introduced into 2 and the potential again determined. The difference between the two readings, in accordance with the Nernst formula, affords a measure of the number of Cl ions bound by the protein. The other concentration-cell was the ordinary gas-chain.

Pt saturated with H_2	Acid	Alkali	Pt saturated with H_2
1	2	3	4

As before, the potential of the chain was first measured for a known concentration of acid in 2, then a weighed amount of protein was added to the acid and the potential of the chain was again determined, the difference between the two readings affording, in this case, a measure of the diminution in the number of H ions due to the introduction of the protein. When it is desired to determine the base-binding capacity of the protein, the latter is added to the alkali in 3, instead of to the acid in 2.

The following are representative of the results obtained by Bugarzsky and Liebermann. In the tables G signifies the number of grams of protein in 100 cc. of solution, p the percentage of acid or base bound by the protein, and r the ratio.

$$\frac{\text{grams of acid or base bound}}{\text{grams of protein}}.$$

I. GAS-CHAIN. EGG ALBUMIN DISSOLVED IN 0.05 *N* HCl

G	p	r	G	p	r
0	0	.....	3.2	60.2	0.034
0.4	9.0	0.042	6.4	96.6	0.027
0.8	18.9	0.044	12.8	99.7	0.014
1.6	33.3	0.038	.....	.....	.....

II. MERCURY-CHAIN. EGG ALBUMIN DISSOLVED IN 0.05 *N* HCl

G	p	r	G	p	r
0	0	.....	1.6	38.0	0.044
0.4	10.7	0.048	3.2	64.0	0.037
0.8	20.2	0.046	6.4	76.0	0.022

III. GAS-CHAIN. EGG ALBUMIN DISSOLVED IN 0.05 *N* NaOH

G	*p*	*r*	*G*	*p*	*r*
0	0	.....	3.2	60.2	0.037
0.8	14.4	0.035	6.4	97.0	0.030
1.6	27.4	0.034	12.8	99.9	0.016

It will be seen that as the concentration of protein rises, i.e., as the proportion of base or acid to protein diminishes, the base- or acid-binding capacity of the protein diminishes. We have already had occasion to note this fact in connection with the results of Erb (11).

Employing a mercury chain containing the element (HgCl (solid), NaCl) instead of the element (HgCl (solid), HCl) used above, and adding to the NaCl solution in this element varying quantities of albumin, the following results were obtained:

IV. MERCURY-CHAIN. EGG ALBUMIN DISSOLVED IN 0.05 *N* NaCl

G	*E* (electromotive force of chain)	*G*	*E* (electromotive force of chain)
0	0.1287	1.6	0.1278
0.4	0.1280	3.2	0.1281
0.8	0.1279	6.4	0.1275

It will be seen that the electromotive force of the chain is but little affected by the addition of egg albumin to the element containing NaCl.

The method employed by Bugarzsky and Liebermann involved, as we have seen, a direct comparison of the ionic concentrations in the protein solution and in the solvent, respectively. This comparison may be avoided and the existence of a variety of protein compounds inferred from the changes in curvature of the curve of ionic concentration consequent upon the admixture with the protein solution of varying proportions of the substance with which compounds are formed. This method, which is essentially the same as that now widely employed in various branches of analytical chemistry (3) (46) (18) (19), was first applied to protein solutions by D'Agostino and Quagliariello (10) and has recently been extensively employed by C. L. A.

Schmidt (45) who, with its aid, has been able to sharply distinguish between the precipitation of a protein through the formation of a true, insoluble compound, and flocculation of the protein through alteration of its physical condition or degree of hydration; the so-called "salting-out" effect.

The details of recent investigations by the potentiometric method and their interpretation will be found in the chapters dealing with the electrochemistry of the proteins.

8. The Method of Catalysis. — In this method, as in the above, direct measurements of the number of hydrogen or hydroxyl ions bound by the protein are secured. The measurement is not, however, altogether a static one, since a foreign substance is added to the solution of the protein, and the rate of change of this substance is the quantity actually measured. When the change in this substance leads to the setting free of an acid (as, for example, in the catalysis of methyl acetate) the method is open to serious objection.

As is well known, many reactions are accelerated by hydroxyl and hydrogen ions, and the velocity-constants of these reactions (such as the inversion of sugar by acids, the saponification of methyl acetate by alkalies, etc.) are directly proportional to the concentration of the hydroxyl or hydrogen ions in the reacting system. If, therefore, we act upon such a mixture with a given concentration of an acid, or of a base, as the case may be, and observe the velocity of the transformation, and, if we then act upon a similar mixture with the same quantity of acid, to which, however, a weighed amount of protein has been added, and again determine the velocity of the transformation, the difference between the two velocities affords a measure of the numbers of H ions (or of OH ions, as the case may be) which have been bound through the introduction of the protein. This method was employed by Cohnheim (8) who states that it was first used for this purpose by Hoffman, at the suggestion of Wilh. Ostwald.

As a reaction which is accelerated by H^+ ions Cohnheim employed the inversion of cane sugar. This reaction, in the presence of free acid, obeys the monomolecular formula

$$\log \frac{A}{A - X} = kt,$$

where A is the initial concentration of the cane-sugar, X is the amount inverted after a time t, and k is a constant (at constant temperature) which varies directly as the hydrion concentration of the solution. The period during which the reaction proceeded was constant (4 hours); hence, if X be the amount of sugar transformed after the addition to its solution of a measured volume of HCl-solution, and X' that after the addition to the same concentration of cane-sugar of the same volume of the HCl-solution, to which, however, protein has been added then:

$$\frac{C}{C'} = \frac{\log A - \log(A - X)}{\log A - \log(A - X')},$$

where C is the concentration of hydrions in the pure acid, and C' that in the acid to which protein has been added. Knowing the value of C that of C' can be immediately calculated. From his results Cohnheim concluded that 0.25 gram of protalbumose, dissolved in 5 cc. of solution containing 0.025 gram of HCl, combines with 4.16 per cent of its own weight of the acid, — similar determinations were made with other albumoses.

Hardy (16) has employed the catalysis of the inversion of cane sugar by H^+ ions in the measurement of the acid-binding capacity of serum-globulin, and that of the saponification of methyl acetate by OH' ions in the measurement of the base-combining capacity of this protein.

In this connection (although, owing to the complexity of the experimental conditions, they have, as yet, but a qualitative significance) the results obtained by a number of observers (12) (7) (22) (23) (2) (1) (53) (35) on the protection conferred by proteins against the destruction of enzymes by acid or alkali may be mentioned. As is well known, the proteolytic enzymes, for example, are very rapidly destroyed by an excess of free acid or base, the action of the acid or base being, probably, that of catalysing the destruction (hydrolysis?) of the enzyme which occurs even in pure water. If, however, protein be added to acid or alkaline solutions containing these enzymes, the rate of destruction of the enzyme is greatly diminished, and this has been attributed by Falk (12), Langley and Edkins (22), Langley and Eves (23) and Vernon (53) to a binding of the free acid or alkali by the added protein. It must not be forgotten, however, that the proteolytic enzymes are, in the presence of protein,

probably combined in some proportion with the protein, which may thus serve in a double manner to protect them from destruction. This fact, however, only adds to the complexity of the conditions, without militating against the correctness of the view urged by the investigators quoted above that the protein protects the enzyme by binding some of the excess of acid or base.

9. The Indicator Method. — This is simply the ordinary method of acidimetry or alkalimetry applied to solutions containing protein, and has been very extensively used; unfortunately, in the past, without a very clear understanding of the exact significance of the data obtained. In order to appreciate this fact it is necessary to recollect that the method, as ordinarily applied, is not a static one. Not only is a foreign substance, namely the indicator, added to the system under investigation, but while acid or alkali is being added to the system to secure neutrality to the chosen indicator, the combining capacity of the protein is continually changing in response to the altering reaction of its solution. The final result obtained in this manner with a given indicator tells us nothing save the condition of equilibrium in the solution at the precise H^+ or OH' concentration at which that indicator changes color; it cannot yield us information concerning the acid or alkali binding capacity of the protein at any other H^+ or OH' concentration. In the further application of this method, the methods of acidimetry and alkalimetry devised by Friedenthal and Salm (14) (13) (44) and elaborated by Sörensen (48) are indubitably destined to prove of the greatest utility, and arouse the hope that the indicator method may be of more service to the protein chemist in the future than it has been in the past.

The use of indicators in protein solutions is, however, accompanied by two notable drawbacks. In the first place, owing to the amphoteric character of the proteins and also to their multiple combining capacities, the changes in the hydrogen- or hydroxyl-ion content of protein solutions, upon the addition of acid or of alkali, over a considerable range, are relatively slight, and, for this reason, sharp end-reactions are rarely obtained with indicators in protein solutions, unless their color changes occur at H^+ or OH' concentrations lying without or upon the boundaries of this range. Then, again, many of the substances commonly used as indicators in acidimetry and alkalimetry combine chemically with the proteins, and the compounds thus formed are not in-

frequently either insoluble or of a different color from the free indicator or its combination with inorganic acids or bases (27) (17) (36).

10. The Method of "Masking" the Physiological Effects of Ions by the Addition of Proteins to their Solutions. — This method although, as yet, only of qualitative importance, is nevertheless of surpassing interest to the physiologist, since the fluids which bathe the tissues contain notable quantities of protein, which may be supposed to modify, in a greater or less degree, the physiological action of the inorganic substances which they contain. This method appears to have first been employed by Loeb (24), and it has since been utilized by Stiles and Beers (50) who, among other observations, have shown that the onset of rigor, which rapidly occurs when frogs' muscles are immersed in solutions of barium salts, is greatly delayed when protein is added to the solutions. These experiments are, however, not conclusive since they are open to the criticism that the added protein may alter the permeability of the tissue for inorganic ions, for example, by clogging up the pores of the external limiting membranes. This criticism, although a serious one from the chemical standpoint, does not, however, detract from the interest to physiologists of such experiments, since, whatever the mechanism may be which leads to modification by proteins of the physiological effects of inorganic substances in solution, the probable importance of such effects in life-phenomena is the same.

La Franca (21) has published the results of a series of experiments which, in the light of the important investigations of Madsen and Nyman (26) hold out the hope that this method may ultimately be employed in a quantitative manner. This observer, employing the method of Paul and Krönig (20) (32), determined the toxicity, for bacteria, of solutions of copper sulphate, mercurous nitrate and silver nitrate to which varying amounts of protein had been added; at the same time he measured the concentration of heavy-metal ions in the solution by the potentiometric method. His results show a satisfactory parallelism between the diminution in the toxicities of these solutions caused by the addition of the protein, and the number of heavy-metal ions bound by the protein, as revealed by the potentiometric measurements.

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CHAPTER V

THE COMPOUNDS OF THE PROTEINS (Continued)

1. Stoichiometrical Relations in Protein Compounds. — With the aid of the various methods outlined in the previous chapter, the existence of a number of compounds of the proteins with inorganic acids, bases and salts has been conclusively demonstrated. Of these compounds, however, comparatively few have been extensively studied, and in equally few cases have the stoichiometrical relations which pertain between the constituents of the compounds been elucidated. This is due in part, no doubt, to the complexity and amphoteric character of the proteins themselves, and in part to the difficulty of sharply characterizing and of isolating the individual proteins in a pure condition, but in the main, I believe, to the fact that physical chemistry has only recently been in a position to supply us with the implements which the investigation of these compounds demands. We have seen that the successful investigation of these compounds requires, as a rule, the employment of static methods of measurement; methods, that is, which do not involve a disturbance of the equilibria in the system while these equilibria are being determined. Many investigators in this field have, in the past, employed methods of determining the existence of protein compounds, which, to our modern perceptions, clearly involved a variable and uncontrolled interference with the very equilibrium which was the subject of investigation. Examination of more modern literature, however, cannot fail to impress the reader with the conviction that in proportion to the adequacy of the chemical or physico-chemical technique employed, stoichiometrical relations between the proteins and the substances with which they combine are revealed or indicated.

The problem of determining the nature of the compounds formed by the proteins with inorganic acids or bases is complicated by the fact that, as a rule, a protein can combine with not only one but several equivalents of a base or acid, so that on adding acid or alkali to a solution of a protein we obtain a continuously varying mixture of the various possible salts. Precisely analogous phenom-

ena are, however, met with among the complex and double salts, and the salts of polybasic acids or polyacid bases in the domain of inorganic chemistry, and they afford no legitimate grounds for the belief that the protein compounds are not molecular in character and consequently do not obey the law of constant combining proportions. All of the accurate data which we possess lend decided support to the view that although the proteins may enter into combination in multiple proportions, these proportions are constant and indicate that under definite conditions of reaction and concentration the protein molecules possess a definite and measurable equivalent weight.

For the investigation of such compounds exactly the type of technique must be employed which has proved to be successful in dealing with the inorganic compounds of analogous complexity, modified, however, by the limitations imposed upon us by the instability of the proteins themselves. From the previous chapter it will be clear that in the majority of instances the characterization of the protein compounds must, for the present and pending further elaboration of our technique, be based upon electrochemical measurements. A discussion of the data derived from measurements of this type must necessarily be deferred until later chapters in which the electrochemical behavior of the protein salts will be taken up in detail. For the present, therefore, except in the case of the protamins, of which the salts with acids lend themselves to sharp characterization by ordinary chemical methods, we shall simply dwell upon the existence and general properties of certain protein compounds without seeking to decide, in any specific instance, how many equivalents of the protein or of the remaining constituent are bound up in one molecule of the salt.

2. The Compounds of the Protamins with Inorganic Acids and Bases. — The protamins occur combined with nucleic acid, in the nuclei of spermatozoa (27) (28) (13). According to Burian (10), the nucleo-protein in the spermatozoa of the salmon is formed by the combination of one molecule of nucleic acid with one molecule of salmin.

The protamins (19) (20) are predominantly basic substances, the acid function being very small in comparison. They are soluble in water, yielding strongly alkaline solutions. The formula of salmin sulphate is a multiple of:

$$C_{30}H_{57}N_{17}O_6,\ 2\ H_2SO_4 + H_2O$$

while that of clupein sulphate is a multiple of

$$C_{30}H_{57}N_{17}O_6,\ 2\ H_2SO_4;$$

but if an insufficient quantity of acid is present to completely neutralize the protamin, "basic" salts, containing fewer equivalents of acid, are readily formed.

The sulphuric acid in these compounds is readily replaced by an equivalent amount of hydrochloric (13), nitric, carbonic, or chromic acids. The nitrates, chlorides and carbonates are readily soluble, the sulphates sparingly soluble in cold, but more soluble in hot, water, while the chromates are insoluble. The chloride of salmin is soluble in methyl alcohol (13).

The formula of sturin sulphate is, according to Kossel, a multiple, either of

$$2\ C_{33}H_{61}N_{17}O_7 + 5\ H_2SO_4$$

or, more probably, of

$$4\ C_{36}H_{69}N_{19}O_7 + 11\ H_2SO_4.$$

Scombrin (derived from mackerel) sulphate possesses, according to Kurajeff (21), the formula:

$$C_{30}H_{60}N_{16}O_6,\ 2\ H_2SO_4.$$

It is soluble in water. The chromate is insoluble in water and possesses the formula:

$$C_{30}H_{58}N_{16}O_5,\ 2\ H_2CrO_4,$$

differing, therefore, from the sulphate, not only in the acid component, but also in the absence of one molecule of water for every two molecules of combined acid. It is to be particularly noted that *the less soluble protein salt contains less water combined with the protein*. Remarkably analogous to this is the fact that whereas salmin sulphate is freely diffusible through parchment paper (53), clupein sulphate, which contains one molecule less of water for every two molecules of combined acid, is almost indiffusible (49). We shall have occasion to refer to analogous facts when considering the mechanism of the coagulation of proteins (Chap. XII).

The protamins also form compounds with chloroplatinic acid (28); these compounds are insoluble in neutral water, alcohol or ether, but are soluble in water containing an excess of acid. According to Goto (13), the formula of the chloroplatinate of salmin is:

$$C_{30}H_{57}N_{17}O_6,\ 4\ HCl,\ 2\ PtCl_4.$$

Although the acid function of the protamins is very weak in comparison with the basic function, salts with inorganic bases are nevertheless formed. The salts of the heavy metals (except cupric salts) produce from alkaline solutions of free protamin precipitates of the corresponding salts of the protein. The compound with divalent copper is soluble in water, and its solutions are violet in color. The compounds with monovalent copper are very sparingly soluble. On adding alkali (sodium hydrate) in excess of one-half the equivalent of the protein (clupein) molecule, to the precipitate of the silver salt, a yellow color is produced, indicating that sodium has displaced some of the silver from the molecule and that the silver protaminate is now mixed with silver oxide.

Other compounds which the protamins form will be considered in the succeeding chapter. The physical properties of the protamins and their salts will be discussed in the chapters dealing with the physical properties of protein systems.

3. The Compounds of Casein with Bases, Acids, and Salts. — Casein, in the dry state, forms a white powder, the general properties of which have been described in Chap. II. Uncombined and purified casein is insoluble in distilled water or in very dilute salt solutions (11) (34) (22) (36). A suspension of casein, thoroughly shaken up in distilled water, reddens litmus paper wherever the suspended particles touch it, but if this suspension be filtered the filtrate contains no detectable casein and is neutral to litmus. Casein is therefore probably soluble to a very slight extent in distilled water, but this slight amount is practically entirely concentrated at the surface of the water which is in immediate contact with the solid particles, owing to the diminution in surface energy which is thus brought about, so that the litmus is only reddened where it touches these surfaces.

Uncombined casein is soluble in warm 5 per cent salt-solution and in hot 50 per cent alcohol. When freshly prepared and warmed it is very plastic and is capable of being drawn out into long, fine threads (59).

Although it is so insoluble in water, uncombined casein, when suspended in water, nevertheless acts as an acid in expelling carbon dioxide from carbonates and bicarbonates, forming a salt with the base (51), and in this way, according to Osborne (34), the ammonium, potassium, sodium, lithium, magnesium, strontium and calcium caseinates can be prepared. The salts of the alkaline

earths can be precipitated without alteration of their composition by the addition of alcohol to their solutions in water.* By direct analysis of this precipitate Van Slyke and Hart (59) have shown that the calcium salt which is formed in this way contains 2.4 per cent of calcium oxide or from 80×10^{-5} to 90×10^{-5} equivalents of calcium per gram (7). This salt, in solution, is neutral to phenolphthalein, and the alcohol precipitate from a lime-water solution of casein which has been neutralized to phenolphthalein contains exactly the same percentage of CaO (unneutralized by mineral acids) as that from the solution of casein in a carbonate-solution. Similar results had previously been obtained by Söldner (51) (52), de Jäger (18) and Laquer and Sackur (22), who, however, employed volumetric methods of measurement. Other figures for the percentage of CaO in this compound which have been obtained by various observers are: Courant (12), 2.91 and Timpe (54), 2.62. These figures are not very divergent, and since the great majority of observers agree in placing the percentage at 2.4, we may assume that this result is not far from correct. The percentage of casein in alkaline solutions can be fairly accurately determined by titration to neutrality to phenolphthalein, upon the assumption that in solutions of this alkalinity (OH concentration) casein is combined with 2.4 per cent of CaO or with an equivalent quantity of other (strong) bases.

If, however, a lime-water solution of casein be neutralized to *litmus* instead of to phenolphthalein, by the addition of mineral acid or of casein, the alcoholic precipitate now contains only 1.5 per cent of CaO (Van Slyke and Hart (59)). The same estimate of the combining-capacity of casein at absolute neutrality (i.e., in solutions neutral to litmus) has been made by Söldner (51) (52) and by myself (42) employing the potentiometric method. The casein from goats' milk combines with bases in the same proportions as the casein from cows' milk (9). The compound of casein with 2.4 per cent of CaO, or an equivalent quantity of any other strong base, is termed by Söldner and by Van Slyke and Hart the "basic" caseinate, by Courant the "tricaseinate" of the base. That with 1.5 per cent of CaO, or an equivalent quantity of other strong bases is termed by Söldner and by Van Slyke and Hart the

* Cf. Chap. II. The "basic" salt of potassium (i.e., neutral to phenolphthalein) is soluble in 98.6 per cent alcohol, but it may be precipitated from this solution by the addition of ether (45).

"neutral" caseinate; by Courant the "dicaseinate" of the base. Both terminologies are objectionable since they imply or suggest hypothetical stoichiometrical relations. Neutrality of its solutions to an arbitrarily chosen indicator is no evidence of the unity of a substance. As we shall see in considering the electrochemistry of these substances, both that containing 2.4 per cent CaO and that containing 1.5 per cent CaO are *mixtures* of two or more individual salts of casein. We shall, however, for the sake of brevity in allusion, employ the nomenclature of Söldner, as being the less objectionable of the two.

At neutrality either to litmus or to phenolphthalein all observers agree that casein binds the alkalies and alkaline earths in equivalent-molecular proportions. Thus regular stoichiometrical relations between the protein and these metals are clearly *indicated* although the above cited data are insufficient to *define* them. With regard to ammonium caseinate, however, some confusion exists since Bechamp (3) states that it contains 1.17 to 1.21 per cent of ammonia (= 1.94 to 1.99 per cent of CaO), while Salkowski (47) states that it contains only 0.35 per cent of ammonia. Salkowski's method of preparing this compound, however, was faulty since he precipitated it from alcoholic solution by the addition of NaCl, a procedure which involved the possibility of double decomposition and the partial substitution of sodium for ammonia in the casein compound.*

The quantitative results for casein, which we have so far cited, may be stated in other words thus: At neutrality to litmus one gram of casein binds 50×10^{-5} equivalent gram molecules of a base, and at neutrality to phenolphthalein 80×10^{-5} equivalent gram molecules.†

It was formerly stated by the author (36) that if a given concentration of alkali be "saturated" with casein, that is, if it be shaken up with excess of casein until no more of the protein will dissolve, and then filtered, the filtrate is always neutral to litmus and contains the quantity of casein required to form the "neutral" caseinate of the base. In later communications (41) (42), I have shown, however, that this conclusion was erroneous, the source of

* Regarding the actual occurrence of such types of interaction between casein salts and inorganic salts, Cf. W. A. Osborne (35) and Van Slyke and Bosworth (57).

† That is, gram molecules divided by the valency of the combining ion.

error lying in the fact that after the attainment of neutrality to litmus additional casein dissolves in the solution with extreme slowness. Many hours of rapid shaking or stirring are quite insufficient, at room temperature, to bring about complete "saturation" of the alkali with casein. Resource was had, therefore, to the device of dissolving weighed amounts of casein in measured volumes of alkali of known concentration, and then neutralizing the excess of alkali by the addition of standardized acid, stirring vigorously the while, until the casein just began to be precipitated. The point at which precipitation began was readily determined, with considerable accuracy, by the change (diminution) of the refractive index of the solution. In this way it was ascertained that at complete "saturation" of an alkali (NaOH or LiOH) one gram of casein binds 11.4×10^{-5} equivalent gram molecules of the base.

These determinations have been repeated by Van Slyke and Bosworth (57), whose results very closely coincide with mine. They find that at the point at which further diminution of the proportion of base to casein results in the precipitation of free casein one gram of casein is combined with between 11.0×10^{-5} and 11.5×10^{-5} equivalents of ammonium, sodium or potassium.

These "saturated" compounds of casein with bases react in solution to indicators as follows (41):

Dimethylaminoazobenzol	yellow
Congo red	red
Sodium alizarinsulphonate	red
Cochineal	rose
Paranitrophenol	colorless
Rosolic acid	yellow

indicating, according to the determinations of Salm (48) an *acidity* (H^+ concentration) of about 10^{-5}. According to Michaelis and Rona (26) and Allemann (1), the optimum reaction for the complete precipitation of casein is 2×10^{-5} H^+.

It is not possible with equal simplicity of technique to determine the combining capacity of casein for the *alkaline earths* at "saturation" of these bases with casein, because the chloride of the alkaline earth, which is necessarily formed in the solution at the same time, precipitates the caseinate.* Thus four grams of casein were dis-

* The "neutral" and "basic" caseinates of the alkaline earths are likewise precipitated by the addition to their solutions of a somewhat greater proportion of the corresponding alkaline earth. Cf. A. S. Loevenhart (24) and Van Slyke and Hart (59).

solved in 100 cc. of 0.048 N $Ca(OH)_2$ solution and then 40 cc. of $N/10$ HCl were cautiously delivered into the solution by means of a pipette of which the opening was held below the surface of the fluid, the fluid being rapidly and continuously stirred meanwhile through the agency of a small motor. The total volume was then made up to 200 cc. and the mixture filtered through soft filter-paper. By measurement of the refractive index of this filtrate it was found that the 0.0008 equivalent of $Ca(OH)_2$ unneutralized by HCl had, under these conditions, only held in solution 0.2 gram of casein; although, as we have seen, in the absence of $CaCl_2$, 0.0008 equivalent of $Ca(OH)_2$ will readily dissolve one gram of casein, rendering the solution neutral to phenolphthalein.

In a subsequent chapter (X) it will be shown that a definite relationship subsists between the initial alkalinity of a solution and the depression of its electrical conductivity which is brought about by the addition to it of a definite proportion of casein. This relationship is of such a character that by exterpolation from the measurements made upon solutions of potassium caseinate containing from 25×10^{-5} to 300×10^{-5} equivalents of base per gram of casein, it would appear that the depression of conductivity caused by casein is zero when the proportion of base is sufficient and only just sufficient to hold the casein in solution (42), namely, 11.4×10^{-5} equivalents per gram. Similar exterpolation from measurements of the depression of the conductivity of $Ca(OH)_2$ solutions consequent upon the addition of casein leads to the conclusion that the depression of conductivity would vanish at an initial alkalinity corresponding to a proportion of 11.9×10^{-5} equivalents of $Ca(OH)_2$ per gram of casein, a value so close to that obtained for the combining-capacity of casein for the alkalies at "saturation" with casein that I have previously inferred that bases dissolve casein in equivalent-molecular proportions (46). Van Slyke and Bosworth have, however, measured the combining-capacity of casein for alkaline earth bases at "saturation" of the base with casein by the more direct method of first dissolving the casein in an excess of the base and then neutralizing the excess with hydrochloric acid and dialysing the mixture until free from soluble chlorides (57). In this way they have found that a compound of casein with calcium hydrate which contains 11.25×10^{-5} equivalents of base per gram exists but is not soluble in water, the lowest proportion of base yielding a soluble compound being

22.5×10^{-5} equivalents per gram, or exactly double the proportion of alkaline earth contained in the insoluble compound. The insoluble compound will, however, dissolve in solutions of NaCl, NH_4Cl, KCl, etc., probably owing to exchange of bases, with the formation of soluble caseinates of the alkalies (56) (57).

Magnesium hydrate forms a similar series of compounds with casein, namely a compound insoluble in water but soluble in 5 per cent NaCl solution and containing 11.25×10^{-5} equivalents of base per gram, a soluble compound containing 22.5×10^{-5} equivalents of base per gram, a compound neutral to litmus containing about 56×10^{-5} equivalents of magnesium per gram and a compound neutral to phenolphthalein containing between 80×10^{-5} and 90×10^{-5} equivalents of magnesium per gram (62).

From the phosphorus content of casein (8), we would infer a minimal combining-weight of 4430. The combining-weight corresponding to the proportion of NaOH or KOH combined with casein at "saturation" of the base with protein is 8888. This corresponds so closely with twice the minimal combining-weight calculated on the assumption that the casein molecule contains only one atom of phosphorus that we may infer, with Bosworth and Van Slyke, that the molecule of casein contains two atoms of phosphorus.

The *maximum* combining-capacity of casein for bases (KOH) has been measured by the author (42), employing the potentiometric method. In the presence of a considerable excess of KOH, the combining-capacity of casein attains the constant maximum of 180×10^{-5} equivalents per gram. In passing from its minimum to its maximum, therefore, the combining-capacity of casein is multiplied sixteen times.

The compounds of casein with *acids* have not been so extensively studied as those with bases. Hammarsten (14) held that there is no true combination between casein and acid, because by prolonged trituration with water he could remove all of the acid contained in the casein. Our modern conception of balanced reactions deprives this consideration of its weight (59) (36). Since, however, it has been frequently applied to other protein compounds,* it may be worth while to dwell briefly upon the implications of such a deduction.

* For example by Hans Trunkel (55): When so much of the published evidence on behalf of the "adsorption theory" is of this unfortunate and inconclusive character, advocates of "adsorption" cannot complain if their hypotheses are viewed with scepticism by physical chemists.

When any two or more substances, for example, A and B react to form other substances A' and B', equilibrium is reached, i.e., no further change occurs in the composition of the system, when the reaction

$$A + B \rightleftarrows A' + B'$$

proceeds at exactly the same velocity from left to right as it does from right to left. Since the velocity with which a chemical reaction proceeds varies directly as the active masses of the reacting substances (Guldberg and Waage's law) the velocities of the two opposing reactions gradually approach each other as the reaction proceeds, until they become equal, when the reaction apparently ceases. According to the relative magnitude of the proportionality-factors expressing the ratio of mass to velocity of each of the reactions concerned, equilibrium may occur so far to the left or right that, with our limited precision of measurement, the reaction appears to be complete or not to occur at all as the case may be, or equilibrium may occur when the active masses of the reacting components on either side of the equation are nearly equal; and, of course, every type of equilibrium intermediate between these extremes is found to occur. If, after the attainment of equilibrium, one of the reacting components be wholly or partially removed from the system the reaction will recommence more or less rapidly, restoring a certain proportion of the abstracted component, and attaining a fresh station of equilibrium determined by the new relative masses of the components.

If, now, after the attainment of equilibrium in such a reaction as:

$$\underset{\text{(Insoluble)}}{\text{Casein}} + \underset{\text{(Soluble)}}{\text{HCl}} \rightleftarrows \underset{\text{(Insoluble or sparingly soluble)}}{\text{Casein hydrochloride}}$$

even if only a small proportion of free HCl and casein exist in the system, and HCl be removed by trituration with water, the reaction will recommence, now proceeding from right to left, and restore a proportion of the HCl which has been washed away. If trituration be prolonged and the velocity-constant (proportionality between mass and velocity) for the decomposition of the casein hydrochloride be tolerably high, it is easy to understand how the compound might ultimately be completely decomposed and deprived of its combined acid. Similarly, the acid components of substances so universally admitted to be chemical compounds as mercuric sulphate and cuprous chloride can be completely removed

from them by prolonged trituration with water (23) (16). To argue from this fact that these combinations are not chemical in character would be analogous to concluding that, because salicylic acid can be completely removed from its solution in water by repeated extraction with ether, therefore the salicylic acid was not truly dissolved in the water.

To return to the more specific problem of the occurrence of combinations between inorganic acids and casein. Van Slyke and Hart (59) and Van Slyke and Van Slyke (60) have described a series of compounds of casein with inorganic acids which are insoluble in distilled water. When solid casein is suspended in a dilute solution of an inorganic acid, the protein abstracts some of the acid from the solution, forming these insoluble compounds. Van Slyke and Van Slyke measured the velocity with which the acid is abstracted from the solution and the final equilibrium attained by abstracting portions of the well-stirred suspension, from time to time filtering, and measuring the electrical conductivity of the filtrate. The decrease in conductivity, from the moment of introduction of the casein, afforded a measure of the amount of acid bound. They found that the acid is bound at first rapidly and later more slowly (Cf. Chap. XII) and at equilibrium the ratio

$$\frac{\text{acid bound by one gram casein}}{\text{acid in one cc. of solution}}$$

is nearly constant between the limits of concentration of the acid employed ($N/125$ and $N/1000$) for hydrochloric acid at constant temperatures. When sulphuric acid is employed the ratio increases with dilution of the acid, the quantity of acid bound by one gram varying as the square root* instead of directly as the concentration of the acid. The equilibrium is the same from whichever direction it is approached, whether by suspending the protein compound in more dilute acid than that in which it was formed, in which case it yields acid to the solution, or by suspending uncombined casein directly in the solution of acid. When one gram of casein is suspended in 100 cc. of $N/500$ acid at 0° C., it takes up 17.4×10^{-5} equivalents of sulphuric, 11.9×10^{-5} equivalents of hydrochloric, 8.9×10^{-5} equivalents of lactic or 5.3×10^{-5} equivalents of acetic

* The exact exponent obtained by Van Slyke and Van Slyke is 1/1.95; this experimental value is so nearly $\frac{1}{2}$ that it affords no grounds for preferring the experimental value to that which is indicated by stoichiometry.

acid. Increase in temperature increases the rate at which equilibrium is approached but decreases the final amount of acid taken up.

It is evident that the concentration of acid was not sufficient in any of these solutions to bring about complete neutralization of the casein. When more concentrated solutions were employed, however, a proportion of the casein was dissolved, and the methods of estimation utilized by Van Slyke and Van Slyke could not be employed. From considerations which follow below (Cf. Chap. XII), it appears possible that the compounds of casein with acids which are formed in experiments such as these are in reality soluble, but that while a sufficient proportion of casein remains uncombined they are mechanically hindered from passing out of the casein particles into the solvent (38) (40). Van Slyke and Van Slyke, however, believe that the binding of casein by acids, under these conditions, is an "adsorption" phenomenon (60) (61).

If one stirs excess of casein in 0.1 *N* HCl (or more dilute) rapidly and continuously for over an hour, at room temperature very little or none of the casein passes out into the solvent; the solution remains perfectly clear on filtration and its refractive index is only very slightly changed. If, however, the casein be previously dissolved in dilute NaOH and then precipitated with HCl, the addition of the slightest excess of HCl will then carry it into solution. In other words, wet, freshly precipitated casein dissolves rapidly in dilute acid, but the physical properties of dry, granular casein, hinder the compounds which are formed from passing out of the particles.* Dry serum globulin similarly dissolves with extreme slowness in acids, although, when wet and freshly precipitated, it dissolves in dilute solutions of acids with great readiness.

I have determined the acid-equivalent of casein at "saturation" of the acid with protein in the following manner (41): Weighed amounts of casein were dissolved in measured volumes of alkali of known concentration; the excess of acid over that sufficient to

* The fact that the casein hydrochloride which is formed is in reality soluble, although it does not pass out into the solution, explains the dependence of the amount formed upon the dilution of the acid, which was observed by Van Slyke and Van Slyke. Were the casein salt *insoluble*, as these authors point out, the amount formed should be invariable, or else equivalent to the total amount of acid in the system.

neutralize the alkali, which it was necessary to add to the solution in order to *just* redissolve the casein was then determined. The point of complete re-solution of the casein was determined by noting the point at which the refractive index of the filtered mixture attained a constant (maximum) value. It was found that at "saturation" in solutions containing 1.25 per cent of casein, 1 gram of casein = approximately 32×10^{-5} equivalent gram molecules of HCl. The acid-equivalent in more dilute solutions appears to be somewhat higher, although I have not measured it directly.* This is attributable to the fact that, as the observations of Van Slyke and Van Slyke show, the hydrochloride of casein tends in some measure to decompose into casein and hydrochloric acid, and, since free casein is insoluble, an excess of acid over that actually combined with the casein is necessary in order to diminish this (hydrolytic) dissociation sufficiently to keep the casein in solution. The same phenomenon, as we shall see, is encountered in acid solutions of serum globulin.

Schlossmann (50) and Arny and Pratt (2) have described compounds of casein with alums which, especially that with ferric alum, are but sparingly soluble. Upon this fact Arny and Pratt have based a method of estimating casein volumetrically.

Paracasein, the product of the action of rennet upon casein, resembles casein very closely, save in the greater insolubility of the calcium salt in the presence of an excess of calcium ions. Calcium caseinate is precipitated by calcium chloride, a smaller amount of calcium chloride being required to bring about complete precipitation, the smaller the proportion of base in the caseinate (i.e., the more acid the solution). This precipitation is still more readily accomplished at higher temperatures (24) (59). Calcium paracaseinate differs from calcium caseinate in that it is precipitated by a smaller excess of calcium ions, and, by a given excess, at a lower temperature (24). Paracasein differs, however, still more markedly from casein in the proportion of base which it binds at "saturation" of the base with protein, i.e., when the proportion of inorganic base is sufficient and only just sufficient to

* The accuracy of this estimate of the combining-capacity of casein for acids at "saturation" of the acid by protein cannot be relied upon, since the solution necessarily contained a certain quantity of the chloride of the alkali employed to dissolve the casein, and the compounds of casein with acids are very readily precipitated by salts.

hold the paracasein in solution. We have seen that the proportion of alkali which just suffices to hold casein in solution in water is about 11.25×10^{-5} equivalents per gram. Paracasein, however, requires exactly double this proportion of alkali to hold it in solution (57) indicating, as Van Slyke and Bosworth believe, that the molecule of paracasein is one-half the weight of the molecule of casein. From this it would follow that the molecule of paracasein contains but one atom of phosphorus (58).

4. The Compounds of Serum Globulin with Inorganic Acids and Bases. — The compounds of the globulin which is precipitated from diluted serum by the passage of CO_2 through it, and which, in the free condition, is insoluble in distilled water, have been extensively studied by W. B. Hardy (15). This investigator has measured the quantities of various acids which are required to dissolve one gram of globulin at various concentrations. The mean values, measured in gram molecules, taking the quantity of HCl required to dissolve one gram of the globulin as unity, are quoted below:

HCl.....1.0	H_2SO_4.....1.91	Citric.....3
HNO_3.....0.995	Tartaric.....1.994	H_3PO_4.....2.9
$CHCl_2COOH$....1.0	Oxalic.....1.9	H_3BO_3.... very great excess
$CH_2ClCOOH$....1.05		
HCOOH.....1.25		
CH_3COOH.....5.2		
CH_3CH_2COOH...7.56		

It is evident that the acid-equivalent of serum-globulin is the same for all of the *strong* monobasic acids investigated, although it is much higher for the weak acids. This equivalent is, for one gram of the protein, about 18×10^{-5} gram-equivalents (mean value). It is, however, somewhat higher the more dilute the globulin, and this effect is more marked with the weak than with the stronger acids. Thus:

Concentration of protein, per cent	Gram-equivalents of acid necessary to dissolve one gram of protein	
	Acetic acid	Hydrochloric acid
0.28	183×10^{-5}	23×10^{-5}
1.46	56×10^{-5}	15×10^{-5}
4.18	40×10^{-5}	13×10^{-5}

We have already had occasion to discuss the interpretation of similar phenomena which are displayed by the compounds of casein with acids.

The acid-equivalent of serum-globulin for dibasic (strong) acids is twice that for monobasic acids, and for tribasic (strong) acids it is three times that for monobasic acids. Hence serum-globulin combines with acids, at "saturation" in *molecular* and not *equivalent molecular* proportions.

Solutions of acids "saturated" with serum-globulin are faintly acid to methyl orange.

Employing the potentiometric method (37) (39) I have determined the quantity of acid neutralized by globulin in solutions of hydrochloric acid of various concentrations. The following are among my results, the concentration of globulin in each solution being 0.496 per cent.

Concentrations of HCl in which globulin was dissolved	Concentration of H^+ ions in solution containing globulin	Equivalents of HCl neutralized per gram of globulin
38.5×10^{-4}	12.4×10^{-4}	53×10^{-5}
57.8×10^{-4}	23.8×10^{-4}	69×10^{-5}
77.0×10^{-4}	36.8×10^{-4}	81×10^{-5}
120.3×10^{-4}	72.7×10^{-4}	96×10^{-5}

We see that the acid-equivalent of serum-globulin rises with increasing acidity of its solution.

The compounds of serum-globulin with bases have also been investigated by Hardy. He finds that at "saturation" of solutions of bases with serum-globulin the bases are bound by the globulin in *molecular*, not *equivalent molecular* proportions. Solutions of monacid bases "saturated" with globulin are neutral to litmus, the alkali equivalent for monacid bases being approximately 10×10^{-5} gram-equivalents of the base per gram of globulin, that for di-acid bases being approximately 20×10^{-5} in the same units.

At neutrality to phenolphthalein, however, that is, at an alkalinity corresponding to an OH' concentration of 20×10^{-7} (Cf. Salm (48)), the bases are bound in *equivalent* molecular proportions, such that one gram of globulin binds 20×10^{-5} gram-equivalents of the base. Thus the point of complete saturation of $Ba(OH)_2$ solutions by globulin and that of neutrality to phenolphthalein coincide, while solutions of the *monacid* bases which are

saturated with globulin contain only half the proportion of base to protein which is contained in those which are neutral to phenolphthalein. Hardy has suggested, in explanation of this, that globulin has at least two replaceable hydrogens and that its acid salts of sodium or potassium are soluble, while its acid salt of barium is relatively insoluble, the "neutral" salt being soluble and neutral to phenolphthalein. Since complete solution of the globulin is just attained at neutrality to litmus and the ratio of the alkali-binding capacity of serum at neutrality to phenolphthalein to its capacity at neutrality to litmus is 2 : 1, this hypothesis, although based only upon results obtained with arbitrarily chosen indicators, would appear to be justifiable. We shall have occasion to further discuss this question from a somewhat different point of view in Chap. IX.

Employing the potentiometric method (37) I have determined the quantity of alkali neutralized by globulin in solutions containing an excess of KOH. The following were the results obtained, the concentration of globulin in each solution being 0.208 per cent.

Concentration of KOH in which globulin was dissolved	Equivalents of KOH bound by one gram of globulin
12.9×10^{-4}	47×10^{-5}
16.1×10^{-4}	46×10^{-5}
19.3×10^{-4}	50×10^{-5}
25.8×10^{-4}	45×10^{-5}

The alkali-equivalent of the globulin, therefore, remained appreciably constant throughout the range of alkalinities mentioned. Its mean value is 47×10^{-5} gram-equivalents of KOH per gram of globulin, which may therefore be regarded as the alkali-equivalent of globulin *in the presence of excess of base.*

The ratio of the alkali to the acid-equivalent of globulin at "saturation" is, according to Hardy's determinations 10 : 18. He considers that the true ratio is probably 1 : 2.

The compounds of globulin with salts have been studied by Hardy (15) and by Mellanby (25). But as these compounds have not yet been clearly defined, and their discussion involves a consideration of the general nature of the mechanism of the solution and precipitation of protein by salts, the description of the results obtained by these observers will be deferred to the succeeding chapter.

5. The Compounds of Fibrin with Inorganic Acids and Bases. — Employing methods similar to those utilized by Van Slyke and Bosworth in the investigation of the compounds of casein with inorganic bases, Bosworth has investigated the combining-capacity of fibrin for inorganic bases and acids (6). He finds that at neutrality to phenolphthalein one gram of fibrin neutralizes 61.4×10^{-5} equivalents of NaOH or 61.6×10^{-5} equivalents of $Ca(OH)_2$. Fibrin, therefore, neutralizes bases, in the presence of excess of the base, in equivalent-molecular proportions. At "saturation," i.e., when the proportion of base is only just sufficient to hold the protein in solution, fibrin combines with 15.2×10^{-5} equivalents of NaOH, and the precipitate produced on addition of HCl to this solution is free from sodium. In other words no insoluble salts of sodium are formed. The case is quite otherwise with calcium which forms with fibrin an insoluble salt (soluble, however, in 5 per cent NaCl solution), containing 30×10^{-5} equivalents of the base, and a soluble salt containing 45×10^{-5} equivalents of the base. The proportion of 1 : 2 : 3 encountered in these various compounds is irresistible evidence of the existence of definite stoichiometrical relations between fibrin and the bases with which it combines.

The sulphur-content of fibrin would indicate a minimal combining-weight of 6751. The proportion of base in the "saturated" compound of fibrin with NaOH would indicate a combining-weight of 6667.

Calcium and barium fibrinates yield a precipitate of fibrin on the passage of CO_2 through them (6) (17), sodium, potassium and ammonium fibrinates do not. Fibrin does not decompose $CaCO_3$, however, as casein does on trituration with this salt in the presence of water.

Fibrin forms a compound with hydrochloric acid, which is just soluble in water, containing 15×10^{-5} equivalents of the acid.

6. The Compounds of the Vegetable Proteins with Inorganic Acids and Bases. — These have been investigated by T. B. Osborne (29) (30) (31) (32), who has found evidence of combination between inorganic acids and the following vegetable proteins: Edestin, legumin, excelsin, amandin, corylin, phaseolin, gliadin, hordein and zein. He finds that when edestin, deposited from salt-solutions, is suspended in water and made neutral to phenolphthalein, edestin itself remains undissolved, while the added alkali carries into solution its equivalent of the acid or acids which

had been united with the edestin. The character of the acid united with the edestin varies with that of the salt employed for solution of the edestin. When the salt is sodium chloride, the acid is HCl, when it is ammonium sulphate, the acid is H_2SO_4. The combination is apparently facilitated by a weakly acid reaction, not necessarily induced by the same acid as that with which the edestin combines. Osborne attributes this phenomenon to the strength of edestin as a base; the compound which is formed, however, must be practically undissociated so far as Cl′ ions are concerned, otherwise, having regard to the extremely low equivalent-concentration of protein in the solutions in which it is formed, we should attribute to edestin a basic function of a magnitude wholly inconsistent with the fact that it is an amphoteric acid. Therefore, it does not follow that these proteins are stronger bases than they are acids because they can displace hydrochloric acid from its combination with sodium hydrate, as Osborne assumes, for this phenomenon may be interpreted by merely supposing that the protein can combine with either constituent of the organic salt, but that the compound with HCl produces fewer Cl′ ions than the Na^+ ions produced by the compound with NaOH. A further discussion of this phenomenon will be found in a later chapter. (Chap. VI, section 6.)

The fact that the occurrence of decomposition of neutral mineral salts through the agency of *animal* proteins has not been generally recognized, is probably attributable to the fact that it has not been carefully looked for, and that the proteins which are most readily obtained in the pure condition are not usually prepared under conditions favoring the formation of protein compounds through the decomposition of inorganic salts.

Two compounds of edestin with acid (hydrochloric) appear to exist. The one, which Osborne terms the monohydrochloride, contains an amount of acid-equivalent to 0.7 cc. $N/10$ HCl per gram of the protein, and is insoluble in water; it is, however, soluble in salt solution and is deposited therefrom in crystals on dialysis. The other compound of edestin with hydrochloric acid contains just sufficient acid to hold the protein in solution, and is termed by Osborne the dihydrochloride. The quantity of HCl required to just hold one gram of edestin in solution is 1.4 cc. of $N/10$ HCl. The acid-equivalent, for monobasic acids, at "saturation" of the acid with edestin is therefore 14×10^{-5} gram-equivalents per gram. The quantity of *alkali* which will

just hold one gram of edestin in solution is equivalent to the quantity of acid with which it combines to form Osborne's "edestin monohydrochloride," that is, 7×10^{-5} equivalents. Hence the ratio of the alkali- to the acid-equivalent at "saturation" is 1 : 2.

From the observations of Osborne it would appear that acids dissolve edestin, as they do serum-globulin, in molecular proportions, not in equivalent molecular proportions.

Osborne has also determined the hydrochloric acid equivalents at neutrality to *tropæolin* (10^{-2} to 10^{-3} H^+ according to Salm (48)), of a variety of vegetable proteins. That of edestin was found to be 127×10^{-5} gram-equivalents per gram, of excelsin 124×10^{-5}, of legumin 129×10^{-5}, of amandin 103×10^{-5}, of crystallized egg-albumin 90×10^{-5} in the same units. Recollecting that these determinations are made in the presence of a considerable excess of acid they may be considered as furnishing, possibly, a measure of the constant maximum acid-equivalents of these proteins in the presence of excess of acid. However this may be, it is evident that the acid-equivalent of edestin increases very considerably with the acidity of its solution.

The compounds which edestin and gliadin form with cupric hydroxide (33) have already been discussed in Chap. I.

7. The Compounds of Ovomucoid with Acids and Bases. — These have so far chiefly been studied by electrochemical methods (43). Uncombined ovomucoid is freely soluble in distilled water, but on adding small quantities of acid or alkali to this solution a proportion of the acid or base is neutralized by the protein. Employing the potentiometric method I have shown that at neutrality to litmus (i.e., absolute neutrality) 1 gram of ovomucoid neutralizes 7×10^{-5} equivalents of HCl. Its basic function, therefore, predominates over its acid function.

The maximum (constant) combining-capacity of ovomucoid for KOH, in solutions containing excess of the base, is about 50×10^{-5} equivalents per gram.

The maximum combining-capacity of ovomucoid for HCl was not attained in any of the solutions investigated, but is probably in excess of 100×10^{-5} equivalents per gram.

In the chapters dealing with the electrochemistry of the proteins the reader will find a more detailed discussion of these compounds.

8. Summary of Some of the Results Cited in this Chapter. — Some of the more important determinations which have been cited in this chapter are summarized in the following table:

	Casein	Serum-globulin	Fibrin	Edestin	Ovomucoid
Acids dissolve	.....	In molecular proportions	.....	In molecular proportions	Soluble in water
Bases dissolve	In molecular proportions	In molecular proportions	Three equivalents of diacid base per equivalent of monacid base	.....	.....
Bases neutralize to litmus	In equivalent-molecular proportions	In molecular proportions (saturated)	.....	.....	.....
Bases neutralize to phenolphthalein	In equivalent-molecular proportions	In equivalent-molecular proportions	In equivalent-molecular proportions	.....	.....
Acid-equivalent at "saturation" (monobasic acids)	32×10^{-5} gram-molecules per gram	18×10^{-5} gram-molecules per gram	15×10^{-5} gram-molecules per gram	14×10^{-5} gram-molecules per gram	.....
Alkali-equivalent at "saturation" (monacid bases)	11.4×10^{-5} gram-molecules per gram	10×10^{-5} gram-molecules per gram	15×10^{-5} gram-molecules per gram	7×10^{-5} gram-molecules per gram	.....
Ratio of alkali-equivalent at saturation to acid-equivalent at saturation	1 : 3	1 : 2	1 : 1	1 : 2	.....
Alkali-equivalent at neutrality to litmus	50×10^{-5} equivalent gram-molecules per gram	10×10^{-5} gram-molecules per gram	.....	.....	.....
Acid-equivalent at neutrality to litmus	.....	.....	.....	.....	7×10^{-5} equivalent gram-molecules per gram
Alkali-equivalent at neutrality to phenolphthalein	80×10^{-5} equivalent gram-molecules per gram	20×10^{-5} equivalent gram-molecules per gram	61.5×10^{-5} equivalent gram-molecules per gram	.....	.....
Maximum alkali-equivalent	180×10^{-5} equivalent gram-molecules per gram	47×10^{-5} equivalent gram-molecules per gram	.....	.....	50×10^{-5} equivalent gram-molecules per gram
Maximum acid-equivalent	.....	.....	.....	(127×10^{-5} equivalent gram-molecules per gram?)	.....

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CHAPTER VI

THE COMPOUNDS OF THE PROTEINS (Continued)

1. General Remarks on the Precipitation of Proteins by Inorganic Salts. — It was pointed out by Hardy (24) in his exhaustive communication on globulin that the precipitation of proteins and, indeed, of colloids in general may be of two kinds. The first is clearly accompanied by decomposition of the precipitating agent; it will not occur, as Pauli has demonstrated (56) unless the protein is in some proportion ionized, and relatively small quantities of the precipitating agent are required to bring about the precipitation. The second kind of precipitation, however, whether accompanied by decomposition of the precipitating agent or not, occurs even when the protein is non-ionic and requires relatively large amounts of the precipitating agent. Precipitation of the first kind is, generally speaking, only brought about by electrolytes, while precipitation of the second kind, although as a rule, more readily brought about by electrolytes than by non-electrolytes, may nevertheless be brought about by certain non-electrolytes, for example, by alcohol.

For this latter type of precipitation we shall henceforth, whenever possible, reserve the term coagulation.* Both precipitation and coagulation of a protein may be brought about by one and the same inorganic salt. In such a case the gradual addition of salt to the originally salt-free solution which contains ionic protein, i.e., protein which drifts to one electrode or to the other in an electric field, first brings about precipitation and then resolution of the protein. In this new solution the protein is, accord-

* Much confusion exists in the literature on this subject on account of the fact that the distinction between the *precipitation* of a protein through chemical interaction with the added salt, and its *coagulation* through the change in the nature of the solvent resulting from the further addition of salt has not invariably been recognized.

ing to Hardy, non-ionic and it can be coagulated by still further addition of the salt.*

The first kind of precipitation appears to be undoubtedly chemical in character and in mechanism. The mechanism of *coagulation* is, however, far from clear, and for the attainment of an adequate understanding of this phenomenon we shall doubtless have to wait until the physico-chemical theory of solutions in general has reached a more mature stage of development than it has at present. At least three possibilities exist.

(i) The coagulation of proteins by salts is a purely physical phenomenon due either to an alteration in the electrical condition of the protein (Bredig, Billitzer, Freundlich) or to a physical alteration in the nature of the solvent.

(ii) The coagulation of proteins by salts is partly a physical and partly a chemical phenomenon depending upon the formation of various compounds between the protein and the salt, and upon their varying solubilities in salt solutions (Spiro, Galeotti).

(iii) The coagulation of proteins by salts is indirectly a chemical phenomenon, attributable to a disturbance in the chemical equilibrium between the protein and its solvent (Hofmeister, Pauli).

In reviewing the various factors which have been ascertained to be of importance in determining the precipitation and coagulation of proteins by electrolytes we shall incidentally discuss the applicability of these several hypotheses.

2. Earlier Investigations on the Significance of the State of Hydration of the Proteins in Relation to their Coagulation by Salts. — The fact that proteins can be thrown out of solution by the addition thereto of inorganic salts appears to have first been pointed out by Claude Bernard (2), who employed, among others, magnesium sulphate, sodium sulphate and ammonium carbonate. As early as 1854, Virchow (76) suggested that the inorganic

* An interesting example of the dependence of the ionization of a protein upon concentration (i.e., available mass of water) of the medium in which it is dissolved is afforded by serum-globulin dissolved in solutions of sodium citrate. In solutions containing low concentrations of sodium citrate the protein is ionized and drifts in an electric field, while the same protein when dissolved in more concentrated sodium citrate solution is found to be no longer ionized (9).

salts render proteins insoluble by extracting water from them. This suggestion was again put forward in 1888 by F. Hofmeister (30), who, together with his pupils, advanced in the immediately succeeding years, a number of experimental data in support of his thesis (30) (31) (38) (62) (43).

Kühne (39) and Kauder (38), having shown that not only the concentration of the added salt but also that of the protein is of importance in determining the amount of salt required for its coagulation, care was taken in these and in the majority of succeeding investigations, when comparing the coagulating power of different salts, to maintain the concentration of the protein constant, the procedure being to add to a given volume of protein-containing fluid (e.g., blood serum) varying amounts of different salt solutions, and then dilute the mixture to a standard volume.

Lewith showed that the relative efficacy of the different salts which he employed in coagulating the proteins of blood serum was the same for serum globulins as for serum-albumins, sulphates and acetates being more powerful coagulants than nitrates or chlorides. His results have been tabulated as follows by Gustav Mann (46), a (+) indicating that the salts do, and a (−) that they do not precipitate serum-albumin.

	Potassium	Sodium	Ammonium	Magnesium	Calcium	Barium
Acetate	+	+	−	−	−	−
Chloride	+	+	−	−	+	−
Nitrate	−	+	−	−	+	−
Phosphate	.....	+	.....	.....	.....	.....
Sulphate	−	+	+	+	.....	.....
Sulphocyanate	.....	−	.....	.....	.....	.....
Iodide	−	−	−	−	−	−
Bromide	−	−	−	−	−	−
Chromate	.....	.....	−	.....	.....	.....
Bicarbonate	.....	−	.....	.....	.....	.....

Hofmeister, in 1888, extended and confirmed these observations, employing not only serum-proteins but also egg-albumin, gelatin and other colloids, namely colloidal ferric hydrate and sodium oleate. He found that whatever the colloid employed the relative coagulating power of the various salts was the same. Exceptions to this rule were noted, however, when salts of di- or tribasic acids were employed, the order of their efficacy in coagulating egg-globulin and gelatin being the same, but differing

when ferric hydrate and sodium oleate were employed. Nasse (49), in discussing the hypothesis that the coagulation of proteins is due to the withdrawal of water from the colloid, had raised the objection that the ratio of the concentrations at which the magnesium and ammonium sulphates bring about coagulation of different colloids is not always exactly the same. For different colloids Nasse found the ratio

$$\frac{\text{conc. of } (NH_4)_2SO_4 \text{ just sufficient to coagulate}}{\text{conc. of } MgSO_4 \text{ just sufficient to coagulate}}$$

possessed the following values:

Gelatin	Egg-albumin	Serum-albumin	Hemi-albumose	Peptone
0.84	1.03	0.94	0.85	1.00

Hofmeister, however, pointed out that the *absolute* concentrations which are required to bring about coagulation in these cases are very different, and an exact quantitative relation of this kind could not be expected to hold good, since the condition of the salts in solution, or, as we should now express it, their relative degree of electrolytic dissociation, differs at different absolute concentrations.

Following up the idea that the coagulation of colloids by salts is attributable to the possession by the salts, in the concentrations employed, of a greater power of binding water than that possessed by colloid, Hofmeister (1890–91) took up the study of the swelling or absorption of water by colloids in various solutions, the degree of swelling in different solutions being regarded as a measure of the relative binding capacities of the colloid and of the dissolved salts. He found that in solutions of sulphates, tartrates, acetates, alcohol, cane-sugar or grape-sugar gelatin-plates take up less water than they do when immersed in distilled water, while in solutions of potassium, sodium or ammonium chlorides, sodium chlorate, sodium nitrate and sodium bromide they take up more water than they do when immersed in distilled water. It will be recollected that the sulphates, tartrates and acetates are the most energetic *coagulants* of gelatin. Regarding their high coagulating power as being attributable to their power of abstracting water from the protein, the interpretation of these results of Hofmeister's becomes clear.

In 1898 Pauli (51) (58) published the results of a number of investigations upon the influence of various salts on the gelatinizing- and melting-temperatures of gelatin solutions. He found that effects of different salts upon the gelatinizing and melting of strong gelatin solutions ran parallel with their power of coagulating gelatin and of inhibiting the swelling of gelatin plates. Thus chlorides, bromides, and iodides of potassium, sodium, ammonium and magnesium lower the temperatures of gelatinization and of melting, in the following order, the most effective being placed first.*

Sodium sulphate, magnesium sulphate, sodium citrate, ammonium sulphate, magnesium sulphate, sodium tartrate, sodium acetate. The following is the order in which these salts bring about the coagulation of gelatin, the most effective being placed first:

Sodium sulphate, potassium sulphate, sodium citrate, magnesium chloride, sodium tartrate, magnesium sulphate, ammonium sulphate, sodium acetate, potassium chloride, sodium chloride.

There is evidently a close, although not an absolute parallelism between the two series. Urea and alcohol lowered, but glycerin markedly *raised* the temperatures of gelatinization and of melting.

In a later communication (52) Pauli showed that the order in which various salts affect the coagulation-temperature of egg-globulin is similar to that in which they occur in the above series. The following table, cited after Pauli, gives the order in which the various salts bring about the transformation of the proteins from the dissolved into the solid condition:

Coagulation of egg-globulin (Hofmeister)	Coagulation of gelatin (Pauli)	Heat coagulation of egg-globulin (Pauli)	Gelatinization of gelatin (Pauli)	Swelling of gelatin (Hofmeister)
Sulphates	Sulphates	Chlorides	Sulphates	Sulphates
Acetates	Citrates	Acetates	Citrates	Citrates
Citrates	Tartrates	Sulphates	Tartrates	Tartrates
Tartrates	Acetates	Chromates	Acetates	Acetates
Chlorides	Chlorides	Chlorates	Chlorides	Chorides
Chlorates	.....	Nitrates	Chlorates	Chlorates
Nitrates	.....	Bromides	Nitrates	Nitrates
.....	.....	Iodides	Bromides	Bromides
.....	.....	.....	Iodides	.....

* All of these substances were employed in equimolecular solutions.

The nature of the cation was, however, found to be not wholly without influence, since the temperature at which egg-globulin coagulates is lowest in the presence of ammonium chloride, and highest in the presence of magnesium chloride, the series being as follows: NH_4, K, Na, Li, Ba, Mg. The curves expressing the relationship between the concentrations of the salts employed and the temperature of coagulation are, however, not parallel, and in some cases cut one another, so that at higher concentrations (4 N) the series runs as follows: Na, NH_4, Li, Ba, Mg.

Pauli also investigated the effect of mixtures of two salts, and found, as had already been indicated by the results of Schäfer (71), that the coagulating action of salts upon proteins is additive, that is, each salt exerts its separate effect and the *precipitating (or dissolving) power of the mixture is the algebraic sum of the separate effects exerted by its components* except when the two salts have a common ion and so diminish each other's degree of dissociation. This result has since been more fully confirmed by Pauli (53) and by Mellanby (47).

The globulins, as a class, are insoluble in distilled water, but are soluble in dilute saline solutions. Further addition of salt results in coagulation of the globulin. Non-electrolytes can bring about the coagulation but not the solution of the globulin. From these facts Pauli concluded that the solution of globulin is due to the formation of compounds of the globulin with salts, of the type:

$$\text{Na} - \text{globulin} - \text{Cl}.$$

the further addition of salts to this solution resulting in the precipitation of this compound.

3. The Influence of the Electrical Condition of the Proteins upon their Precipitation and Coagulation by Electrolytes. — The action of very small quantities of salts in bringing about the precipitation of colloids from their solution was first studied by Graham (20). He ascertained, for example, that a solution of colloidal aluminium hydrate prepared by dialysing the chloride is so sensitive to the presence of salts that the mere addition of a few drops of undistilled water suffices to precipitate it. This type of action of salts upon colloids was further investigated by Schultz (73), Prost (66), and Linder and Picton (44).

Defining the precipitating-power of a salt as the reciprocal of the concentration in gram-molecules per litre necessary to coagulate a given solution of the hydrosol of sulphide of arsenic, Schultz found that the relative precipitating-powers of the uni-

valent, divalent and trivalent metals are in the ratios 1: 30: 1650. Prost, employing the hydrosol of cadmium sulphide, obtained a similar relation, while Linder and Picton found that the precipitating powers of different salts of a given metal are proportional to their equivalent conductivities and that the relative precipitating powers of the sulphates of univalent, divalent, and trivalent metals can be expressed by the ratios 1: 35: 1023.

In all of these cases the colloid employed was electronegative, that is, on electrolysis it migrated to the anode. The experiments which we have cited show that in such cases the ion of the added electrolyte which is effective in bringing about precipitation is the cation. In 1899, however, Hardy (21) showed that egg-albumin, modified by heating its solution so as to partially coagulate the protein, may be induced to travel either to the anode or to the cathode by simply changing the reaction of its solutions. In acid solutions the protein behaves like a cation, migrating to the cathode; in alkaline solutions it behaves like an anion, migrating to the anode. In alkaline solutions, the cations of added salts proved to be the active agents in precipitating egg-albumin, while in acid solutions the precipitating power of the cation proved to be altogether subordinate to that of the anion (22). The following illustrate his results:

PROTEIN IN PRESENCE OF TRACE OF ALKALI, ELECTRONEGATIVE

Temperature 16 degrees. Coagulating salt 1 gram-mol. in 80,000 cc.

Coagulated at once	On slightly warming	Did not coagulate
$Al_2(SO_4)_3$	$MgSO_4$	Na_2SO_4
$Cd(NO_3)_2$	$BaCl_2$	K_2SO_4
$CuSO_4$	$CaCl_2$	NaCl
$CuCl_2$	.....	.....

PROTEIN IN PRESENCE OF TRACE OF ACID, ELECTROPOSITIVE

Coagulated instantly	No effect
$Al_2(SO_4)_3$	$CuCl_2$
$CaSO_4$	$Cd(NO_3)_2$
K_2SO_4	$BaCl_2$
Na_2SO_4	NaCl
$MgSO_4$	.....

Similar results were obtained with other colloids. Electronegative colloids are precipitated, if at all, by cations; electropositive colloids, by anions.

Whetham (78) (26) explained these phenomena in the following way: He assumes that at the surface of the colloid particles there exists a double electrical layer. When the particles move towards the anode they are negatively charged, when they move to the cathode they are positively charged. The surface energy of the colloidal phase is reduced by the presence of the electrical double layer and therefore its tendency to contract. The existence of the double layer, therefore, conduces to the stability of a system in which the surface of the colloid is greatly extended, i.e., to the stability of the "colloidal solution." The cations of the added electrolyte, in the case of electronegative colloids, or the anions in the case of electropositive colloids, neutralize this charge and therefore increase the energy of the surface of the colloidal phase and its tendency to contract. Hence the finely suspended colloidal particles unite to form large aggregates having a less extended surface, and these aggregates finally become so large as to assume the properties of matter in mass, and hence are carried out of solution by the action of gravity. In this way the dependence of the precipitating-power of an electrolyte upon its dissociation, found by Linder and Picton and by Hardy, and also the reversal in the relative precipitating-powers of the ions of the added electrolyte upon reversion of the sign of the electrical charge carried by the colloid, found an explanation. In interpreting the valency rule discovered by Schultz, Whetham develops his theory as follows:

"In a solution where ions are moving freely, the probability that an ion is at any instant within reach of a fixed point is, putting certainty equal to unity, approximately represented by a fraction proportional to the ratio between the volume occupied by the spheres of influence of the ions and the whole volume of the solution, and may be written as AC, where A is a constant and C represents the concentration of the solution. The chance that two such ions should be present together is the product of their separate chances, that is $(AC)^2$. Similarly the chance for the conjunction of three ions is $(AC)^3$, and for the conjunction of n ions is $(AC)^n$."

"In order that three solutions, containing trivalent, divalent,

and univalent ions respectively should have equal coagulative powers, the frequency with which the necessary conjunctions should occur must be the same in each solution. We should then have, the constant being assumed equal in each case,

$$A^{2n}C_3^{2n} = A^{3n}C_2^{3n} = A^{6n}C_1^{6n} = \text{a constant} = B.$$

Therefore

$$C_3 = \frac{B^{\frac{1}{2n}}}{A}; \quad C_2 = \frac{B^{\frac{1}{3n}}}{A}; \quad C_1 = \frac{B^{\frac{1}{6n}}}{A},$$

C_1, C_2, C_3, representing the concentrations of monads, diads, and triads in their respective solutions. Thus we get for the ratios of the concentrations of equi-coagulative solutions:

$$C_1 : C_2 : C_3 = B^{\frac{1}{6n}} : B^{\frac{1}{3n}} : B^{\frac{1}{2n}} = 1 : B^{\frac{1}{6n}} : B^{\frac{1}{3n}}.$$

Let us put $B^{\frac{1}{6n}} = \frac{1}{x}$; the ratios can then be written:

$$1 : \frac{1}{x} : \frac{1}{x^2}.$$

The reciprocals of the numbers expressing the relative concentrations of equi-coagulative solutions give values proportional to the coagulative-powers of solutions of equal concentration; so that, calling the coagulative-powers of equivalent solutions containing monovalent, divalent, and trivalent ions respectively, p_1, p_2, p_3, we get:

$$p_1 : p_2 : p_3 = 1 : x : x^2.$$

Let us now take some numerical examples.
Putting $x = 32$ we get the series:

$$1 : 32 : 1024.$$

Which agrees very well with Linder and Picton's results for colloidal solutions of antimony sulphide:

$$1 : 35 : 1023;$$

and putting $x = 40$, we get

$$1 : 40 : 1600,$$

numbers comparable to Schultze's values for sulphide of arsenic" (79).

It is curious that during the discussions which immediately followed these discoveries the fact should have been so generally overlooked that the above hypothesis of Whetham's is simply a statement, in kinetic terms, of the Guldberg and Waage mass-law. According to this law, the velocity with which any given reaction proceeds varies directly as the active masses of each of the reacting molecules. In the case under consideration, presuming that a given number (e.g., one) of molecules of protein react with one molecule of a salt of a monovalent metal to form a compound, then twice as many molecules of the protein may be supposed to react with a molecule of a salt of a divalent metal, and three times as many with a salt of a trivalent metal. Assuming that the active mass of the colloid (the molecular concentration multiplied by the degree of dissociation) is the same in each of these cases (which is also assumed in Whetham's theory) and equal to A, calling the initial velocities of the respective reactions v_1, v_2, and v_3 and the concentrations of the mono-, di- and trivalent ions c_1, c_2, and c_3, we have:

$$v_1 \text{ is proportional to } Ac_1,$$
$$v_2 \text{ is proportional to } A^2c_2,$$
$$v_3 \text{ is proportional to } A^3c_3,$$

whence it follows that if $v_1 = v_2 = v_3$ and the velocity-constants of the three reactions are equal (which is also assumed in Whetham's hypothesis):*

$$\frac{c_1}{c_1} : \frac{c_1}{c_2} : \frac{c_1}{c_3} : : 1 : A : A^2,$$

and $\frac{1}{c_1}$, $\frac{1}{c_2}$ and $\frac{1}{c_3}$, i.e., the dilutions of the mono-, di- and trivalent ions at which combination proceeds with equal velocity are related to one another in the same way. Now in the experiments described above, $\frac{1}{c}$ is defined as p, the precipitating-power of the salt, hence:

$$p_1 : p_2 : p_3 : : 1 : A : A^2,$$

which is exactly the relation deduced by Whetham. The relations found by Schultze, Prost, Linder and Picton and others, are, therefore, just as explicable upon the assumption that the

* In the terminology of Whetham's hypothesis, the term "velocity constant" would read "proportion of effective collisions."

colloid reacts chemically with the precipitating ion as upon the assumption that the precipitating ion acts merely through altering the electrical condition of the colloidal particles. The former view attributes to the colloids no especial qualities which differentiate them from other chemical systems, the latter view necessarily involves assumptions regarding the nature of colloidal solutions which have hitherto proved incapable of verification.

It is not surprising that *velocities* rather than *equilibria* determine the "precipitating-powers" of reagents for colloids, when we recollect, firstly, the enormous part played by the *velocity of change* in the final physical condition of a colloid,† and secondly the method by which these "precipitating-powers" are measured. Linder and Picton measured the precipitating-power of a salt by titration, running the solution of the salt into the solution of arsenic sulphide until coagulation just began to be perceived. They expressly state that unless the time occupied in the titration be kept approximately the same, serious deviations from the above rule occur, "as a quantity of coagulant insufficient to produce coagulation immediately will do so in the course of time." Under these conditions, what is actually measured is the concentration of the precipitating agent which is requisite to bring about a given degree of change (visible precipitation) within a given brief period, that is, a velocity and not an equilibrium. Data regarding the degree of precipitation after varying periods and the equilibria which are attained in such reactions as these are lacking, but Linder and Picton have shown that the cation which brings about the precipitation of colloidal arsenic is bound by the colloid and carried down with it. It cannot be washed out with water, but it can be replaced by another metal. Whitney and Ober (80) obtained similar results and, moreover, showed that different metals are carried down by this colloid in equivalent-molecular proportions.

That these results are susceptible of purely chemical interpretation was pointed out by Hardy (22) in 1900 and this view was further developed by him in his communications on globulin referred to above (24). He showed that in a series of salts of

* According to W. B. Hardy (23), the more slowly the division into two phases occurs the smaller and less curved is the surface of separation. Cf. also Freundlich (15).

the same valency the power of precipitating an electronegative colloid varies directly as the *equivalent conductivity of the salt*, that is, as the active mass of the dissociated metal ions. Since electronegative colloids appear to behave, electrically, like the anions of an acid, and electropositive colloids like the cations of a base, their respective affinities for the basic and acid radicals of salts are readily explicable. This fact appears, however, to have been first clearly pointed out by Loeb (45).

An interpretation of the phenomena attending the precipitation of ionic ("electrically active") colloids by electrolytes practically identical with that of Whetham was afterwards brought forward by Bredig (7).

Modifications of Whetham's theory have been advanced by Billitzer (3) (4) and by Freundlich (15) (16). Billitzer objected to the assumption which was made by Whetham (although as we have seen, it was not at all essential to his hypothesis) that an electrical double layer exists at the surface of colloid particles in aqueous solution and showed that this assumption is inadequate to explain the migration of the particles in an electrical field. The charge on the colloid particles must therefore be supplied by the particles themselves, by giving up oppositely charged ions to the solution, i.e., the colloids must be electrolytes. So far his view is identical with that which is developed above. But he adds to this the assumption that the charge which is carried by the colloid particles is not a full atomic charge. The precipitating action of oppositely charged ions he attributes to the electrostatic attraction of the colloid particles by the oppositely charged crystalloid ion. Since the colloid particle is assumed not to possess a full atomic charge, a number of particles must, he considers, be attracted by one crystalloid ion, thus forming a molecular group large enough to be appreciably acted on by gravity. How the colloid particles come to acquire a fraction of an atomic charge Billitzer does not explain, nor does our experience of the behavior of electrolytes afford any legitimate precedent for such an assumption.

Freundlich believes that the precipitating ion must penetrate (be "adsorbed" by) the surface of the colloid particle and that the surfaces of colloids are permeable to ions possessed of an opposite but not to ions possessed of the same charge as their own. The precipitating powers of various salts are stated to

run parallel with the degree to which they are "adsorbed" by finely pulverized charcoal.

Biltz (5) also considers that the precipitation of colloids by electrolytes and by other colloids is due to the formation of "adsorption compounds."

4. Later Investigations on the Significance of the State of Hydration of the Proteins in Relation to their Coagulation by Electrolytes. — Continuing his investigations, cited above, on the coagulating action of pairs of salts on egg-white, Pauli (53) found that a number of salts which will not coagulate egg-white by themselves will increase the coagulating power of other salts when mixed with them, while others markedly *diminish* the coagulating power of salts which, in their absence, readily coagulate egg-albumin. Moreover certain salts, although very soluble, do not in any concentration cause coagulation of egg-white. The possibility suggested itself that the coagulating action of salts might depend upon two antagonistic factors, respectively attributable to the cations and the anions of the salts. In confirmation of this view it was found that the NH_4 ion, when combined with SO_4 will coagulate egg-albumin, although when combined with acetanion it will not. Acetanion coagulates when combined with sodium, but not when combined with ammonium. Pursuing this line of reasoning and investigation Pauli concluded that if the coagulating powers of a series of cations be indicated by $f, f', f'', \ldots$ and the opposite (solvent) powers of a series of anions by $h, h', h'', \ldots$, then in a mixture of electrolytes the following possibilities exist:

$$\Sigma\,(f, f', f'', \ldots) \gtreqless \Sigma\,(h, h', h'', \ldots),$$

the mixture being such as to coagulate, leave unaffected or inhibit the coagulation of the albumin. Pauli found that in egg-white (in which the protein is electronegative) the cations of added electrolytes are the active agents in inducing coagulation, while the anions inhibit coagulation. In the following table of Pauli's the cations are arranged in ascending order of precipitating-power from left to right, while the anions are arranged vertically, the weakest inhibitor coming first and the strongest last. A (+) indicates that the salt which results from the union of the cation and anion causes coagulation of egg-albumin; while a (−) indicates that it does not.

Anions	Cations				
	Mg	NH_4	K	Na	Li
Fluoride	.....	+	+	+	.....
Sulphate	+	+	+	+	+
Phosphate	.....	+	+	+	.....
Citrate	.....	+	+	+	.....
Tartrate	.....	+	+	+	.....
Acetate	−	−	+	+	.....
Chloride	−	−	+	+	+
Nitrate	−	−	−	+	+
Chlorate	.....	−	−	+	.....
Bromide	−	−	−	−	+
Iodide	.....	−	−	−	.....
Thiocyanate	−	−	−	−	.....

The valency of the coagulating ion would appear to be quantitatively of less importance in the coagulation of proteins by salts than it is in the precipitation of ionic protein by small quantities of salts, since magnesium resembles the alkalies closely in its coagulative power, while lithium approaches the alkaline earths.

The order of effectiveness of the different salts in bringing about the coagulation of *electropositive* protein is, however, exactly the reverse of their order of effectiveness in bringing about the coagulation of electronegative protein, such as the albumin in egg-white. This was first shown by Posternak (64), who employed the reserve-material of the seeds of *Picea excelsa*, dissolved in very dilute hydrochloric acid. Posternak's observation has been confirmed by Pauli (53) and by Hoeber (29). Acidifying a solution of egg-white reverses the functions of the coagulating ions, those which coagulated electronegative protein most strongly now inhibit its coagulation most strongly. Those which inhibited its coagulation now induce coagulation. The series is reversed in every respect; the anions now induce coagulation and the cations inhibit it. The anions precipitate in the order:

$$CNS > I > Br > NO_3 > Cl > \text{Acetate}.$$

while the cations inhibit precipitation in the order:

$$Li > Na > K > NH_4 > Mg.$$

When electronegative protein (egg-white) is acted upon by salts of the alkaline earths "irreversible" precipitates or coagula

are formed, insoluble, that is, on dilution of the system with water. The alkaline earths, moreover, afford a strong contrast to the alkalies, in that the precipitating-power of the cation is *increased* by the anions, in the series which is characteristic for *electropositive* protein (54). In other words, when egg-white is acted upon by salts of the alkaline earths the protein behaves, so far as the action of anions upon it is concerned, as though it were in acid solution. Pauli believes that the alkaline earth reacts with OH groups of the protein to form the comparatively slightly dissociated hydrates of the alkaline earths, the acid which is set free inducing the acid reaction of the medium. In other words, Pauli's view is that protein aids the action of water in bringing about hydrolytic dissociation. Other colloids would appear to act similarly, since Whitney and Ober (80) found that on the addition of neutral halogen salts of the alkaline earths to colloidal arsenic sulphide the reaction of the solution becomes acid.

The influence of added salts of the alkalies and magnesium upon the precipitation of proteins by heavy metals varies with the concentration of salt employed (55). At low concentrations (0.005 m.) the salts inhibit precipitation in the order:

$$SO_4 < Cl < C_2H_3O_2 < NO_3 < Br < I < SCN,$$

while in high concentrations (4 m.) they *encourage* precipitation in the order:

$$SO_4 > Cl > C_2H_3O_2 > NO_3 > Br > I > SCN.$$

This is simply a particular instance of the rule, to which the attention of the reader was drawn in the earlier part of this chapter, that the salts may act as precipitants and as coagulants at low and at high concentrations respectively, acting as solvents at intermediate concentrations. The heavy metal salts afford no exception to this rule. At low concentrations they precipitate, at higher concentrations they dissolve, and at still higher concentrations they coagulate the proteins of egg-white (55). Copper sulphate acting upon egg-albumin would appear to afford an exception since it does not coagulate even in saturated solution. That the exception is apparent and not real is, however, shown by the observation of Galeotti (17) that in sufficiently *supersaturated* solution copper albuminate is coagulated by copper sulphate, addition of merely *saturated* copper sulphate solution redissolves

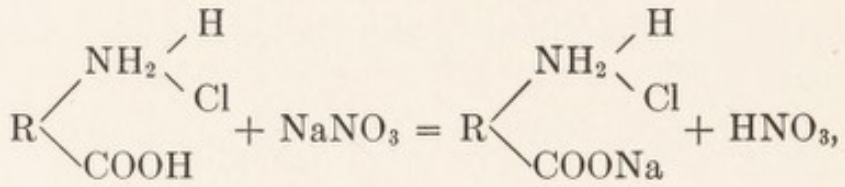
this coagulum, addition of water then results in the *precipitation* of copper albuminate.

The concentration-range throughout which the salt acts as solvent may be evanescent, as it is in the case of silver nitrate acting upon egg-albumin (Pauli).

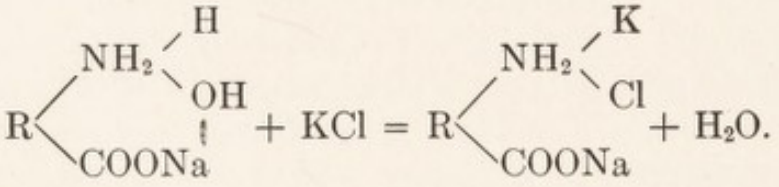
It was already shown in 1833 by Rose (68) that hæmoglobin is coagulated by concentrated mercuric chloride, but may be redissolved by dilution, to be precipitated on further dilution.

The very important observation has been made by Pauli (56) that absolutely electrolyte free egg-albumin is not ionic (i.e., does not drift in an electric field) and that under these conditions it is not precipitable by heavy metals. It is, however, coagulated by highly concentrated salts.*

According to Pauli and Handovsky (57) (60) the number of ionized protein particles in a solution of ionic protein is diminished by the addition of salts; at the same time the viscosity of the solution diminishes and the coagulability of the protein by the usual coagulating agents is increased. When salts are added to a solution of electropositive protein (i.e., protein combined with acid) an increase in the acidity of the solution results (24). They believe that acid-protein reacts with salts as follows:



while alkali-protein reacts as follows:



On the basis of the more probable view of the electrolytic dissociation of proteins outlined in Chap. I, this hypothesis would be represented by the following schematic formulæ:

* Egg-albumin is nearly equally basic and acid. It is probable that the proteins which are either predominantly acid or basic may still, in some measure, be electrolytically dissociated when uncombined with bases or acids.

$$-\mathrm{COH}^{++} + \overset{\mathrm{Cl}}{\underset{\mathrm{H}}{\overset{|}{\underset{|}{\diagdown\!\mathrm{N}-}}}} + \mathrm{NaNO_3} = -\mathrm{CONa} = \overset{\mathrm{Cl}}{\underset{\mathrm{H}}{\overset{|}{\underset{|}{\mathrm{N}-}}}} + \mathrm{HNO_3}$$

$$-\mathrm{CONa}^{++} + \overset{\mathrm{OH}}{\underset{\mathrm{H}}{\overset{|}{\underset{|}{\diagdown\!\mathrm{N}-}}}} + \mathrm{KCl} = -\mathrm{CONa} = \overset{\mathrm{Cl}}{\underset{\mathrm{K}}{\overset{|}{\underset{|}{\mathrm{N}-}}}} + \mathrm{H_2O}.$$

The symmetry of the action of the salts upon the acid and alkali protein compounds would appear, however, to indicate a greater similarity in the structure of the compounds formed than that which is suggested by these formulæ.

5. Applications of the Phase-rule to Protein-salt-water Systems. — In addition to the above investigations attempts have been made to interpret the behavior of the proteins in the presence of salts, in the light of the phase-rule, starting from the view developed by Spiro (74) (10) that the coagulation of proteins by salts might be regarded as a separation of the system into two phases, a solid phase rich in protein, or a protein-salt compound, and poor in salt and water, and a liquid phase poor in protein, rich in salt and water.

The phase-rule, as developed by Willard Gibbs (19), van't Hoff and Roozeboom may be enunciated as follows: A system of r coexistent phases containing n independently variable components is capable of $n + 2 - r$ variations in the temperature, total pressure of the system or concentration of the components of its phases. A "phase" is a portion of the system separated from the rest by a definite surface, the "independently variable components" are the least number of different substances with which it is possible to represent, in a chemical equation, the composition of each phase in the equilibrium (14). Only *equilibria* are contemplated in the derivation of the phase-rule, consequently it is not applicable to systems which by reason of hysteresis or limited reaction-velocity * are in a condition of incomplete equilibrium. A discussion of the theoretical basis of

* Unless the reaction-velocity in question is so small as to be negligible in comparison with the velocity with which the equilibrium under examination is attained; for example, the *hydrolysis* of protein by water in the system protein-salt-water.

the phase-rule would be out of place here, since an exposition of the principles underlying its application can be found in any general work on physical chemistry.

Galeotti (17) (18) has applied the phase-rule to the systems egg-albumin, $CuSO_4$, H_2O, serum-albumin $CuSO_4$, H_2O; serum-albumin, $AgNO_3$, H_2O; egg-albumin, $AgNO_3$, H_2O; while Hardy (25) has applied it to the systems serum-globulin, salts, H_2O. Both observers employ the triangular co-ordinates recommended by Roozeboom, in which the three sides of an equilateral triangle, each equal to unity, represent the components of the system and the distances of a point P from these axes, measured in a direction parallel with the sides of the triangle, express the composition of a ternary mixture corresponding to the point. Since each of the systems investigated by these observers is a system consisting of two immiscible bodies, protein and salt, which are both partially miscible in a third, water, they are comparable with the system water, sodium chloride and succinic nitrile, which has been studied by Schreinemakers (72). The form of the isotherm obtained by Hardy and by Galeotti is exactly that obtained by Schreinemakers in the above-named system.

The temperature and pressure in these investigations being arbitrarily fixed and the components being three in number and in equilibrium with water-vapor at the pressure and temperature employed, the number of degrees of freedom is $3 - r$, where r is the number of phases. From this it follows that for a given temperature and pressure there can be, in general, not more than three phases in equilibrium with water-vapor coexisting in the system at one time.

Now Galeotti, as has been mentioned above, finds that solid $CuSO_4 + 5\ H_2O$, solid protein and water can coexist in the system at the same time, the water being saturated with $CuSO_4 + 5\ H_2O$ and also containing some dissolved protein, as is shown by the fact that dilution of the fluid phase causes further precipitation of protein. *From this it follows that the protein, while in solution, does not constitute a separate phase and is not divided off from the solvent by a surface enwrapping molecules which are not in physical contact with the fluid*, for otherwise there would be four coexistent phases; this simple consideration appears to have been overlooked by the majority of writers on colloids and by many of the advocates of "adsorption."

The general thermodynamic conditions of equilibrium in systems in which the protein or other colloid forms a region divided from the remainder of the system by a boundary at which abrupt change of properties takes place (as in a suspended coagulum or a jelly) have been elaborated by Tolman (75).

In his first communication (17) Galeotti arrived at the conclusion that proteins do not form unions of definite composition with copper, since the copper can be partially and progressively removed from the protein by washing. I have dwelt in a previous chapter (Chap. V) upon the fallibility of this reasoning. In his second communication (18), however, Galeotti obtained data which indicate that at the moment of precipitation of egg-albumin by $AgNO_3$, a compound is formed of perfectly definite molecular weight and solubility in distilled water. These data will be more fully discussed in the succeeding chapter.

Keeping the quantities $AgNO_3$ and water constant and altering the concentration of protein (egg-albumin) Galeotti finds that as protein is successively added a rapid diminution in the number of silver ions occurs, but with the appearance of a precipitate the diminution proceeds more slowly and finally tends to a constant maximum.

6. The Chemical Mechanics of the Precipitation and Coagulation of Proteins by Salts. — We have seen that in order that *precipitation* of a protein by salts may occur the protein must be ionized, but for coagulation this condition is not requisite. In determining the rate of precipitation the valency of the precipitating ion is of prime importance, in determining the rate of coagulation it is of comparatively subordinate importance. For precipitation very low concentrations of the precipitating salt suffice, for coagulation high concentrations of the salt are required. This latter fact, and the fact that the presence of coagulating salts aids coagulation by alcohol and by heat suggests, as it did to Hofmeister, that coagulation is dependent upon *dehydration* of the protein.

Starting from the observation of Jones and Ota (35) that certain salts, when dissolved in water, produce an abnormal depression of the freezing-point, Jones and his pupils have built up a very large body of evidence for the existence of hydrates (or "solvates") of substances in solution (32) (37). These investigators find that both ions and undissociated molecules can

form "solvates," and that these hydrates or "solvates" are readily decomposed at temperatures which approach the boiling point of the solvent and by the presence of other agents in the solution which compete for the solvent.* An interesting attempt has been made, upon the basis of this hypothesis, to explain the color changes which many salts undergo in the presence of varying amounts of water or of dehydrating agents (37) (33) (36) (42) (34). As is well known, anhydrous cobalt chloride is blue, but on taking up water it becomes violet or red. Ostwald (50) believed that the undissociated cobalt chloride is blue, while the cobalt ion is red. Since, however, the color of a concentrated solution of cobalt chloride can be changed from purplish red to blue by the addition of small amounts of calcium, or still smaller amounts of aluminium chlorides, or by the addition of a few drops of alcohol (1) Lewis concludes that this change in color is due to dehydration of the cobalt chloride molecule in solution, by the abstraction of water from it by the added substance, similar conclusions had previously been reached by other observers (70) (63) (81) (40) (27) (28). Similarly the progressive change in color of cupric chloride solutions, from blue to greenish brown, on concentration or dehydration is attributed to the loss of water on the part of cupric chloride-water complexes. Lewis finds that if various bromides be added to concentrated solutions of cupric bromide the copper salt is dehydrated (turned brown) by the salts of monovalent metals in the order $Li > (Na, NH_4) > K$. For the chlorides the order was $Li > Na > NH_4 > K$. Divalent metals dehydrate more strongly, the order being $Mg >$

* A very striking experiment illustrating the formation of "solvates" is that cited by Pickering (6). If a mixture of propyl alcohol and water be placed in a semipermeable vessel and surrounded with water, it is found that water enters the cell, but that no propyl alcohol escapes. If, however, the same semipermeable vessel, containing the same mixture of alcohol and water be immersed in propyl alcohol, propyl alcohol enters the cell and water does not leave it. In other words the vessel is permeable to either propyl alcohol or water when these are pure, but it is impermeable to mixtures of the two, the inference being that large molecular complexes are formed on mixing these reagents, which cannot pass through the pores of the vessel. From these and similar experiments Poynting (65) concludes that osmotic pressure is an expression of the diminution in the active mass of the solvent due to the formation of compounds with the dissolved substance. For a fuller discussion of these and similar hypotheses the reader is referred to current works on physical chemistry.

Ca > Sr > Ba while trivalent metals (Al) act still more energetically. In opposition to this view Donnan (11) (12) (41) (48) (13) advances the hypothesis that the blue color of concentrated solutions of cobalt chloride is due to the formation of complex ions of the type $CoCl_2.Cl_2''$. Lewis points out, however, that this view is inconsistent with the fact that it is possible to change the color of the solution from blue to red by mere dilution, without altering the active mass of any component of the system except water. Other objections against Donnan's view have been urged by Hartley and H. C. Jones.

The peculiar interest to the biological chemist of the possibility thus indicated, that substances dissolved in water form loose combinations with the solvent, lies in the especial significance of water in relation to the protein and polypeptid structure. As indicated in the first chapter, dehydration of a protein may result in the following reactions:

$$R\begin{matrix}\diagup NH_3OH \\ \diagdown COOH\end{matrix} + \begin{matrix}HOOC \diagdown \\ HOH_3N \diagup\end{matrix} R = R\begin{matrix}\diagup NH_3.OOC.R.NH_3OH \\ \diagdown COOH\end{matrix} + H_2O$$

$$R\begin{matrix}\diagup NH_3OOC.R.NH_3OH \\ \diagdown COOH\end{matrix} = R\begin{matrix}\diagup NH.OC.R.NH_3OH \\ \diagdown COOH\end{matrix} + H_2O$$

$$R\begin{matrix}\diagup NH.OC.R.NH_3OH \\ \diagdown COOH\end{matrix} = R\begin{matrix}\diagup NH.OC.R.NH_2 \\ \diagdown COOH\end{matrix} + H_2O$$

$$R\begin{matrix}\diagup NH.OC.R.NH_2 \\ \diagdown COOH\end{matrix} = R\begin{matrix}\diagup NH.OC.R.NH \\ \diagdown CO\text{———}|\end{matrix} + H_2O$$

and hydration, of course, may result in the reversion of this series of changes.

That proteins may be thrown out of solution in two very different conditions of hydration is evident from the researches cited in the previous sections of this chapter; it is even more clearly shown by the following experiments (67):

Anhydrous casein dissolves readily in cold anhydrous * formic acid; still more readily in hot formic acid If, to a two per cent solution of casein in formic acid, we add a fairly concentrated

* Anhydrous, that is, save for traces of moisture derived from the atmosphere.

solution of cupric chloride, the mixture is at first green, indicating the presence of lower hydrates of cupric chloride, but on adding more of the solution it becomes blue, and *simultaneously with the appearance of a pure blue color, but not before, precipitation of cupric caseinate occurs.* If, to 5 cc. of a 2 per cent solution in formic acid, we add $1\frac{1}{2}$, 2, or $2\frac{1}{2}$ cc. of a saturated solution of cupric chloride, no precipitation of the caseinate occurs, but on diluting this mixture with water a precipitate results, and the appearance of this precipitate coincides with the attainment of a clear blue color on the part of the mixture.

About six cubic centimetres of water are required to produce a permanent precipitate. This precipitate redissolves on heating and *the mixture simultaneously becomes green;* on cooling the blue color reappears and with it the precipitate. If formic acid be added to the mixture the precipitate redissolves as soon as the mixture becomes green. If the precipitate be very slight it will redissolve on adding alcohol. It cannot be urged that the formation of cupric caseinate requires the presence of a sufficient concentration of cupric ions, because green solutions of cupric chloride contain an abundance of ions * and casein will react with very small amounts of metal ions, for although it is itself insoluble it will drive carbonic acid out of the sparingly soluble calcium carbonate to form a freely soluble caseinate of calcium (*vide* Chap. V).

If instead of adding *water* to a mixture of 5 cc. of 2 per cent casein in formic acid and 2 cc. of saturated $CuCl_2$, we add *alcohol*, no coagulation occurs until the mixture changes in color from green to brown when a *coagulum* of cupric caseinate is produced which redissolves on adding water.

Similar results are obtained when a 2 m. solution of cobalt chloride is employed instead of a saturated solution of cupric chloride. If to 5 cc. of a 2 per cent solution of casein in formic acid we add 2 to 3 cc. $CoCl_2$ we obtain a blue-purple solution;

* Green solutions containing, probably, a mixture of the anhydrous brown salt and the fully hydrated blue salt. Even on the basis of the hypothesis urged by Donnan (11), (12), therefore, a considerable number of cupric ions must exist in green solutions. It is important to notice that the precipitation, as stated above, does not occur until the solution is *pure* blue in color; mixtures so slightly green that they appear wholly blue until viewed alongside pure blue mixtures produce no precipitate.

on adding water to this mixture it changes in color from blue-purple through red-purple to clear pink. Not until a pure pink color is obtained does a precipitate result. If, instead of adding water, we add a considerable volume of alcohol (10 volumes) the mixture rather abruptly changes to a clear pale blue and then, but not before, we obtain a *coagulum* of cobalt caseinate.

Electronegative casein is not precipitated by the salts of the alkalies, though it is readily precipitated by salts of the alkaline earths. Electropositive casein (i.e., casein dissolved in acids) is, however, very readily precipitated by salts and these precipitates are not soluble upon dilution. Thus if 2 cc. of $N/10$ HCl be added to 5 cc. of a 1 per cent solution of casein in 0.008 N KOH, a clear acid solution of casein results. The casein is precipitated from this by the addition of four drops of a saturated solution of sodium chloride, or by one drop of a saturated solution of ammonium sulphate; this latter precipitate does not dissolve on diluting the mixture to one-sixteenth.

Casein formate is no exception to the other salts of casein with acids, but *the precipitation will only occur in the presence of a sufficiency of water.* If to 5 cc. of a 2 per cent solution of casein in formic acid we add a saturated solution of ammonium sulphate, 3 cc. of this solution just suffice to produce a coagulum; this becomes more abundant on adding water, and redissolves on adding formic acid. If, however, instead of adding 3, we add 2 cc. of the saturated ammonium sulphate solution, a clear solution is obtained. *On adding water to this a precipitate results which redissolves on heating and reappears on cooling.*

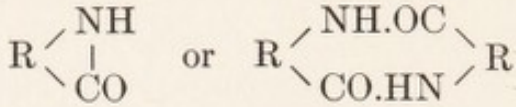
Analogous results may be obtained with ovomucoid.

It is clear, therefore, that protein may be thrown out of solution by electrolytes in two grades of hydration, the one of high, the other of very low hydration. The former process is what we have termed precipitation, the latter we have defined as coagulation. At grades of hydration intermediate between these extremes the protein may be soluble. Dehydration, partial or complete, leading to resolution or to coagulation, may be induced by heat, by non-electrolytes possessing an affinity for water or by electrolytes.*

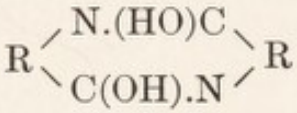
The importance of a high degree of dehydration in the pro-

* See also Chick and Martin (10).

duction of *coagula* irresistibly suggests that this phenomenon is dependent upon the formation of anhydrides * analogous to the diketopiperazines (*vide* Chap. I) and of the general formula:



Such bodies may exist either in the keto form, illustrated by the above formulæ, or in the enol form, such as:



If this be granted then the fact that alcohol throws down the protein salts in an unaltered condition (*vide* Chap. IV) lends strong support to the view which I have advanced (Chap. I) regarding the mode of formation and structure of the protein salts, since according to that view the metal ions in a protein-metal compound would be bound up in $-COH.N-$ groups previously to dehydration, which could only affect terminal $-NH_2$ or $-NH_3OH$ and $-COOH$ groups and would leave the union between the protein and the metal unaffected.

In this connection, and with reference to the "solvate" theory of Jones, it is of interest to note that the relative efficacy of the various salts in bringing about the coagulation of electronegative protein is exactly their relative efficacy in bringing about diminution of the solubility of phenylthiocarbamid (69) (53). On the other hand, evidence of the affinity of proteins for water is afforded by the observation of Pauli and Samec (59) that the solubility of highly soluble inorganic salts, such as ammonium chloride, magnesium chloride or ammonium sulphocyanate in water is diminished by the presence of gelatin or of blood-serum proteins. The solubility of very slightly soluble substances (calcium sulphate, phosphate, or carbonate and uric acid) is, however, definitely increased.

As regards the *precipitation* of proteins by salts; it appears probable that acid and alkali protein react with salts as follows:

* Cf. also Gustav Mann (46).

$$H_2N.R.COH^{++} + \begin{array}{c} H \\ | \\ \diagdown N.R.COOH \\ | \\ Cl \end{array} + 2\,NaNO_3$$

$$= H_2N.R.CONa = \begin{array}{c} Na \\ | \\ N.R.COOH \\ | \\ Cl \end{array} + 2\,HNO_3 \qquad \text{(i)}$$

$$H_2N.R.CONa^{++} + \begin{array}{c} H \\ | \\ \diagdown N.R.COOH \\ | \\ OH \end{array} + NaCl$$

$$= H_2N.R.CONa = \begin{array}{c} Na \\ | \\ N.R.COOH \\ | \\ Cl \end{array} + H_2O \qquad \text{(ii)}$$

Since proteins, dissolved in salt solutions, are electrically neutral (Hardy), it appears probable that this compound undergoes internal neutralization thus (*vide* Chap. I):

$$H_2N.R.CONa = \begin{array}{c} Na \\ | \\ N.R.COOH \\ | \\ Cl \end{array} = H_3N.R.CONa = \begin{array}{c} Na \\ | \\ N.R.COO \\ | \\ Cl \end{array} \qquad \text{(iii)}$$

It will be observed that this hypothesis is a slight modification of that advanced by Pauli and Handovsky (57) in that, in the first place, cognizance is taken of the fact that the proteins ionize, not at terminal $-NH_2$ or $-COOH$ groups but at internal enol groups and, in the second place, the compounds which are formed with acid- and alkali-protein respectively are symmetrical in structure, so that the symmetry of the effects of salts in dissolving and coagulating these compounds, which has been observed by Pauli, is readily accounted for.

In dilute solutions, that is, solutions in which the *active mass of water* is great, these compounds undergo hydrolytic dissociation in the following way:

$$H_2N.R.CONa=\begin{matrix}Na\\|\\N\\|\\Cl\end{matrix}.R.COOH + H_2O$$

$$= H_2N.R.CONa=\begin{matrix}H\\|\\N\\|\\Cl\end{matrix}.R.COOH + NaOH. \quad \text{(iv)}$$

This decomposition will, naturally, take place more readily in dilute acid than in alkaline solution; more concentrated acid would, of course, abstract the sodium from the compound and convert the whole of it into the acid-protein compound. In either case the neutral compound which results may be insoluble. When the active mass of water is diminished, however, for example by the addition of a dehydrating agent or of a salt with an affinity for water, this hydrolytic decomposition is prevented, and the complex salt:

$$H_3N.R.CONa=\begin{matrix}Na\\|\\N\\|\\Cl\end{matrix}.R.COO$$

may pass into solution.

Further dehydration leads to the loss of $-H$ and $-OH$ by terminal $-NH_2$ and $-COOH$ groups, as depicted above, and the formation of complex insoluble anhydrides.

This hypothesis furnishes an explanation of the following facts:

(i) That the addition of small concentrations of neutral salts to a solution of acid-protein increases the acidity of the solution, while the addition of salts to alkali-protein solutions does not increase their alkalinity (*vide* equations i and ii).

(ii) That acid-protein is precipitated by cations, alkali-protein by anions (*vide* equation iii).

(iii) Since the union between the salt and the protein is chemical in character, Schultzes' valency-rule would apply to the *rate* of precipitation (Cf. section **3**).

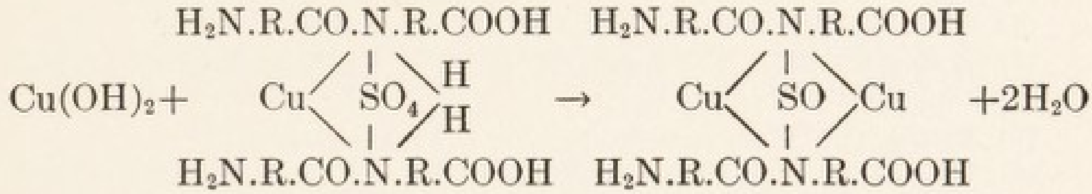
(iv) The reaction (acidity or alkalinity) of the system being maintained constant, the precipitation of the protein depends only upon the active mass of water and not upon the active

mass of salt, provided this is sufficient to enter into combination with the protein (*vide* equation iii), i.e., it is possible to bring about precipitation by mere dilution, the relative masses of protein and salt being unaltered.

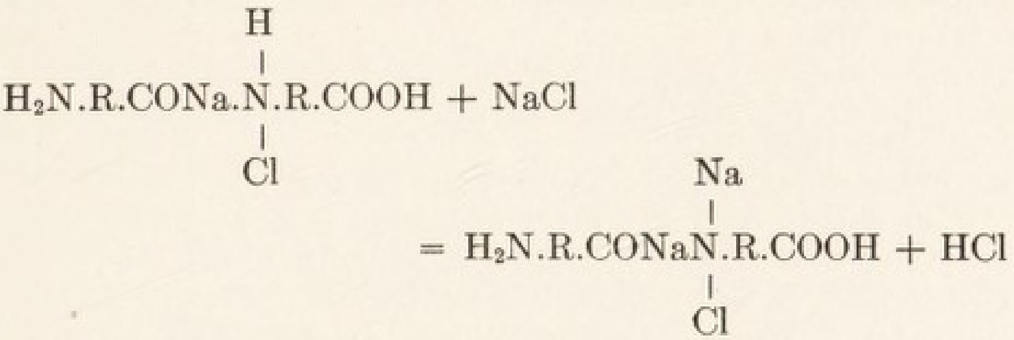
(v) The precipitation of proteins by salts occurs more readily in acid than in alkaline solutions (*vide* equation iii).

(vi) The observation of Bonamartini and Lombardi (6)* that egg-albumin in neutral solution combines with both the basic and the acid radicals of copper sulphate in equivalent proportions to form an insoluble compound but that in alkaline solution it combines with excess of copper to form a soluble compound (*vide* equation iv). In alkaline solution hydrolytic dissociation of the complex salt is pushed back in accordance with the following equations.

$$CuSO_4 + 2KOH \rightarrow K_2SO_4 + Cu(OH)_2$$



(vii) The observation of Osborne (Cf. Chap. V) that edestin crystallized from concentrated salt-solutions will decompose the salt, binding the base, is probably attributable to the reaction:



the complex salt being in this case insoluble.

(viii) The observation of Pauli that *precipitation* of a protein by salts when it is non-ionic is impossible. For, when the protein is not ionized the nitrogen is bound up in undissociated $-COH.N-$ groups and is not attached to H^+ and OH' groups replaceable by the ions of the salt.

* The egg-albumin employed by these observers is not ash free; it must, therefore, according to Pauli have been ionic.

This fact also explains the observation of Bugarszky and Liebermann (8) that uncombined and, presumably, unionized proteins do not combine with salts in neutral solutions. These observers used egg-albumin, which, according to Pauli, is electrically neutral when uncombined with acids or bases.

(ix) The observation of Hardy and Pauli that *coagulation* of a protein by salts is possible whether it be ionic or not, since the dehydration of terminal $-NH_2$ and $-COOH$ groups does not depend upon the dissociation of $-COH.N-$ groups.

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CHAPTER VII

THE COMPOUNDS OF THE PROTEINS (Continued)

1. Compounds with the Heavy Metals. — From what has been said in the previous chapter concerning the mechanism of the precipitation and coagulation of proteins by salts, it will be clear that the percentage of a heavy metal which is bound by protein must depend very intimately upon the mode of preparation of the compound. For instance, in the presence of silver nitrate under conditions involving moderate hydration (e.g., a fairly high concentration of the silver nitrate or the presence of some other dehydrating agent), or in alkaline solution, the following reaction may occur:

$$\begin{array}{l} \qquad\qquad\quad H \\ \qquad\qquad\quad | \\ H_2N.R.CONa.N.R.COOH + 2\,AgNO_3 \\ \qquad\qquad\quad | \\ \qquad\qquad\quad OH \end{array} \qquad \begin{array}{l} \qquad\quad Ag \\ \qquad\quad | \\ = H_2N.R.COAg.N.R.COOH + NaNO_3 \\ \qquad\quad | \\ \qquad\quad NO_3 \end{array}$$

in an acid medium, or under conditions involving less hydration, this compound could not exist and the reaction

$$\begin{array}{l} \qquad\quad Ag \\ \qquad\quad | \\ H_2N.R.COAg.N.R.COOH + H_2O \\ \qquad\quad | \\ \qquad\quad NO_3 \end{array} \qquad \begin{array}{l} \qquad\quad H \\ \qquad\quad | \\ \rightarrow H_2N.R.COAg.N.R.COOH + AgOH \\ \qquad\quad | \\ \qquad\quad NO_3 \end{array}$$

must occur. In a still more acid medium the silver would be altogether abstracted from this compound, while in a fairly strongly alkaline medium the compound

$$\begin{array}{l} \qquad\quad H \\ \qquad\quad | \\ H_2N.R.COAg.N.R.COOH \\ \qquad\quad | \\ \qquad\quad OH \end{array}$$

would doubtless be formed. In the majority of investigations upon this subject no care has been taken to maintain a neutral or otherwise constant reaction in the medium from which the protein-heavy metal compound is thrown out, each observer has prepared these compounds under different conditions, and no clear distinction has been made between the *precipitation* of the protein by the heavy metal salt and its *coagulation*. It is not matter either for surprise or bewilderment, therefore, that different observers have obtained very conflicting results in determining the heavy metal content of these compounds. In the light of our present knowledge the majority of these results are perceived to be meaningless, since they were obtained with compounds, or mixtures of different compounds, prepared under inadequately controlled conditions.*

From the observations of Galeotti (30), we may infer that *at the moment when precipitation begins*, the compound of egg-albumin with silver (or silver nitrate) *is of constant molecular weight and solubility in water*. This investigator has applied to the system protein, egg-albumin, water, the law of Jahn, that the influence which an electrolyte exerts upon the solubility of another substance (its dehydrating or hydrating action) is a function of three variables, namely, the concentration of the undissociated molecule of the electrolyte, that of its cation, and that of its anion. Galeotti measured the percentage of protein precipitated and also the concentration of silver ions, determining the latter directly with the aid of a concentration-chain (potentiometer). Calling C the *molecular* concentration of a saturated solution of the protein-silver-nitrate, and C' its solubility in the presence of silver nitrate, applying Jahn's law we have

$$\log_{\text{nat.}} \frac{C'}{C} = \frac{2m}{R} [C_o \phi_{01} + C_k (\phi_{11} + \phi_{21})],$$

in which R is the gas-constant; ϕ_{01} (at constant temperature and pressure) is the constant ratio between the concentration of the non-dissociated molecule of $AgNO_3$ and its effect upon the solubility of the protein-compound; ϕ_{11} and ϕ_{21} are similar factors for the cation and the anion of the silver nitrate; and C_o and C_k are the concentrations, respectively, of the undissociated and the

* For a review of the older literature upon the heavy metal compounds of proteins, Cf. O. Cohnheim (25).

dissociated $AgNO_3$, the concentration of the cation being assumed to be equal to that of the anion.

Calling C_g the *total* concentration of silver nitrate, then $C_o = C_g - C_k$, and we have

$$\log_{10}\frac{C'}{C} = \frac{2m}{R}[C_g\phi_{01} + C_k(\phi_{11} + \phi_{21} - \phi_{01})],$$

where C is the *molecular* concentration of the protein-silver-nitrate in saturated watery solution (a quantity which naturally, cannot be measured directly) and C' is the molecular concentration of the protein-silver-nitrate in the $AgNO_3$ solution.

Assuming that the molecular weight of the compound, in saturated solution, is always the same, whether excess of silver nitrate be present or not, we can take C' as the *percentage* concentration of the compound, since the *ratio* $\frac{C'}{C}$ will then not involve the molecular weight of the compound.

Putting

$$-\frac{1}{\log C} = \alpha;\quad \frac{2m}{R}\cdot\frac{\phi_{01}}{\log C} = \beta;\quad \frac{2m}{R}\cdot\frac{(\phi_{11} + \phi_{21} - \phi_{01})}{\log C} = \gamma,$$

we have, from the above equation,

$$\alpha \log C' + \beta C_g + \gamma C_k + 1 = 0,$$

in which α, β, and γ are constants, and C', C_g, and C_k are all directly measurable. The following results of Galeotti's demonstrate the validity of this equation, the most probable values of the constants ($\alpha = -1.507$; $\beta = +37.27$; $\gamma = -72.06$) having been determined from all the observations by least squares.

$\alpha \times \log C'$	$\beta \times C_g$	$\gamma \times C_k$	Sum
−0.207	+0.933	−1.741	−1.015
−0.136	+1.465	−2.341	−1.012
+0.140	+1.759	−2.872	−0.973
+0.501	+2.250	−3.741	−0.990
+0.741	+3.063	−4.813	−1.009
Average.....	.....	.....	−1.000

From this result we may draw the following conclusions:

(a) The substance precipitated is a *compound* and not the free protein, since the value of α in the above equation indicates a

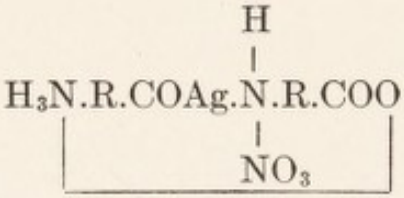
limited solubility in water and free egg-albumin is miscible with water in all proportions.*

(b) This compound is of the same molecular weight in all of the solutions investigated, otherwise the ratio $\frac{C'}{C}$ would include a factor expressing the relation between the molecular weight of the compound and the excess of $AgNO_3$ in its saturated solution. Also the constancy of C (i.e., the constancy of α) shows that it is always the *same substance* that is present in solution at the moment preceding precipitation.

(c) This compound is probably not ionized, since Jahn's law applies only to the influence of electrolytes upon the solubility of non-electrolytes.

(d) The quantity of NO_3' bound by the protein just before precipitation must be the same as the quantity of Ag^+ which is bound by it at the same moment, otherwise the concentration of silver ions in its saturated solution would not be the same as that of the NO_3' ions.

All of these conclusions obviously answer to the formation of the electrically neutral compound:



and its precipitation.

The last of the above conclusions also agrees with the finding of Bonamartini and Lombardi (22), that in neutral solutions egg-albumin binds the entire $CuSO_4$ molecule to form an insoluble compound, although in alkaline solutions it binds an excess of copper, forming a soluble compound.

Rohmann and Hirschstein (105) have described compounds of casein with silver, or silver nitrate, formed by acting upon solutions of casein, neutral to phenolphthalein, with excess of $AgNO_3$. The resultant precipitate when prepared from solutions of sodium caseinate contained 85×10^{-5} equivalents of silver per gram, while that prepared from ammonium caseinate contained 77×10^{-5} equivalents of silver. It will be recollected (Cf. Chap. V) that at neutrality to phenolphthalein casein neutralizes $80 \times$

* For it not only dissolves in water, but swells in water. Cf. Chap. XII.

10^{-5} equivalents of base. This corresponds, therefore, with the direct interaction of one molecule of silver nitrate with one molecule of the caseinate, according to the equation:

$$\begin{array}{l} \qquad\quad H \\ \qquad\quad | \\ H_2N.R.CONa.N.R.COOH + AgNO_3 \\ \qquad\quad | \\ \qquad\quad OH \end{array} \qquad \begin{array}{l} \qquad\quad H \\ \qquad\quad | \\ = H_2N.R.COAg.N.R.COOH + NaNO_3. \\ \qquad\quad | \\ \qquad\quad OH \end{array}$$

This compound is sparingly soluble in water (0.47 per cent) and its solution requires the addition of a small amount of NaOH before it becomes neutral to phenolphthalein.* If to this solution, neutralized to phenolphthalein, more $AgNO_3$ be added, a fresh precipitate is obtained containing a higher percentage of silver than the first, and the additional silver content is exactly equivalent to the quantity of alkali which was added to the first to render it neutral to phenolphthalein. *The active mass of caseinate in this reaction is directly proportional to the number of* $-COH.N-$ *groups which have been opened up by combination with a base, i.e., which are electrolytically dissociated.* Conversely, undissociated $-COH.N-$ groups cannot react with neutral silver nitrate.

The compounds which proteins form with zinc sulphate have been studied by Lippich (72) who considers that the reaction between zinc sulphate and protein results in an equilibrium which is determined by a variety of factors amongst which he enumerates ionization, hydrolytic dissociation, complex salt formation, etc. He finds that serum proteins combine with zinc in alkaline solution to form insoluble salts which dissolve in excess ($\frac{1}{4}$ saturated) of ammonium sulphate. This soluble salt he believes to be of the type $ZnSO_4$-protein, the insoluble salt formed at a higher degree of hydration (absence of a dehydrating agent such as ammonium sulphate) being of the type Zn-protein.

The compounds which copper salts form with amino-acids,

* Since AgOH is a very weak base, a caseinate of silver corresponding to the caseinate of a strong base which is neutral to phenolphthalein, would necessarily be acid to phenolphthalein.

peptids, peptones and proteins have been extensively investigated by Kober (57) (58) (59) (60) and, more recently, by Osborne and Leavenworth (88). Kober has shown that the copper salts of amino-acids in alkaline solutions yield their copper quantitatively in the form of a precipitate of cupric hydrate on heating the solution or on addition of an excess of alkali. Under similar conditions the peptones and peptids yield little or no precipitate. Osborne and Leavenworth have shown that the maximum amount of cupric hydrate which edestin or gliadin will hold in solution exactly corresponds with the number of $-COHN-$ groups in the molecule of these proteins, assuming that one atom of copper combines with each nitrogen atom. This fact would appear to be at variance with the view expressed by Kober and Sugiura (59) that the pink "biuret" color which alkaline solutions of the peptone compounds of copper yield on heating is due to a union of copper with four nitrogen atoms, for if this were the case the development of the pink color would lead to the precipitation of an excess of cupric hydrate previously held in solution by the $-COHN-$ groups of the peptone.

Neumann (84) has studied the compounds which ovomucoid forms with various metal salts ($ZnCl_2$, $CuCl_2$, $MgCl_2$, $AlCl_3$, $FeCl_3$) and finds that in alkaline solutions this protein forms insoluble compounds with the heavy metal, the weight of the metal combined being proportionate to the molecular weight of its hydroxide. In other words, ovomucoid and the various hydroxides of the heavy metals combine in stoichiometrical proportions.

Rona and Michaelis (106) have shown that ferric hydrate will displace $Ca(OH)_2$ from its combination with casein, forming insoluble ferric caseinate. Benedicenti and Rebello-Alves (15) (16) have shown that the magnetic properties of iron in solution or even of finely pulverized iron suspended in water are "masked" by the presence of proteins. They believe that in the latter case this phenomenon is attributable to a direct fixation of the metallic ions by the protein.

Henze (42) has described an interesting chromogenic protein in the blood corpuscles of ascidians in which vanadium would appear to play the part of the iron in the hæmoglobin of mammalian corpuscles.

2. The Compounds with the Phosphoric Acids. — It was pointed out by Graham in 1861 that metaphosphoric acid unites

with gelatin to form an insoluble compound (34), 100 parts of gelatin uniting, according to this observer, with 3.6 parts of metaphosphoric acid. Graham suggested that the difference between the behavior of metaphosphoric and other phosphoric acids in this respect might be connected with the possibly colloidal character of the latter.* This suggestion has more recently been reiterated by Mylius (82). There appears, however, no adequate reason for adopting such a conclusion. Metaphosphoric acid differs from orthophosphoric acid in other respects, and these differences are not attributable to the "colloidal" character of its solutions. As Graham pointed out, the low equivalent of metaphosphoric acid which combines with gelatin suggests that the metaphosphoric acid enters into the compound in a crystalloid and not a colloidal (polymerized) form. Colloidal *silicic* acid combines with gelatin in its colloidal form and the compound contains nearly equal weights of gelatin and of silicic acid (Graham, loc. cit., p. 206).

The compounds of metaphosphoric acid with the proteins were at one time regarded with peculiar interest on account of their supposed identity with the nucleo-proteins (69) (74). Pohl (96) and Kossel (61), however, showed that these compounds differ fundamentally from the true nucleins in that, on hydrolysis, they yield no purin bases. Thanks to the work of Miescher, Kossel and their pupils, it has now been shown that the nucleins are not protein salts of phosphoric or of metaphosphoric acid but of a complex substituted phosphoric acid, nucleic acid (6).

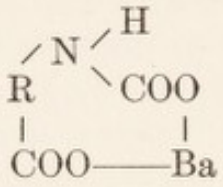
The compounds of the protein with metaphosphoric acid appear, as a rule, to be unstable save in the presence of excess of the acid (Graham, loc. cit., Malfatti, loc. cit.); if this excess be removed by washing then the compound undergoes decomposition, liberating metaphosphoric acid. Progressive removal of the free acid by washing, therefore, results finally in complete decomposition of the product. Conversely, in the presence of a variable excess of metaphosphoric acid the quantity which is bound by a protein is greater the greater the excess (28).

Bechhold (14) has described compounds of egg-albumin with

* "It will be an interesting inquiry whether metaphosphoric acid is a colloid, and enters into the compound described in that character, or is a crystalloid, as the small proportion and low equivalent of the acid would suggest," loc. cit. page 221.

orthophosphoric acid prepared by treating crystallized egg-albumin, dissolved in NaOH, with $POCl_3$.

3. Compounds of the Proteins with Carbonic Acid. — Siegfried (113) has described a special form of combination between inorganic salts and amino- or polyamino-acids which is probably destined to assume considerable importance in the eyes of physiologists. He observed that if CO_2 be passed through solutions of various amino-acids in barium hydrate, provided that the total concentration of $Ba(OH)_2$ is not greater than twice that of the amino-acid no precipitate of barium carbonate is obtained. Similar results are obtained with $Ca(OH)_2$, and the peptones and the proteins of the blood behave in a manner analogous to the simple amino-acids. On standing, $BaCO_3$ or $CaCO_3$, as the case may be, is slowly liberated, and this process is accelerated by heating. Direct analysis of the products obtained when $CaCO_3$ acts upon glycocoll and other amino-acids showed that the compounds can be represented by the general formula



being the barium salts of carbamino-acids.

For the monoamino-acids the ratio

$$\frac{\text{molecules } CO_2 \text{ bound}}{\text{atoms of N}}$$

is 1, indicating that the $-NH_2$ group reacts quantitatively with the carbonate. For diamino-acids, such as lysin, the ratio is also 1, showing that both $-NH_2$ groups react quantitatively. For arginin, which contains 4 atoms of nitrogen, the ratio is $\frac{1}{4}$, indicating that only one of the $-NH_2$ groups and neither of the imino-groups react. For the different dipeptids the ratio varies between

$$\frac{1}{1.63} \text{ and } \frac{1}{1.79}.$$

If the $-N.HOC-$ groups did not react at all the ratio would be $\frac{1}{2}$, if they reacted quantitatively it would be 1. For tripeptids the ratio is $\frac{1}{2.57}$, whereas it would be $\frac{1}{3}$ if the $-N.HOC-$ groups

did not react at all. For the tetrapeptid triglycyl glycin the ratio is $\frac{1}{3.29}$.

When CO_2 is passed into a solution of a protein in $Ca(OH)_2$ the conductivity of the mixture diminishes. From this Siegfried draws the conclusion that the proteins react with carbonates to form carbamino acids. From what has been said in the previous chapter concerning the mode of combination of proteins with salts, however, the reader will gather that the validity of this conclusion does not admit of being established in so simple a manner.

4. Compounds of the Proteins with the Alkaloidal Reagents, Dyes, Alkaloids, etc. — In order that a protein may react with an acid which is insufficiently dissociated to break up its $-N.HOC-$ groups, some stronger acid must be present, combined with the protein, and which can then be displaced by the weaker acid through the agency of one of its more strongly ionized salts. Thus:

$$-N.HOC- + HA = -\overset{\overset{H}{|}}{N''}\overset{\overset{|}{A}}{} + \overset{++}{H}OC-$$

$$-\overset{\overset{H}{|}}{N''}\overset{\overset{|}{A}}{} + \overset{++}{H}OC- + BA' = -\overset{\overset{H}{|}}{N''}\overset{\overset{|}{A'}}{} + \overset{++}{H}OC- + BA$$

HA being a strong acid, HA′ a very weak acid, and BA′ a salt of that acid. Similar remarks apply, of course, to very weak bases. Hence it is observed that proteins will combine with very weak acids or with the acid radical of salts more readily in acid than in neutral or alkaline solution, and with very weak bases or the basic radical of salts in an alkaline rather than a neutral or acid medium. Similarly a protein may not be able to decompose a salt of an acid, binding the acid (or base as the case may be), but, in the presence of a free acid it may be able to do so, partly because the first acid is partially set free from its salt by the second, but also because the ionization of the protein is increased.

Hence, on adding free picric, molybdic, tungstic, phosphotungstic, tannic, stearic or chromic acids to a neutral solution of protein a precipitate or coagulum is, as a rule, formed immediately. But if these acids be added in the form of their (neutral) salts then no precipitate results until the *reaction* of the solution is rendered acid, when the protein at once combines with the acid radical of the salt. Similarly, neutral lead acetate will not, as a rule, precipitate protein (egg-albumin) from neutral solutions, but it will precipitate it readily from faintly alkaline solutions (77) (solutions, that is, in which the protein is in the form of an ionized compound with a base).

Upon these facts probably depend certain phenomena which are encountered in the tanning of leather. As is well known, chromium unites firmly with the hide-substance only in the form of its cation Cr^{+++}, and not when it is united with oxygen to form an anion as in chromic acid. Now it is observed that firm union only occurs when the chromium salt is "basic" (such as $Cr_2Cl_3(OH)_3$), i.e., when the salt is neutral in reaction or even, at the moment of union, faintly alkaline (97). Further addition of alkali results in a displacement of the chromium by the added base. On the other hand, tannic acid unites with the hide substance in a faintly acid medium.

Similar principles apply to the union of dyes with proteins (77) (39). Proteins which are about equally acid and basic in character, such as gelatin and egg-albumin, combine with basic dyes only in faintly alkaline solutions. Predominantly acid proteins, such as casein or the nucleins, will combine with basic dyes even in neutral or faintly acid solutions (80) since (ionized) salts of these proteins with bases can exist even in solutions acid to litmus (Cf. Chap. V); on the other hand, they combine with acid dyes with great difficulty. Lilienfeld has shown that so long as nucleic acid is not saturated with albumins to form neutral nuclein an insoluble compound with methyl green is formed when this dye is added to a solution of the nuclein, but after saturation with protein the nucleins show a greater affinity for acid dyes (71).

An interesting compound of azolitmin and mucoid has been obtained by Rosenbloom and Gies (109). I have found that in faintly alkaline solution trypsin, or some constituent of Gruebler's trypsin (nach Spateholz), forms an insoluble compound with safranin (99) (44).

Evidence of the existence of protein dye compounds even when these are freely soluble is afforded by the influence of added protein upon the distribution of the dye between water and some other appropriate solvent (100). Thus if erythrosin in acidulated water is shaken up with ethyl acetate it passes completely into the ethyl acetate, forming a yellow solution and leaving the water colorless; if, however, neutral sodium caseinate be added to the water, the watery layer remains pink upon shaking it up with erythrosin. Similarly, when gentian violet in alkaline watery solution is shaken up with ethyl acetate a large proportion of the gentian violet passes into the ethyl acetate, forming a red solution but leaving the water blue; if, however, casein or gelatin be added to the water very little of the gentian violet passes into the ethyl acetate and that which does so only stains it faintly violet. Similarly neutral red can be shown to combine with casein, and bismarck brown with casein and gelatin. I have observed that casein forms insoluble compounds with acid fuchsin and orange G, gelatin with crystal violet and acid fuchsin, and protamin with orange G and carminic acid. The compound of protamin with carminic acid is very interesting because it is of a very different color from the free acid or its compounds with inorganic bases; as is well known, free carminic acid, in solution, is golden in color, while its salts with inorganic bases have the familiar carmine tint; the compound with protamin is, however, *deep violet*. I have observed in several cases, also, that the colors of solutions of dyes to which the proteins have been added are not identical with the colors of pure watery solutions.

Many authors have doubted the chemical nature of the process of staining of tissues, preferring to regard it as a physical phenomenon of "solid solution" or "adsorption." Having regard, however, to the unquestionable chemical combinations between dyes and proteins which occur, as we have seen, *in vitro*, and recollecting that, as Mathews has shown, even proteins coagulated by fixatives show similar phenomena, there can remain very little doubt that similar combinations must frequently occur between the dyes used by cytologists and the protein constituents of the tissues.*

* For a discussion of this interesting question Cf. Gustav Mann (75), also Pelet-Jolivet, L., " Die Theorie des Farbeprozesses," Dresden (1910).

That casein will combine with various alkaloids to form water-soluble compounds has been shown by W. A. Osborne (89). From the marked effects of caffeine upon the viscosity and conductivity of gelatin in hydrochloric acid solutions Pauli and Falek (93) infer that caffeine enters into combination with gelatin hydrochloride. The compounds which the various dyes form with *amino-acids* have been studied by Suida (116).

5. The Compounds of Proteins with Soap and Lipoids. — It has been pointed out by Rona and Michaelis (106) that the power of soaps, such as sodium oleate, to reduce the surface tension of water, is much diminished by the presence of proteins, and they infer that the soaps form compounds with protein. No such effect was observed when esters, such as amyl acetate, tributyrin, propyl acetate or ethyl butyrate were employed instead of soaps. These results have been confirmed by Berczeller (17). It has long been known that it is an extremely difficult matter to free proteins from contamination with lecithin, and the existence of lecithin-protein compounds has frequently been inferred from this fact (48) (87) (95). More decisive evidence of the existence of such compounds has recently been brought forward by Feinschmidt (27) and Allemann (5) who have shown that the optimal H^+ concentration for the precipitation of various proteins is very considerably modified by the presence of lecithin.

Fatty acid compounds of the peptones have been prepared by Izar and di Zuattro (54) by the action of chlorides of the fatty acids upon peptones at low temperatures. The compounds thus prepared contain from 18 to 20 per cent of fatty acid.

6. The Compounds of Proteins with Proteins and their Possible Significance in Life Phenomena. — It has been shown by Kossel (62) that the protamins, which, it will be recollected, are strongly basic proteins, when added to weakly alkaline solutions of other proteins give rise to precipitates. Kossel believes that the compounds which are thus formed between the protamins and less basic proteins are analogous to the naturally occurring histones (63) (66). Hunter (53) has further investigated these compounds and finds that while crystallized egg-albumin, casein, hemi-elastin, gelatin, edestin, heteroalbumose, protalbumose, "alkali-albuminate" and histone sulphate yield a precipitate in alkaline solution upon the addition of clupein.

Elastin, peptone, deuteroalbumose, histopeptone and several peptids fail to yield a precipitate. Upon digestion of these precipitates with pepsin the protamin is set free * and the remainder of the compound is converted into deuteroalbumose and peptones which are not precipitable by picric acid. The protamin which was held in the compound before digestion can now be determined quantitatively by precipitation with picric acid. The following are among the results which were obtained by Hunter:

Protein combined with clupein	In 100 parts of nitrogen in the compound the protamin moiety contains	Weight of protein which combines with one part by weight of clupein
Alkalialbuminate	34.36	.....
Ovalbumin	29.31	4.1
Gelatin	22.79	4.8
Hemi-elastin	22.71	5.2
Casein	39.17	2.5
Edestin	13.95	8.5

An attempt made by Gay and Robertson (32) to prepare protamin (salmin) caseinate by the method of Hunter failed to yield a product containing the high proportion of protamin which he describes. The nitrogen content of the compound protein indicating a protamin content of less than ten per cent. The origin of this discrepancy has been ascertained by af Ugglas (117) who has shown that the preparations described by Hunter contained a considerable excess of the protamin precipitated in a condition of admixture with the compound. The compound of protamin (clupein) with casein prepared by af Ugglas contained 94 per cent of casein and 6 per cent of the protamin while the compound with hæmoglobin contained 5 per cent of protamin. Schmidt, who has prepared protamin (salmin) edestinate (110) (116), finds that it contains about ten per cent of the protamin.

When globin and casein are mixed in faintly acid solution (110) a precipitate of globin caseinate is produced which is soluble in excess of acid or in dilute alkalies (103). That this precipitation is accompanied by true compound formation has been demonstrated by the method of electrometric titration (Schmidt

* The protamins, although readily digestible by trypsin, are not digestible by pepsin.

(110) (111)). The precipitate produced by admixture of an excess of globin with sodium caseinate in solution contains about 34.5 per cent of casein. A compound of globin with deutero-albumose has been prepared by Schmidt (111).

Thymus histone combines with hæmoglobin, according to af Ugglas (117), in the proportion of one part of thymus histone to two of hæmoglobin, and with casein to form a compound containing about 30 per cent of the histone.

A particularly interesting compound protein is the hæmoglobin caseinate which has been prepared by af Ugglas. To a solution of casein in alkali an excess of hydrochloric acid is added until the precipitate of free casein which is at first formed is redissolved. The casein hydrochloride is precipitated from this solution by the addition of sodium chloride and the precipitate redissolved and reprecipitated until the washings from the precipitate are perfectly neutral. A solution of this substance added to an excess of a solution of hæmoglobin, produces a precipitate containing 33 per cent of casein and 66 per cent of hæmoglobin. The commonly accepted molecular weight of hæmoglobin, which has now been confirmed by a variety of measurements (Cf. section **11**), is about 16,700. We have seen that the minimal molecular weight of casein is about 8800 (Chap. V). It would, therefore, appear that casein and hæmoglobin combine with one another in molecular proportions.

From important observations of Hardy's it appears extremely probable that many of the protein constituents which may be isolated from the various tissues and tissue fluids do not pre-exist there but are bound up in complex compounds of proteins with proteins. I quote from the appendix to Hardy's article on globulin (35).

"The facts of the case are these. The proteins of serum are electrically inactive. Neither the whole nor any fraction moves in a field. It is not possible to detect a trace of 'ionic' protein. Dialysis or dilution disturbs the equilibrium and 'ionic' globulin appears, and can be swept out of the general mass of proteins as an opalescent cloud before dialysis has been pushed to the point where precipitation occurs. The development of even minute quantities of 'ionic' globulin can be detected in this way. The direction of the movement is towards the anode, therefore the globulin which appears is the anion of alkali-globulin."

"Serum which has been dialyzed against water with very low carbonic acid content until it ceases to give any precipitate, but which can still give with acid a large yield of globulin, is in a most interesting condition. The whole remaining protein is now ionic. It moves towards the anode quite uniformly, therefore it behaves as a whole as the protein ion of an alkali protein compound."

"Now what does this mean? The presence of other proteins does not interfere with the movement of ionic protein. This point cannot be too much insisted on.* It lies at the root of the evidence. Therefore one starts with protein which behaves uniformly and is electrically inactive, one ends with protein which behaves uniformly and is electrically active, and in the final stage there is no evidence of more than one kind of protein ion. But this residue of uniform character still contains a globulin fraction. It can be split by saturation with a neutral salt or by acidulation into fractions differing in properties according to the mode of separation."

"When in the cell used for these experiments, which is described on p. 289,† the upper layer of fluid is a solution of $\frac{1}{800}$th normal acetic acid and the lower layer is serum and a current is passed for 24 hours, the serum protein becomes slowly charged in such a way that it is repelled from both poles. Therefore in the anodic limb it becomes charged positively, in the cathodic limb negatively. The result of the double repulsion is that the protein is condensed into a hard plate of rubber-like consistency just midway between the electrodes."

"The phenomena would be explained, on the theory which has been outlined, in this way. In the anodic limb an excess of hydroxyl ions are liberated owing to the electric convection, and these, reacting with the serum protein, convert it into cationic protein. In the cathodic limb the converse reaction with hydrions occurs and is possible owing to the amphoteric nature of proteins. The entire mass of serum protein is thus thrown into the ionic state and in this condition moves with uniform motion, the one half as cationic protein, the other half as anionic protein. The electric current, the most subtle of analyses, detects only one substance, and this substance, owing to its amphoteric nature can exist in either the cationic or the anionic state."

* Cf. also Chap. XIII. (Author.) † Cf. Hardy's paper. (Author.)

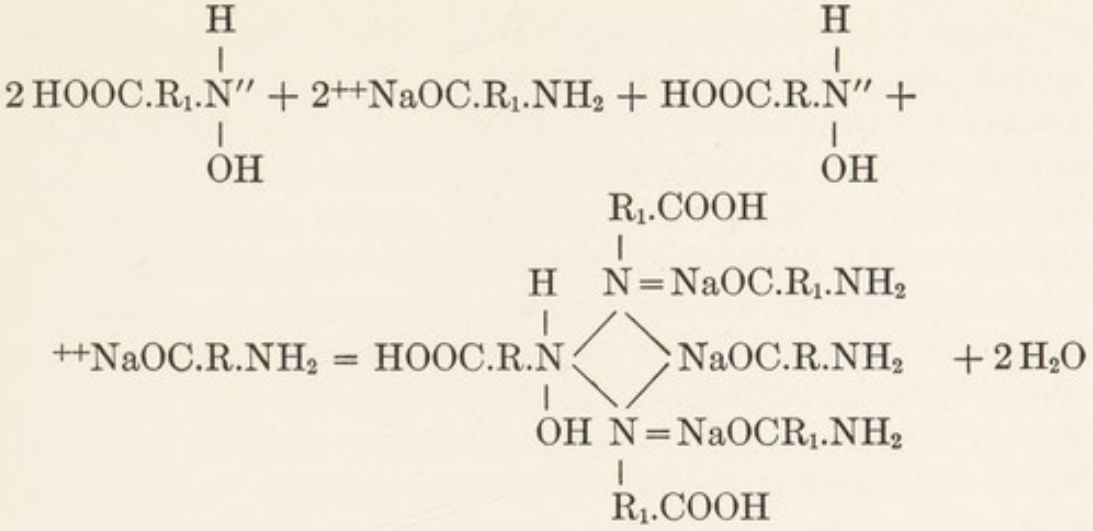
"The concentrated hard rubber-like mass of protein obtained in this way cannot be discriminated from serum protein. It is easily soluble in distilled water and in dilute salt solutions. From the solution in water a globulin fraction can be precipitated by acid, or by saturation with magnesium sulphate. From the solution in dilute salt solution a globulin fraction can be precipitated by saturation with salt (NaCl, or $MgSO_4$). The filtrate, after removal of the globulin by saturation, contains a protein which is precipitated by acetic acid."

"The position is that in serum one has protein which can be thrown into the ionic state and which then moves in a field as a single substance. From it an electrically active fraction, namely globulin, can be split off, and the protein thereby becomes electrically heterogeneous."

"Now the globulin fraction has an abiding characteristic. In all its solutions its molecular state is so gross as to cause the molecules to be arrested by a porous pot. They will not pass such a filter even under pressure. In this it is sharply distinct from the parent serum protein, which is readily filterable. If globulin be present as such in serum it is not only non-ionic, but the agent which dissolves it must be something more than alkali and salt since either alone or together they will not produce so high a grade of solution (78)."

"The difference in the molecular grade of globulin when once separated, and the electrical homogeneity of serum protein and of the fraction (still capable of further subdivision by salting out) which remains after the alkaline globulin fraction which most readily appears has been removed suggests that serum protein is a complex unit. If such a unit exists it is not saturated with globulin. Fresh ox-serum has an extraordinary power of dissolving globulin, it will take up almost its own volume of the thick cake at the bottom of a centrifuge tube; and in ox-serum so saturated there is not a trace of alkali globulin nor of any ionic protein."

The phenomena observed by Hardy appear to admit of interpretation on the view (102) that the protein-complex in serum is formed by the union of a number of alkali protein compounds, the union taking place in a manner strictly analogous to the combination of neutral salts with protein (Cf. Chap. VI), alkali protein molecules behaving like inorganic salt molecules thus:



Hydrolytic dissociation of this complex, just as in the case of the salt-alkali-protein compounds described in the previous chapter, would result in its decomposition, partial or complete, and hence, if the complex were non-ionic (and, as we have seen, the homologous compounds with inorganic salts are non-ionic) mere dilution of its solution or the removal of dehydrating agents (salts) might result, as in Hardy's experiments, in the splitting off of fraction after fraction of ionic protein. A very faintly alkaline reaction would probably favor its stability, a mere trace of acid might be expected to disrupt the complex (Cf. equation iv, Chap. VI).* It will be found, I believe, that the presence of such protein-complexes as these, in the tissues and tissue-fluids, affords

* The fact that this complex will pass through the pores of a porcelain filter, while the simpler, *ionic* protein will not, is due to its non-ionic character. The thesis will be developed in a later chapter (Chap. XI), upon a very extensive experimental basis, that the colloidal, non-filterable, viscous character of solutions of ionic protein is attributable *not to the size of the protein particles but to the size of the associated complex of water molecules*, which is much larger in solutions of ionic than in solutions of non-ionic protein. The non-filterable character of ionic globulin is to be interpreted in the same way as the non-permeability of a porous pot for *mixtures* of propyl alcohol and water, although it is permeable to both water and propyl alcohol when they are pure (S. U. Pickering (94)). Just as, in Pickering's experiment, propyl alcohol, when dissolved in water, will not pass out through the walls of a porous pot into the surrounding water, not because the wall of the pot is impermeable to propyl alcohol, for pure propyl alcohol will pass through the pot, but because it is impermeable to the molecule of propyl alcohol *plus* its associated complex of water molecules, so ionic globulin will not pass through porcelain because the porcelain is impermeable to the globulin ion plus its associated complex of water molecules.

a simple and readily intelligible explanation of an otherwise exceedingly puzzling fact. I refer to the *individuality* of the tissues and tissue-fluids. Despite the fact that the individual proteins which are found in the tissue-fluids of tolerably nearly related animals (e.g., the mammalia) appear, on analysis, to be identical with one another,* yet the tissue-fluids of one mammal, when injected into the circulation of another, are treated as foreign intrusions and give rise to "antibodies"; while the individual protein constituents of these fluids are also treated as foreign matter when introduced into the circulation, giving rise to "precipitations" and to phenomena such as "anaphylaxis" (120).

On the basis of the view developed above, however, not only the constitution of the individual components of the protein complex is of importance in determining its characteristics, but also the relative proportion of these components.† Two protein-complexes of this type might well be built up out of identical units and yet differ fundamentally owing to differences in the combining proportions and, consequently, in the mode of linkage of these units. Any one constituent of the complex would, of course, be a totally different substance from the complex itself and its introduction into the tissues or tissue-fluids would result in a more or less extensive disruption of the equilibrium of which the complex is an expression and which lends it, in each tissue, tissue fluid and species, its own distinctive character.

In pursuance of this idea Gay and Robertson (32) (33) and C. L. A. Schmidt (110) (111) have investigated the antigenic properties of several compound proteins. If compound proteins differ in their biological specificity from their constituents then

* Thus the casein of human milk is chemically identical with the casein of cows' milk and with that of goats' milk (2) (1). The fibrin of ox-blood appears to be identical with that of horse-blood (4). The serum albumins and globulins of goose-blood are identical with those of horse-blood (3). Whether chemically identical proteins derived from different animal species are also antigenically identical or not has not yet been satisfactorily established.

† Just as the different proteins and polypeptids are built up out of very similar amino-acids and yet differ widely from one another in their characteristics because the proportions in which the amino-acids enter into the molecules are different. These protein-complexes may be regarded as bearing the same relationship to the simple proteins which they yield on decomposition as the polypeptids do to the amino-acids.

a compound protein should represent a new antigen giving rise to antibodies for itself as distinguished from the antibodies for its constituents. Unfortunately a formidable technical difficulty stands in the way of clearly recognizing the presence of antibodies which are specific for the compound protein. This is the difficulty which is constituted by the fact that any protein which is capable of being split by hydrolysis into moieties which are still proteins (in the sense that they are antigenic) gives rise on injection into animals to antibodies, not only for itself, but also for these split-products (31). Analogously, a compound protein gives rise to antibodies for its constituent parts and it is only possible to distinguish between these, which would appear in the blood of immunized animals after injection of the separate constituents, and any antibodies which may be formed for the compound as a whole in those doubtless exceptional instances in which the antibody for the compound reacts with a constituent which is not normally antigenic.

We have therefore investigated from this point of view certain compound proteins in which one constituent is non-antigenic, such as protamin caseinate, of which the protamin constituent is non-antigenic and toxic while the casein constituent is antigenic and non-toxic, and globin caseinate of which the globin constituent is toxic and non-antigenic.*

Protamin caseinate displays no antigenic characteristics which enable it to be distinguished from casein. It is non-toxic, but whether this lack of toxicity is attributable to the masking of the toxic properties of protamin by its combination with casein, or to the smallness of the proportion of protamin contained in the compound has not yet been definitely established. It gives rise to antibodies for casein by virtue of its casein-content, just as casein gives rise to antibodies for its split-product paranuclein, but it does not give rise to antibodies for protamin. Similarly, protamin edestinate (111) is non-toxic and gives rise to antibodies for edestin but does not give rise to any antibody which will react with its protamin constituent. Globin caseinate, however,

* It is asserted by C. H. Browning and G. H. Wilson (23) that globin is antigenic. The negative results of Gay and Robertson which have been repeated and confirmed by Schmidt show that the antigenic character of the globin prepared by Browning and Wilson must have been due to contamination by protein impurities.

differs very markedly in its antigenic behavior from either of its constituents. In the first place it is non-toxic, and the failure to exhibit toxicity can hardly be attributable to dilution of the globin constituent by admixture with casein since, as we have seen, globin caseinate contains 65.5 per cent of globin (103). Still more striking is the fact that it yields antibodies which react (i.e., display alexin-fixation) not only with the casein constituent of the compound, but also with the globin constituent. It would appear evident, therefore, that injection of globin caseinate into animals gives rise to an antibody which does not appear in response to separate injections of its constituents. Schmidt (111) has investigated the antigenic behavior of a compound of globin (toxic and non-antigenic) with deutero-albumose (non-toxic and non-antigenic). The compound retains the toxicity of globin and is non-antigenic.

7. Compounds of the Proteins with Toxins, Antibodies, Ferments, etc. — Very extensive evidence has been advanced by numerous authors * in support of the view that the antitoxins are true proteins. If this be true, and the probabilities are immensely in favor of its truth, then the entire series of toxin-antitoxin reactions are reactions in which proteins play a leading part. To consider these here would be manifestly out of place; for an analysis of the physico-chemical laws which govern these reactions the reader is referred to Arrhenius (8).

The question of the occurrence of combinations between the proteolytic ferments and proteins and their significance in the mechanism of protein hydrolysis will fall under consideration in a later chapter (Chap. XVI) but the reader's attention is here drawn to the work of Hedin (37) (38) upon this subject.

8. Methyl and Benzoyl Derivatives of the Proteins. — According to Rogozinski (104), methylation of clupein causes profound alteration in the composition of the molecule, reducing especially the proportion of arginin yielded on subsequent hydrolysis. Skraup and Krause (114) have shown that not only is arginin reduced but also tyrosin, lysin and histidin, while the glutamic acid and leucin yields are unaffected.

Blum and Umbach (19) have prepared benzoyl derivatives of native and iodized proteins.

* Cf. for literature Carl Oppenheimer (86).

9. The Halogen and Nitro Substitution-Compounds of the Proteins. — Besides the combinations of the proteins with inorganic substances which are formed through the ionization of $-N.HOC-$ groups, other types of combination between proteins and inorganic substances are unquestionably possible. Among these the best known are the substitution-compounds of the proteins with the halogens and with NO_2.

According to Blum (18) (20) and to Hofmeister (43) (112) (41) (85), the halogen compounds are formed by the replacement of hydrogen atoms in the aromatic groups of the proteins by halogen atoms. The best known examples, both artificially prepared and naturally occurring, are the iodoalbumins. They can be prepared (Cf. papers just cited) by allowing a mixture of potassium iodate and potassium iodide to act upon proteins at a moderately high temperature (40 degrees). Hopkins and Brook (46), however, prepared iodoproteins by allowing powdered iodine to act directly on the protein, in solution. When the former method of preparation is employed, part of the iodine enters into combination with the protein and part is liberated as HI. Hence excess of a carbonate, of an alkali, or of an alkaline earth must be added to the mixture to neutralize the hydrogen iodide set free, and prevent it from hydrolysing the protein, through the catalytic action of hydrogen ions. The iodo-proteins are, in the dry condition, similar in appearance to ordinary proteins; they are insoluble in water or alcohol, and they appear to be more predominantly acid than the non-iodized proteins from which they are derived, since they are readily soluble in dilute solutions of alkalies or of alkaline carbonates, but are soluble in acid only in the presence of a considerable excess.

The percentage of iodine contained in the iodo-proteins varies, as might be expected, with the nature of the protein, — it also appears to vary somewhat with the mode of preparation or subsequent treatment, since two types of compounds are formed, the one containing a very high percentage of iodine, partly in firm and partly in loose combination (the per-iodo-casein of Liebrecht (70)), and the other a lower percentage of iodine entirely in firm combination.

Improved methods of preparing iodized proteins have recently been described by Oswald (92).

The bromine and chlorine substitution-products of the proteins

have been specially studied by Hopkins (45) (46) (47) and Blum and Vaubel (20). The following were the percentages of the different halogens found by Hopkins and by Blum in firm combination with egg-albumin.*

Hopkins	Blum
6.2 per cent iodine. 2.84 per cent bromine. 1.93 per cent chlorine.	6–7 per cent iodine. 4–5 per cent bromine. 2 per cent chlorine. 1.2 per cent fluorine.

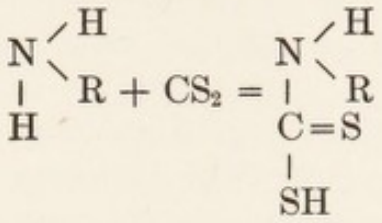
As in the case of the iodo-proteins, two series of bromine compounds exist: the one in which the bromine is firmly bound and the percentage of bromine is low, the other in which a part of the bromine is loosely bound and the percentage is high. Hopkins and Brook found in this latter type of brominated egg-albumin 14.89 per cent of bromine. These compounds, as also the chlorinated egg-albumin, are soluble in hot absolute alcohol, the halogen-protein being precipitated from this solution by the addition of ether (Hopkins and Brook).

The best-known naturally occurring halogen-protein is the thyreoglobulin of the thyroid gland. It contains only 1.75 per cent of iodine (91). The physiological action of this protein does not appear to be primarily dependent upon its iodine content (108) and it is doubtful whether this is the physiologically active iodine-containing principle of the thyroid (56). An iodized keratin, gorgonin, is found in corals (26) (40) (79) (81). It contains a high (about 8) percentage of iodine. Sponges also contain iodo-proteins (36) (52) (115).

Nitro substitution-products of the proteins were first prepared by Loew (73). Von Fürth (29) prepared them by acting upon the protein with nitrous acid, at the same time adding urea to prevent the formation of nitric acid. He obtained a product containing 1.78 per cent of NO_2. The nitro derivatives of the protamins have been very extensively studied by Kossel (65) (64) (67) (68) (119), who believes that the nitration of protein leads to the entry of NO_2 into the guanidine radical of the arginin. The nitrated proteins therefore yield nitroarginin on hydrolysis. On treatment with alkali the nitro-guanidine radical breaks up yielding carbon dioxide, nitrous oxide and ammonia.

* Cited after Gustav Mann (76).

10. The Compounds of Proteins with Sulphur. — Uhl (118) has prepared sulphur compounds of the proteins by utilizing the reaction:



which occurs in alkaline solution. These compounds combine with heavy metals; the salts thus formed contain a high proportion of the heavy metal and are soluble in water.

11. The Compounds of Proteins with Oxygen. — The best known compound of this type is that of oxygen with the coloring matter of the blood, hæmoglobin. This has been especially studied by Hüfner (50) who employed the spectrophotometric method of measurement, and found that at high pressures (by interpolation) the amount of oxygen bound up by one gramme of hæmoglobin is 1.338 cc., reduced to standard temperature and pressure. Carbon monoxide combines with hæmoglobin in equivalent proportions. Hüfner considered that the reaction between hæmoglobin and oxygen is a balanced reaction, namely, $Hb + O_2 \rightleftarrows HbO_2$. From this, if the concentration of reduced hæmoglobin be Cr and of oxyhæmoglobin C_o and that of oxygen

$$P_o \times \frac{\alpha_t}{760},$$

where P_o is the partial pressure of oxygen and α_t the absorption-coefficient of the gas at temperature t it would follow that if K is the velocity constant

$$\frac{C_o}{C_r \times P_o} = \frac{K\alpha_t}{760},$$

the quantities on the right of this equation being constant at constant temperatures, the relation between the oxygen tension and the percentage of hæmoglobin converted into oxyhæmoglobin should be expressed by a rectangular hyperbola. Bohr (21), however, found that the curve of dissociation of oxyhæmoglobin is not a rectangular hyperbola, and he explains his results by supposing that the hæmoglobin, in the presence of oxygen, first splits off a hæmatin-containing moiety and that this then sub-

sequently unites with the oxygen. Barcroft and Roberts (9) (10) (11) (12) (13) have, however, shown that the irregularities obtained by Bohr were due to the presence of electrolytes in the hæmoglobin solutions; in dialysed solutions the curve of dissociation is exactly that demanded by Hüfner's theory. The *velocity* of dissociation of oxyhæmoglobin obeys the equation indicated by the mass law. The variations of the equilibrium-constant (K) with change of absolute temperature follow the van't Hoff equation

$$\frac{1}{K} \cdot \frac{dK}{dt} = \frac{-q}{2\,T^2},$$

where q is constant and equal to 28,000 calories. This is therefore the heat of combination of one gram-molecule of hæmoglobin with oxygen. Since the amount of heat which is actually given out when one gram of hæmoglobin unites with oxygen is 1.85 calories, the molecular weight of hæmoglobin, in dialysed solution is, according to these results, 15,000. The *minimum* weight indicated by Hüfner's results, cited above, is 16,669. A similar figure is indicated by the iron-content of the hæmoglobin molecule (55) (121) (49) (50) and by the osmotic pressure measurements of Hüfner and Gausser (51). Weymouth Reid (98), however, found a value, as indicated by osmotic pressure measurements, three times as great. Barcroft and Hill (11) suggest that hæmoglobin may possibly exist as a polymer of itself, under certain conditions, and that the irregularities observed in the curve of dissociation of oxyhæmoglobin in the presence of electrolytes may be due to the breaking down of such aggregates. The temperature-coefficient of the dissociation of oxyhæmoglobin is large, about 4 per 10 degrees rise in temperature.

These masterly investigations of Barcroft effectually demonstrate two things; in the first place that the union between hæmoglobin and oxygen is not an "adsorption combination" as Wo. Ostwald has suggested (90) (101) (13) and, in the second place that, since the derivation of the van't Hoff law (reaction isochore) involves the assumption that the gas laws apply strictly to the system under consideration,* hæmoglobin, although a colloid, does not form a separate phase within its solution,† but

* Cf. W. Nernst (83).

† It may be remarked that under such conditions hæmoglobin cannot present any surface with which to "adsorb."

is molecularly distributed throughout the solution in accordance with the law of Avogadro. Again we see, therefore, as we saw in considering the copper albuminates (Chap. VI, section **5**), that the distinction which has been built up by certain authors between the crystalloids which form homogeneous, and the colloids which are supposed to form heterogeneous, solutions, is entirely artificial and not in accordance with the facts.

The results obtained by Barcroft and his co-workers have received decisive confirmation at the hands of Butterfield (24) who, using the spectrophotometric method, has shown with a very high degree of precision, that the absorption-spectrum of a solution containing a mixture of oxyhæmoglobin and reduced hæmoglobin does not alter upon dilution. The solubility of oxygen in water not being appreciably affected by the presence of hæmoglobin the relative proportion of the reacting substances (oxygen, hæmoglobin and oxyhæmoglobin) is not altered by dilution and therefore in accordance with the mass law the proportion of oxygen in combination remains likewise unaffected.

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PART II

THE ELECTROCHEMISTRY OF THE PROTEINS

CHAPTER VIII

THE FORMATION AND DISSOCIATION OF PROTEIN SALTS

1. Compounds of the Proteins with Inorganic Bases and Acids; the Non-Dissociable Character of the Inorganic Radical. —We have already had occasion to incidentally dwell upon the fact that the inorganic radical of the protein base and protein acid compounds is not electrically dissociated as such. The most direct proof which we possess of this fact is that which was obtained by Bugarszky and Liebermann (4). These observers employed the potentiometric method, using two different concentration chains. The first was the ordinary gas-chain:

Pt saturated with H_2	Acid (HCl)	Base (NaOH)	Pt saturated with H_2
1	2	3	4

the potential being measured, first with pure acid in 2 and then with acid plus protein; the difference between the two readings * yielding by computation from the Nernst formula, the number *of hydrogen ions bound by the protein.* The second concentration-chain was built up as follows:

Hg	HgCl (solid), HCl	NaCl	NaBr, HgBr (solid)	Hg
1	2	3	4	5

and enabled them to estimate, in a similar way, *the number of Cl' ions bound by the protein.* Now the *number of Cl' ions bound by a given mass of protein dissolved in dilute HCl was found to be exactly equal to the number of H^+ ions which it binds.* The following data are compiled from those obtained by Bugarszky and Liebermann:

* Less a correction expressing the potential between 2 and 3.

EGG-ALBUMIN IN 0.05 *N* HCl.

Grams protein in 100 cc.	Per cent of H^+ bound by the protein	Per cent of Cl′ bound by the protein
0	0	0
0.4	9.0	10.7
0.8	18.9	20.2
1.6	33.3	38.0
3.2	60.2	64.0
6.4	96.6	76.0

The slight irregularities are, save in the last observation, no greater than might have arisen out of the uncertain magnitude of the correction for the potential between the elements 2 and 3 in the gas-chain.

These striking results have been confirmed by Rohonyi (31) employing Merck's crystallized ovalbumin and Witte's peptone; the following were the results obtained, the substances being dissolved in *N*/20 HCl solution:

Substance	Per cent	Per cent of chlorine ions bound	Per cent of hydrogen ions bound
Ovalbumin	1.9	21.9	22.6
Albumose	1.8	37.2	35.9
Alanin	1.5	37.9	66.2

A decided difference between the modes of combination of proteins and an amino-acid with hydrochloric acid is thus very clearly revealed.

Manabe and Matula (17) and Blasel and Matula (2) have shown that at low H^+ ion concentrations a greater proportion of H^+ is bound by serum albumin or gelatin than of Cl′. Ringer (33) has confirmed this observation for albumoses, but he also finds that in higher concentrations of hydrochloric acid (1 per cent of albumose in 0.1 *N* HCl) the H^+ and Cl′ are bound equally. The same tendency is shown in the observations of Bugarszky and Liebermann which are quoted above, for in the solution containing the lowest proportion of hydrochloric acid to protein the Cl′ bound by the protein was about 20 per cent less than the proportion of H^+ which was bound.

It has been shown by Blasel and Matula (2) that deaminized

gelatin, prepared by the action of nitrous acid upon gelatin, notwithstanding the absence of end NH_2 groups, still retains the power of binding Cl′ ions in solutions of hydrochloric acid.

Oryng and Pauli (20) have shown that serum albumin, gelatin and deaminized gelatin, dissolved in solutions of potassium chloride, bind a definite proportion of Cl′ ions, and this proportion is greatly increased by the addition of acids (such as sulphuric acid) to the solution.

Confirmatory evidence is not lacking. Loevenhart (15) and others * have found that rennet will not coagulate calcium caseinate unless a small amount of a dissociable salt of calcium is present. The calcium bound by the casein itself is not available for this purpose, but if a small quantity of acid be added then a proportion of the calcium is freed from its combination with casein and, if it forms a *dissociable* salt with the added acid, it is able to bring about coagulation.

W. A. Osborne (21) has shown that if calcium caseinate be placed inside a dialysing tube which is then immersed in a very dilute solution of mercuric chloride the mercury diffuses into the tube and is there held in an undissociated form, since the concentration of mercury *within* the tube is found, after some time, to considerably exceed that of the mercury in the outside fluid.

Similarly, it has been shown by Moore, Roaf and Webster (18) that if casein dissolved in dilute sodium hydroxide be placed inside an osmometer of which the membrane is permeable to crystalloids but not to colloids and the concentration of NaOH be rendered initially equal on both sides of the membrane, then NaOH will move into the osmometer *against* the osmotic pressure gradient and actually lead to a pronounced increase in the pressure within the osmometer. It is evident, therefore, that not only OH′ but also Na^+ ions must have been bound by the protein, since otherwise no movement of Na^+ across the membrane could have occurred.

* The statement of van Dam (6) that the extent of coagulation depends upon the quantity of Ca *bound* by the casein is not irreconcilable with Loevenhart's results. As is well known, the calcium bound in casein as calcium caseinate does not suffice to bring about coagulation. A dissociable salt of calcium must also be present. That this salt may combine with the calcium paracaseinate to form a double salt analogous to those described in the previous chapters, is not at all unlikely.

Rohmann and Hirschstein (30) have shown that solutions of silver caseinate contain no silver ions, since they fail to yield a precipitate on adding sodium chloride.

The most obvious conclusions to be drawn from these results are (*a*) that the protein-base or protein-acid compounds are not subject to dissociation at all; or else (*b*) that they dissociate the positive and negative ions of the inorganic constituent in equivalent proportions, i.e., undergo hydrolytic dissociation.

That these assumptions are incorrect, however, is shown by a large number of experiments which demonstrate that the protein compounds with inorganic bases and acids are true electrolytes, independently of any hydrolytic dissociation which they may undergo in solution. For example, Sjöqvist (35) has shown that if egg-albumin be dissolved in dilute hydrochloric acid, as the concentration of albumin is increased, keeping that of the HCl-solution constant, the molecular conductivity (calculated for 0.025 *N* HCl) diminishes until it reaches a constant minimum value, which is attained when about four grams are dissolved in 100 cc. of 0.025 *N* HCl. Now the above quoted results of Bugarszky and Liebermann show that in this solution at least 97 per cent of the hydrochloric acid is bound by the egg-albumin. The observed "molecular" conductivity (67×10^{-3}) is at least 7 times greater than could be accounted for by the maximum possible residuum of unneutralized hydrochloric acid and must therefore be due to the protein-acid compound.

Solutions of the caseinates of the alkalies and alkaline earths can be obtained which are neutral or even acid to litmus (Cf. Chap. V), these solutions therefore contain no free base; nevertheless they are excellent conductors of electricity (34) (23) (27) (28) since a 2 per cent solution of potassium caseinate which is neutral to litmus possesses a conductivity of 92.7×10^{-3} reciprocal ohms per equivalent of base neutralized at 30° C. That this conductivity is not attributable to associated impurities, inorganic or otherwise, is shown by the following facts:

(i) It bears a definite relation to the amount of base neutralized by the protein (27).

(ii) The conduction of electricity is accompanied by migration of the casein to the anode, and the amount of casein transported to the anode is directly proportional to the quantity of electricity which is transported through the solution (29).

Similarly, solutions of the serum-globulinates of the alkalies and alkaline earths may be obtained which are neutral to litmus and which nevertheless conduct electricity, the passage of a direct current through these solutions being accompanied by transport of the protein to the anode (10).

Only one conclusion is left open to us, therefore, namely, that the salts which proteins form with inorganic acids and bases do not dissociate at the point of union of the inorganic radical with the protein, but elsewhere, *within the protein molecule itself*, yielding, not an inorganic and a protein ion, but two or more protein ions, in one or more of which the inorganic radical is bound up in a non-dissociable form.*

On examining the details of the behavior of the protein salts, as electrolytes, we are speedily compelled, by inference, to reach precisely the same conclusion.

In the first place, the conductivity of solutions of certain protein salts, for example potassium caseinate, is not at all affected by the presence, in the solution, of an excess of the ions of the inorganic radical. This fact is very clearly shown by the following experiments (27).

Two and a half grams of pure casein were dissolved in solutions containing varying amounts of KOH of which, in each instance, so much was neutralized by 0.1 *N* HCl as to leave the equivalent of 25 cc. of 0.1 *N* KOH unneutralized by the acid. These solutions were then each diluted to 250 cc., so that the final solutions consisted of 1 per cent casein dissolved in 0.01 *N* KOH plus varying amounts of KCl. The conductivities ($= x$) of these solutions (at 30 degrees) were then determined and also the conductivities ($= x_1$) of solutions similarly made up without the introduction of casein. The conductivity of the original solution, before the introduction of casein, is the sum of two quantities, i.e., the conductivity of 0.01 KOH + the conductivity of the KCl; that of the solution of the caseinate is the sum of three quantities, i.e., the conductivity of the *unneutralized* KOH and the conductivity of the KCl + the conductivity of the caseinate. Subtracting the latter from the former, therefore, we obtain $x_1 - x$, which is the conductivity of the 0.01 KOH *minus* the conductivity of the KOH unneutralized by 1 per cent casein and the conductivity of the caseinate itself; in other words, the

* Cf. also Chap. IX, **3**.

conductivity of a solution of the *neutralized* KOH minus the conductivity of the caseinate. If we subtract this quantity $(x_1 - x)$ from the conductivity of the neutralized KOH, therefore, we obtain the conductivity of the caseinate itself. Now in these solutions, as determined by the gas-chain, the concentration of *neutralized* KOH was 0.00993, and the conductivity at 30 degrees of a solution of free KOH of this concentration would be 280×10^{-5} reciprocal ohms. Subtracting $x_1 - x$ from 280×10^{-5}, therefore, we obtain the conductivity, in each of the solutions investigated, of the caseinate itself. In the following table some experimental results are given. In the first column is given the concentration of KCl which was present in the solution of caseinate; in the second the conductivity $(= x_1)$ of the solution containing no casein; in the third the conductivity $(= x)$ of the solution containing casein; in the fourth the conductivity $(= 280 \times 10^{-5} - (x_1 - x))$ of the caseinate itself; in the fifth the deviation of this conductivity from its average value in the different solutions; in the sixth the experimental error of the conductivity measurement itself, exclusive of any possible deviations arising out of errors in weighing, measurements of volume, or decomposition of the casein.

Concentration of KCl	x_1 (recip. ohms)	x (recip. ohms)	Conductivity of caseinate itself	= Deviation from average	= Experimental error
0.00 *N*	277.4×10^{-5}	81.2×10^{-5}	83.8×10^{-5}	-0.5×10^{-5}	$\pm0.7\times10^{-5}$
0.01 *N*	417.0×10^{-5}	222.6×10^{-5}	85.6×10^{-5}	$+1.3\times10^{-5}$	$\pm1.8\times10^{-5}$
0.02 *N*	548.0×10^{-5}	360.5×10^{-5}	91.8×10^{-5}	$+7.5\times10^{-5}$	$\pm3.5\times10^{-5}$
0.03 *N*	698.0×10^{-5}	494.5×10^{-5}	76.1×10^{-5}	-8.2×10^{-5}	$\pm5.9\times10^{-5}$

It is evident that the estimates of the conductivity of the caseinate itself which were thus obtained are appreciably constant, the deviation from the average being irregular and less or only very slightly greater than the purely metrical error of the observation. In other words, whether the concentration of KCl in its solution is 0.00 or 0.03 the share taken by the potassium caseinate itself in conducting the current is the same within the experimental error, that is, at the very highest estimate, within 10 per cent.

Now suppose that potassium caseinate, containing this proportion of potassium, yields, on dissociation, only one potassium

ion and one casein ion, then applying the ordinary laws of dissociation, since in the solution containing no KCl the total concentration of K ions must have been very nearly 0.01 and calling A the concentration of protein ions in this solution, C the dissociation-constant of the caseinate and X the concentration of the undissociated caseinate we have, very nearly:

$$0.01\,A = KX$$

while the solution containing 0.04 N potassium (0.03 N KCl) we have, very nearly,

$$0.04\left(A - \frac{A}{10}\right) = K\left(X + \frac{A}{10}\right)$$

whence $A = 26\,X$, or the caseinate must, in the first solution, have been $\frac{26}{27}$ths dissociated. Now it should be particularly observed that this is most decidedly a minimal estimate of the degree of dissociation of this salt, if the assumption upon which we have proceeded is correct. For free casein is insoluble and 1 gram of casein is just carried into solution by 11.4×10^{-5} equivalents of base, whereas these solutions contained as we have seen nearly nine times this amount of *neutralized* KOH. If we suppose that, in reality, nine COOH groups in the casein molecule have been neutralized by KOH in these solutions, so that the caseinate yields nine K^+ ions, then the above solution (i.e., that which contained no KCl) must have been no less than $\frac{23}{23}\frac{59}{59}\frac{23}{28}\frac{26}{27}$ths dissociated. Hence, at the very lowest estimate, if we assume that it dissociates potassium ions, a solution of potassium caseinate containing 99×10^{-5} equivalents of potassium per gram of caseinate must be $\frac{26}{27}$ths dissociated.

Upon dilution, therefore, its conductivity could only increase by $\frac{1}{26}$th, or 4 per cent and, moreover, no solution of potassium caseinate could be possessed of a greater *equivalent* conductivity than that of the above solution plus 4 per cent, i.e., about 90×10^{-3} at 30° C.

But, as we shall see in succeeding chapters, the equivalent conductivity of a 1 per cent solution of caseinate in 0.01 N KOH increases very much more than 4 per cent on dilution, nor can this increase, as we shall see, be attributed to hydrolytic dissociation. Moreover solutions of potassium caseinate can readily be obtained which are *acid to litmus* and which possess an equivalent

conductivity (calculated upon the basis of the potassium which they contain) of over 120×10^{-3} or 30 per cent more than that of the solution considered above.

Hence we have no resource but to conclude that our initial assumption was erroneous and that potassium caseinate dissolved in water *does not yield potassium ions.**

Again, we may reach the same conclusion by quite a different process of reasoning and from very different experimental data. The conductivity of solutions of the caseinates and globulinates of the alkalies and alkaline earths and of the salts which ovomucoid forms with acids does not decrease in direct proportion with the dilution, but more slowly, indicating a progressively increasing dissociation of the caseinate on dilution. From the curve expressing the relation between the equivalent-conductivity (calculated on the basis of the inorganic radical) and the dilution we can, by exterpolation, estimate the maximum equivalent conductivity, i.e., the equivalent conductivity at infinite dilution of the salt; this we can do rather accurately, since at readily attainable dilutions the equivalent conductivity already increases very slowly with dilution and obviously tends to approach a constant maximum. Now, as is well known, this maximum bears a constant proportion to the sum of the *equivalent conductivities of the ions into* which the salt dissociates.

If the inorganic radical is dissociated as such, the equivalent conductivity of these salts cannot be less than that of the inorganic radical itself, but must exceed it by a quantity equal to the equivalent conductivity of the protein ion or ions. In the following table are compared the observed equivalent conductivities (at infinite dilution) of a number of protein salts at 30 degrees,† and those of the inorganic radicals which they contain, calculated from the data given by Kohlrausch and Holborn (12).‡

* It may be mentioned in passing, that in the solutions investigated potassium caseinate evidently does not form a double salt with potassium chloride. Were such a double salt formed, and we have seen that in many cases salts of this type may be formed in protein solutions, then, of course, the KCl would not be without effect upon the conductivity of the caseinate.

† Cf. data cited in Chap. X.

‡ The migration-velocities of the ions at 18 degrees (p. 200) increased by 2 per cent per degree to reduce to the temperature employed and multiplied by the proportionality between ionic velocity and reciprocal ohms per cc., i.e., 96.44.

Salt	Temp., Degrees C.	Equivalent conductivity at infinite dilution, equivalent conc. taken as that of the inorganic radical. Reciprocal ohms per cc. per equivalent per litre	Equivalent conductivity at infinite dilution of the inorganic radical. Reciprocal ohms per cc. per equivalent per litre
Sodium caseinate (80×10^{-5} equivs. per gram)	25	63.5×10^{-3}	50.6×10^{-3}
Ammonium caseinate (80×10^{-5} equivs. per gram)	25	79.4×10^{-3}	73.2×10^{-3}
Potassium caseinate (80×10^{-5} equivs. per gram)	30	80.6×10^{-3}	81.0×10^{-3}
Calcium caseinate (80×10^{-5} equivs. per gram)	30	35.9×10^{-3}	65.7×10^{-3}
Strontium caseinate (80×10^{-5} equivs. per gram)	30	30.7×10^{-3}	67.0×10^{-3}
Barium caseinate (80×10^{-5} equivs. per gram)	30	42.1×10^{-3}	71.1×10^{-3}
Potassium serum-globulinate (20×10^{-5} equivs. per gram)	30	51.0×10^{-3}	81.0×10^{-3}
Calcium serum-globulinate (20×10^{-5} equivs. per gram)	30	23.5×10^{-3}	65.7×10^{-3}
Strontium serum-globulinate (20×10^{-5} equivs. per gram)	30	27.5×10^{-3}	$67.\times10^{-3}$
Barium serum-globulinate (20×10^{-5} equivs. per gram)	30	23.4×10^{-3}	71.1×10^{-3}
Ovomucoid chloride (45×10^{-5} equivs. per gram)	30	196.0×10^{-3}	81.7×10^{-3}

It is evident that in many cases the equivalent conductivity of a protein salt is actually less, in the salts of the alkaline earths very considerably less than that of its inorganic radical alone. On the supposition that the protein salt splits off the inorganic radical as an ion, not only the inorganic radical but also the protein must be participating in the conduction of electricity through its solution, and its equivalent conductivity, when completely dissociated, must be greater than that of the inorganic radical by the amount contributed by the protein ion. Hence the assumption that the salts of the proteins split off the inorganic radical as an ion must be incorrect.

We have seen, in considering the constitution of the protein molecule (Chap. I), that the protein molecule does not contain a sufficient number of terminal $-NH_2$ and $-COOH$ groups to account for its high combining capacity for acids and bases, and the suggestion was put forward that the true point of union with acids and bases is the $-N.HOC-$ group, and that union with

bases and acids and the dissociation of the resultant salts take place according to the following schemes:

$$-\text{N.HOC}- + \text{NaOH} = -\text{N}''(\text{H})(\text{OH}) + \overset{++}{\text{Na}}\text{OC}-$$

$$-\text{N.HOC}- + \text{HCl} = -\text{N}''(\text{H})(\text{Cl}) + \overset{++}{\text{H}}\text{OC}-$$

or in accordance with modifications of these schemes arising out of the participation of dicarboxylic- and diamino-radicals in the reactions.

In the succeeding pages the endeavor will be made to interpret the electrochemical behavior of the proteins and their salts with the aid of this hypothesis.

2. The Electrolysis of Protein Salts. — It was shown by Hardy (9) in 1899 that if a trace of acid be added to a solution of dialysed white of egg, modified by dilution and boiling, on passing a direct current through the solution the whole of the protein finally moves over to the cathode, where it is precipitated. If a trace of alkali is added, however, instead of acid, the whole of the protein finally migrates, under the influence of the current, to the *anode*. He later showed that serum-globulin in solution behaves similarly (10). Under such circumstances, therefore, the protein behaves like the anion or cation of a salt; like the cation when combined with an acid; like the anion when combined with a base.

Here an apparent, but not a real difficulty confronts us in the application of the hypothesis outlined above to the electrolysis of protein solutions. On the basis of this hypothesis the protein should migrate in both directions, the cation, when the protein is combined with a base, and the anion, when the protein is combined with an acid, carrying the inorganic constituent with it. At first sight it might appear as if the protein should be deposited at both electrodes, but not when we look more closely into the matter. In both of the cases just cited the free, uncombined

protein is insoluble, while the combined protein is soluble. Consider the electrolysis of a compound of such a protein with a base. The anion

$$\begin{array}{c} H \\ | \\ R.N'' \\ | \\ OH \end{array}$$

will migrate to the anode, and after neutralizing any excess of base which may be present (since the ion, obviously, must contain $-N.HOC-$ groups and therefore, like the entire molecule, is amphoteric) must eventually, when the film in immediate contact with the electrode has become saturated with protein, be precipitated as the uncombined and therefore insoluble protein. The case is very different with the cation $R.CONa^{++}$, for this, on arriving at the cathode, will bring it an excess of base and the cathode region must become alkaline and, therefore, free protein cannot be precipitated there. Similarly, in the electrolysis of a salt of such a protein with an acid the uncombined protein must be precipitated at the cathode, but not at the anode. If the protein is of such a type that the free protein is soluble, for example ovomucoid, I have observed that no deposition of protein occurs at either electrode, but only the evolution of gas, presumably hydrogen at the cathode and oxygen at the anode. That under such conditions the protein nevertheless migrates to *both* electrodes has been shown by Stirling and Brito (36) and by Howell (11).

If a direct current of about one milliampere be passed through a solution of potassium caseinate, which is neutral to litmus, gas may be observed to be evolved at both electrodes, but a firm white spongy precipitate is deposited upon the anode, the cellular texture of which is attributable to entangled bubbles of gas, presumably oxygen (29).

A quantity of this precipitate was collected. The anode consisted of a spiral of platinum wire some 9 cm. long. This wire, when coated with the precipitate, was well washed in a stream of distilled water, and the precipitate was then scraped off into 99.8 per cent alcohol. The precipitate which was thus collected under alcohol was washed with alcohol and ether and dried at 30 degrees over sulphuric acid for 48 hours.

The precipitate proved to be uncombined (base free) casein.

In fact so nearly was it devoid of mineral content as to practically realize that elusive ideal, the "ash-free protein," for one gram of the precipitate yielded less than two milligrams of ash. Yet this casein, in every way in which it was tested, proved to be perfectly normal. The possibility was thus indicated of estimating *the electrochemical equivalent of casein.*

Solutions of potassium caseinate were placed in a U-tube of about 30 cc. capacity, 25 cc. of solution being employed in each experiment. The anode consisted of a spiral of platinum wire about $\frac{1}{2}$ mm. thick and 9 cm. long, the diameter of the spiral being about $\frac{1}{2}$ cm. and its "pitch" about 45 degrees. The cathode simply consisted of a platinum wire dipped in the fluid in the other arm of the U-tube. The U-tube was provided at the bottom with a 3-way stop-cock, which could either be turned so as to provide fluid communication between the two arms of the tube, or else so as to permit the contents of the anodal limb to escape into a receptacle. In this way it was possible to investigate separately, if desired, the contents of each arm of the U-tube. It was found that after electrolysis the fluid in the anodal arm, in which the deposition of casein occurred, was practically unaltered in reaction, although its casein-content was much diminished. In the cathodal arm, not only did the casein-content diminish, but the alkalinity of the fluid was markedly increased.

The current was led from the terminals of a 110-volt circuit through a 16 candle-power lamp, a milliamperemeter, a silver titration voltameter containing 30 cc. of $N/100$ $AgNO_3$, and through the solution of caseinate.

The silver was then determined by titration with a $N/100$ ammonium thiocyanate in the presence of a constant excess of nitric acid, a constant quantity (5 cc. of saturated solution) of ferric alum being employed as indicator.

The amount of casein which had been precipitated by the current was estimated by determining the refractive indices of the original and of the electrolysed solutions at the same temperature; the differences between the refractive indices divided by 0.00152 yielding the decrease in the *percentage* of casein contained in the solution consequent upon electrolysis.* The quantity of solution employed was always 25 cc. Hence the decrease

* Cf. Chap. XIV. Also T. Brailsford Robertson (24).

in the percentage-content of casein, divided by 4, was the amount of casein precipitated by the current.

The experiments were all conducted at 30° C.

Varying amounts of casein were dissolved in 100 cc. each of KOH solutions of varying concentrations so that the proportion of base to casein was 50×10^{-5} equivalents per gram (neutral to litmus),* or 80×10^{-5} equivalents per gram (neutral to phenolphthalein),† or 100×10^{-5} equivalents per gram. In estimating the current employed, the electrochemical equivalent of silver, in grams per coulomb, is taken as 0.001118 (8).

It was found that the solutions containing the higher proportions of base to casein yielded an apparently lower electrochemical equivalent for the casein. This was speedily traced to *resolution* of the casein from the electrode after precipitation. The anode, after having been coated with casein by the action of a current of one milliampere passing through a 3 per cent solution of casein, neutral to litmus, was washed in water, alcohol and ether, dried and weighed. It was then immersed in a solution of casein (3 per cent) neutral to litmus for 2 hours, then withdrawn and washed, dried and weighed as before. It was found to have lost 11 milligrams in weight. Similar experiments were conducted in which the solutions in which the coated wire was immersed were neutral to phenolphthalein and alkaline to phenolphthalein. The following were the results obtained:

Current employed for precipitation	Solution	Loss after two hours
1 milliamp.	3 per cent casein in 50×10^{-5} equivs. KOH grm.	11 mg.
1 milliamp.	3 per cent casein in 80×10^{-5} equivs. KOH grm.	54 mg.
2 milliamp.	3 per cent casein in 80×10^{-5} equivs. KOH grm.	59 mg.
1 milliamp.	3 per cent casein in 100×10^{-5} equivs. KOH grm.	97 mg.

Hence doubling the rate of deposition makes very little, if any, difference to the rate of resolution of the casein, but increasing alkalinity of the solution in which the deposition occurs increases the rate of resolution very markedly.‡

* Cf. Chap. V. Also T. Brailsford Robertson (27).

† Cf. Chap. V.

‡ If the anode be much less than 9 cm. in length there is a tendency, after prolonged electrolysis, to what may be termed "flocculent deposition," or precipitation of the casein within the body of the fluid in the anodal arm and

Assuming the rate of resolution to be constant, since the contents of the anodic arm are not appreciably altered in reaction in the electrolysis, it is possible to calculate from the above data for each of the solutions and periods employed the loss due to resolution. In the column headed "Loss due to resolution" in the accompanying tables these quantities are given. On adding them to the amounts of casein lost from the solution from which the "apparent" values of the electrochemical equivalent are estimated one obtains the "corrected" values corresponding to the total precipitation actually induced by the passage of the current.

The possible error in the refractometer reading is 1′ of the angle of total reflection, this corresponds to an error of, ± 0.00010 in the refractive index, that is, to an error of ± 0.07 in the estimated decrease in the percentage of casein due to electrolysis of the solution, and to an error of ± 0.0175 in the estimate of the amount of casein deposited by the current. The possible error in each estimate of the electrochemical equivalent, arising from this source, is indicated in the tabulated results.

The average values for the electrochemical equivalent in the different solutions are obviously, within the experimental error, identical. Now in solutions containing 50×10^{-5} equivalents of base per gram, the combining capacity of casein is 50×10^{-5} equivalents per gram, in solutions containing 80×10^{-5} equivalents per gram, it is 80×10^{-5} equivalents per gram, and in solutions containing 100×10^{-5} equivalents per gram it is between 99 and 100×10^{-5} equivalents per gram.* In the solutions investigated, therefore, the combining weight of the casein varies 100 per cent, yet the electrochemical equivalent, measured in this way, remains the same.

These facts are to be interpreted as follows: We have seen that the casein anion, which is free from base, must migrate to the anode. There it may be presumed to react with water, liberating oxygen and free casein, which combines with the excess

not upon the wire. Under these conditions, resolution is apparently more rapid and even if the flocculent deposit be filtered off from the anodal fluid before the alkaline cathodal fluid is mixed with it, the values of the electrochemical equivalent are low. No such phenomenon was observed when the anode was of sufficient length.

* Cf. Chap. IX. Also T. Brailsford Robertson (27).

TABLE I

50×10^{-5} Equivalents of KOH per Gram of Casein

Per cent of casein	Current in amperes	Time of passage	Grams casein lost from solution	Apparent electrochemical equivalents in grams per coulomb	Loss due to resolution, milligrams	Corrected electrochemical equivalents
6	11.51×10^{-4}	2 hrs. 15 min.	0.2155±0.0175	0.0231±0.0019	12	0.0244±0.0019
4	9.47×10^{-4}	2 hrs. 0 min.	0.1645±0.0175	0.0241±0.0026	11	0.0257±0.0026
4	18.05×10^{-4}	2 hrs. 15 min.	0.3815±0.0175	0.0261±0.0012	12	0.0269±0.0012
3	10.54×10^{-4}	2 hrs. 0 min.	0.1810±0.0175	0.0238±0.0023	11	0.0253±0.0023
3	17.36×10^{-4}	2 hrs. 0 min.	0.3125±0.0175	0.0250±0.0014	11	0.0259±0.0014
2	11.22×10^{-4}	2 hrs. 0 min.	0.1810±0.0175	0.0224±0.0022	11	0.0238±0.0022
2	15.85×10^{-4}	1 hr. 25 min.	0.1678±0.0175	0.0208±0.0022	9	0.0219±0.0022
					Average..	0.0248±0.0020

TABLE II

80×10^{-5} Equivalents of KOH per Gram of Casein

Per cent of casein	Current in amperes	Time of passage	Grams casein lost from solution	Apparent electrochemical equivalents in grams per coulomb	Loss due to resolution, milligrams	Corrected electrochemical equivalent
3.75	10.82×10^{-4}	2 hrs.	0.1480±0.0175	0.0190±0.0022	54	0.0259±0.0022
3.75	16.03×10^{-4}	2 hrs.	0.1973±0.0175	0.0171±0.0015	59	0.0222±0.0015
3.75	8.01×10^{-4}	4 hrs.	0.1810±0.0175	0.0157±0.0015	108	0.0250±0.0015
3.75	8.74×10^{-4}	4 hrs.	0.2468±0.0175	0.0196±0.0014	108	0.0282±0.0014
3.75	18.31×10^{-4}	2 hrs.	0.1973±0.0175	0.0150±0.0013	59	0.0194±0.0013
3.75	10.56×10^{-4}	2 hrs.	0.1052±0.0175	0.0138±0.0023	54	0.0209±0.0023
3.75	19.50×10^{-4}	1 hr.	0.1315±0.0175	0.0189±0.0025	30	0.0230±0.0025
					Average..	0.0235±0.0018

TABLE III

100×10^{-5} Equivalents of KOH per Gram of Casein

Per cent of casein	Current in amperes	Time of passage	Grams casein lost from solution	Apparent electrochemical equivalent in grams per coulomb	Loss due to resolution, milligrams	Corrected electrochemical equivalent
3	2.03×10^{-4}	2 hrs.	0.0988±0.0175	0.0114±0.0020	97	0.0226±0.0020
2	12.56×10^{-4}	1 hr.	0.0493±0.0175	0.0109±0.0039	49	0.0217±0.0039
					Average..	0.0222±0.0030

of base until the proportion of base to casein in the film in immediate contact with the anode falls to that which obtains at "saturation" of the base with casein. Any additional casein thus migrating into the film in contact with the anode must be precipitated as uncombined casein. The cations, containing the potassium, migrate to the cathode and there react with water, liberating KOH, casein and hydrogen, the casein reacting with the excess of KOH to again form potassium caseinate and to again participate in carrying the current in each direction.

Hence the electrochemical equivalent which is actually measured in solutions of all reactions is that of casein at "saturation" of the base with the protein.

Rejecting the data obtained in the solutions *alkaline* to phenolphthalein, on account of the possible error arising from hydrolytic decomposition of the protein due to the excess of alkali, the average of all the determinations yields the value 0.0242 ± 0.0019 for the electrochemical equivalent of casein.

Multiplying this by the Faraday constant, 96,530, we obtain the weight of casein in grams which transports one atomic charge. This is 2336 ± 183.

Now at "saturation" of a base by casein, the proportion of base to casein is 11.4×10^{-5} equivalents per gram,* corresponding, if at this reaction casein combines with only one molecule of base, with the molecular weight of 8772. If we assume that at "saturation" of the base with casein two, three, or four, etc., molecules of base are bound up in one molecule of caseinate the molecular weight of the casein would be two, three, or four, etc., times 8772. Either of two assumptions may now be made:

(i) The potassium caseinate dissociates into potassium and casein ions. If this be the case then the weight of the casein anion must be that of the molecule of casein, i.e., a multiple of 8772, and the *valency* of the casein ions must be a multiple of

$$\frac{8772}{2336 \pm 183}$$

i.e., of 3.8 ± 0.3, or, in round numbers, 4.

(ii) The potassium caseinate dissociates into two protein ions of approximately equal weight. If this be the case then the weight of the casein anion must be half that of the molecule of

* Cf. Chap. V. Also T. Brailsford Robertson (24) (27).

casein, i.e., a multiple of 4386, and the *valency* of the casein ions must be a multiple of $\frac{4386}{2336 \pm 183}$, i.e., of 1.9 ± 0.15, or, in round numbers, 2.

Since, as we have seen in the earlier part of this chapter, the former of these two assumptions is inadmissible, we may conclude *that the valency of the casein ions, in solutions of a base "saturated" with casein, is a multiple of* 2. This obviously corresponds with the view that the caseinate dissociates into two protein ions in accordance with the scheme:

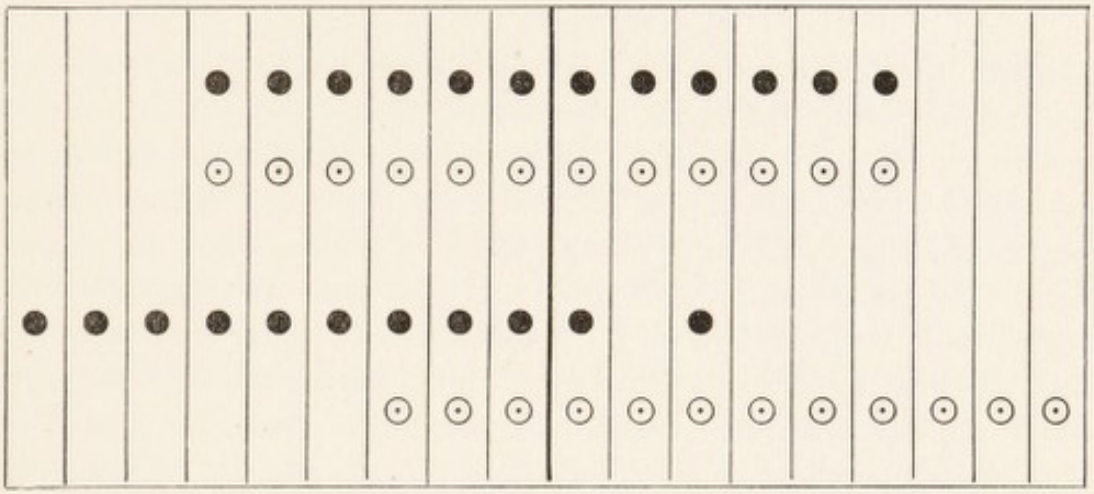
$$R_1.N = \underset{\underset{OH}{|}}{C.R_2} + KOH = \underset{\underset{OH}{|}}{\overset{\overset{H}{|}}{R_1N''}} + \overset{++}{K}OCR_2$$

3. The Relative Masses of Protein Anions and Cations. — From the above experimental results it appears that the masses of the protein cation and anion must be nearly equal, for otherwise the weight of one ion would not be one-half but some other fraction of the entire molecule and the valency, deduced from the above experimental data, would not be a whole number but some fraction. Since the valency of an ion is necessarily a whole number and not a fraction, the weights of the cations and anions which the protein molecule yields must be nearly equal. The above data do not enable us, however, to decide whether or not the weights of the cations and anions are *exactly* equal, since the precision attained in these experiments is not sufficient to reveal with certainty a difference of less than 10 per cent between the weights of the two ions.

It has been shown by Bredig (3) that the equivalent migration-velocities * of very heavy ions under unit potential gradient at constant temperature tend to approach a minimal constant value of about 20×10^{-5} cm. per sec. at 18° C. The conception developed above, therefore, of the mode of dissociation of the salts of a protein leads to the conclusion that the velocities of migrations of both the cation and the anion of a protein salt must be

* That is, the migration-velocity under unit force. For a divalent ion under unit potential gradient the force exerted is twice as great as that which is exerted on a univalent ion; the *absolute* velocity is, therefore, twice as great as that of a univalent ion, but the *equivalent* velocity is the same.

equal. Equal numbers of protein ions must therefore migrate to the anode and to the cathode respectively. Now since only one of these ions is precipitated it would appear possible to determine whether or not they are of equal weight by measuring the change in concentration at the anodal and cathodal regions. Employing Hittorf's method of representation, in the accompanying diagram let the region to the right represent the cathodal and that to the left the anodal region. Let the black spots represent anions and the white cations. The initial state of the solution is represented by the first double row of spots and its final condition, after the decomposition of six molecules by the current, by the second double row of spots.



Since the *velocities* of the ions are presumed to be equal, the number of undecomposed molecules of the original caseinate must, after the passage of the current, be the same on each side. The ions which have separated to the left (anions) have been precipitated while those which have migrated to the right have remained in solution. It is clear from the above diagram that on the right six anions have been lost and six cations have been gained. If they were equal in weight, therefore, it would appear as if the concentration of casein in the right (cathodal) half should remain unaltered by the passage of electricity. A moment's consideration, however, will suffice to show that this is not correct. In attaining this conclusion the assumption has been made that the casein cations after reaching the cathode, no longer participate in the carrying of current. Unquestionably this is not the case. As we shall see in the two succeeding chapters, the combining capacity of casein increases markedly with increasing

alkalinity of its solution, at first in direct proportion, each fresh equivalent of combined alkali splitting another $-N.HOC-$ union. Therefore the potassium or other metal which is transported along with the casein to the cathode, on being transformed there into the hydrate, will split the ion with which it has travelled into two, thus:

$$H_2N.R_1.COH.N.R_2.C\overset{++}{O}K + 2\,H_2O = H_2N.R_1.COH.NR_2.COOH + KOH + H_2$$

$$H_2N.R_1.COH.N.R_2.COOH + KOH = H_2N.R_1.C\overset{++}{O}K + \begin{array}{l} H \\ | \\ \diagdown N.R_2.COOH \\ | \\ OH \end{array}$$

the resultant cation may be presumed to be retained in the cathode region while the anion migrates to the anodal region. Each cation which the cathodal region gains, therefore, yields up, provided the masses of the cations and anions are in every case equal, one-half of its mass to the anodal region again. Referring to the diagram again, therefore, the true gain by the cathodal region will be only half of that represented, equal in weight to only three of the six ions which have been deposited on the anode, while its loss will have been three molecules, together equal to the total weight of casein deposited on the anode. Provided, therefore, the anions and cations in a solution of potassium caseinate are equal in weight, no matter what their *absolute* weight, then it is clear that *the loss of casein from the cathodal region must be exactly half the loss from the anodal region.*

I have endeavored to test this theoretical conclusion by determining the loss of casein from the anodal and cathodal limbs of the U-tube employed in the experiments described in the preceding section of this chapter. The following are some experimental results. The measurements of the amounts of casein were made by means of the refractometer, as described in the preceding section.

(1) Solution of 3.75 per cent casein in 0.03 *N* KOH; neutral to phenolphthalein.

Electrolysis for 2 hours at 30 degrees. Current approx. 1 milliampere.

Gram of casein lost from the anodal arm	0.062 ± 0.007
Gram of casein lost from the cathodal arm	0.036 ± 0.009
Ratio	1.9 ± 0.7

(2) Solution of 4 per cent casein in 0.02 *N* KOH; neutral to litmus.
Electrolysis for 2 hours at 30 degrees. Current approx. 1 milliampere.

Gram of casein lost from the anodal arm	0.093 ± 0.008
Gram of casein lost from the cathodal arm	0.045 ± 0.007
Ratio	2.15 ± 0.55

(3) Solution of 3.75 per cent casein in 0.03 *N* KOH; neutral to phenolphthalein.
Electrolysis for 2 hours at 30 degrees. Current approx. 1 milliampere.

Gram of casein lost from the anodal arm	0.070 ± 0.008
Gram of casein lost from the cathodal arm	0.045 ± 0.009
Ratio	1.65 ± 0.55

Within the experimental error, therefore, the ratio of the anodal to the cathodal loss is 2, as demanded by theory. The experimental error in determining the ratio is, however, large, as the above figures reveal, and although these results may be taken as confirmatory of the general correctness of the above outline of the mechanism of electrolysis in these solutions, yet they do not suffice to enable us to determine whether or not the protein anions and cations are absolutely equal in mass. Further elaboration and refinement in the technique of these measurements will doubtless enable us in the future, however, to measure the relative masses of the protein anions and cations with considerable precision.

In passing it may be pointed out that these results afford a striking confirmation of the view which I have developed above that the protein salts in solution in water do not yield protein and inorganic ions but only protein ions. Referring to the Hittorf diagram again it will be evident that if the cations were potassium ions the loss of casein from the anodal region should be at least four times that from the cathodal region, since the equivalent velocity of potassium ions is at least four times that of heavy organic ions. The experimental fact that the loss from the anodal region is only about twice as great as that from the cathodal region can only be interpreted by assuming that protein material is transported into the cathodal region by the current, *in other words, that the current is transported in both directions by protein ions.*

4. The Migration-Velocity of Protein Ions. — W. B. Hardy endeavored to measure the migration-velocities of serum-globulin ions directly (10) by placing solutions of serum-globulin, combined with acids or bases, at the bottom of a U-tube and

placing above the solutions, in both arms of the U-tube, a solution of the acid or base employed to dissolve the protein. A potential gradient was placed across the U-tube and it was observed that in acid solutions *both* boundaries of the protein solution migrated towards the cathode, while in alkaline solutions *both* boundaries of the protein solution migrated towards the anode. The rate of migration varied between 7 to 10×10^{-5} cm. per sec. for unit potential gradient when strong acids or bases (HCl and NaOH) were employed and about 20×10^{-5} cm. per sec. when a weak acid (acetic acid) was employed. Following the view which I have developed of the mode of dissociation of protein salts, it would appear as if the boundaries should move in *opposite* directions at approximately equal velocities. A moment's consideration of the method of measurement employed by Hardy shows, however, that under the conditions of his experiment this would not occur. At the boundary of the protein solution (nearly neutral) and the HCl solution (for example) a difference of potential of considerable magnitude would exist owing to the much more rapid diffusion of H^+ into the protein solution than of Cl'. This difference of potential would lower the potential gradient at the cathodal boundary and raise it at the anodal boundary. Bearing in mind the fact that the two protein ions are possessed of the same equivalent migration-velocities under unit potential fall, the effect of these inequalities in potential gradient must have been, at the anodal boundary, to urge the protein anions toward the anode more rapidly than the cations were repelled from the boundary, and, at the cathodal boundary, to repel the protein anions from the boundary into the protein solution more rapidly than the cations crossed the boundary. The net result of these processes would be, obviously, a migration of the protein, as a whole, towards the anode. The contact-differences of potential at the boundaries would be equal in magnitude but opposite in sense and hence both boundaries would migrate at the same velocity, but in the same direction. The fact that the highest velocities were obtained when weak acids (acetic) were employed, in which the difference between the equivalent velocities of the ions (H^+ and acetanion) is greatest, strongly supports this interpretation of Hardy's results. Alkali-globulin, in contact with alkaline solutions would, of course, move as a whole, but somewhat more slowly (since the

difference between the velocity of OH' and that of Na^+ is not so great as that between H^+ and Cl') towards the cathode. This accords with the experimental observations of Hardy. The velocities measured by Hardy, therefore, do not afford a measure of the migration-velocities of protein ions under a uniform potential gradient.

5. Objections to the above Theory of Protein Ionization. — Several objections to the hypothesis of protein ionization which has been presented above have been urged by Pauli, Samec and Strauss (22). These objections may be summarized as follows:

(i) The hypothesis involves the splitting in solution of a polyamino-acid into parts which are not amino-acids. Enzymatic splitting of a protein, if it took place in the same way, would not yield amino-acid end-products. This objection is really analogous to that which was originally advanced against Arrhenius' theory of the electrolytic dissociation of inorganic substances. It will be remembered that at the time that that hypothesis was first advanced, it was argued that the ionization of KCl into K^+ and Cl' would imply the presence of free potassium and chlorine in the solution, whereas the characteristic properties of these substances are not displayed by a solution of KCl. The answer to this was simply that the properties of an ion are not to be estimated in terms of those of a molecule and furthermore the ability of ions in a solution to display properties independent of those of the oppositely charged ion is limited by electrostatic forces. We may well suppose that protein ions, like other ions in solution, cannot react with other substances independently of the corresponding ion of opposite charge. In considering the reaction between proteins and water which leads to the decomposition of the polyamino-acid chains and is accelerated by enzymes we must consider the two ions of the protein molecule together, and not each ion separately. Undoubtedly the hydrolytic decomposition of a protein must involve the interaction of the protein ions with accompanying transfer of a labile hydrogen atom from the cation to the anion in accordance with the scheme:

$$-\underset{\underset{OH}{|}}{C}=N- + HO.H = -\underset{\underset{O}{\|}}{C}-OH + \overset{\overset{H}{|}}{N}-$$

The presence of a labile atom in a molecule leads, not unusually, to the development of color in solution, due to the absorption of light-vibrations by the vibrating atom. The development or non-development of absorption-bands in the visible or photographic spectrum will, of course, depend upon the ratio of the frequency of vibration of the labile atom to the frequency of the light-waves which impinge upon it. That the presence of absorption-bands in the visible spectrum is not an inevitable consequence of the presence within the molecule of a labile hydrogen atom is shown by the analogous instance of an equilibrium between the keto- and enol-forms in the colorless hydantoins (5).

(ii) The above-mentioned authors still consider that electrophoresis experiments show the presence of only one protein ion. Now this is unquestionably the reverse of the fact. While it is true that a superficial consideration of Hardy's results, alluded to in the preceding section of this chapter, might encourage such a supposition, we have seen that more careful analysis of the conditions of the experiment shows that his results admit of a very different interpretation, while the experiment cited at the end of section **3** showing that in solutions of casein in which the minimal valency of the casein ion, on the supposition that potassium caseinate yields only one protein ion, must be 4, the loss of protein in the cathodal arm during electrolysis is only one-half the loss in the anodal arm, is totally inconsistent with the view that protein, during electrolysis, migrates in only one direction.

Direct proof, however, of the simultaneous migration of protein to both poles under the influence of an electric current is fortunately available. Stirling and Brito (35) have shown that if a direct current be allowed to traverse a solution of hæmoglobin, deposition of crystals of hæmoglobin occurs at both electrodes. An alternating current is without effect. Howell (11) has furthermore shown that if a direct current be passed through a solution of fibrinogen, the fibrinogen increases in concentration at both poles although, as might be anticipated, the solutions of fibrinogen obtained from the neighborhood of the two poles differ in some particulars from one another in their behavior towards thrombin. Since hæmoglobin is a crystalline protein and fibrinogen, as Howell has recently shown, is converted by thrombin into a crystalline protein (fibrin), it cannot be urged that in these cases we are dealing with the opposite migrations of two distinct

and oppositely charged colloids. The phenomena can only be interpreted to mean that solutions of these proteins contain oppositely charged but otherwise similar moieties, i.e., oppositely charged protein ions.

(iii) Pauli, Samec and Strauss also urge the objection that under certain conditions (a low proportion of acid to protein) H^+ and Cl' may not be bound equally in solutions of protein in hydrochloric acid. This, however, does not really affect the question of the mode of ionization of the protein molecule but rather the question of the relative affinity of the nitrogen atom for H^+ and Cl' ions. It is not inconceivable that some measure of "Zwitter ion" or doubly and oppositely charged ion formation may occur in protein solutions (37). Yet this is not the normal, but rather the exceptional form of ionization and it is especially remarkable, and quite inconsistent with the view that proteins normally dissociate into protein and non-protein ions, that the inferiority of Cl' binding to H^+ binding is dependent solely upon the *proportion* of HCl to protein and not at all upon the *dilution* of the system, although the equivalent conductivity of protein solutions is very decidedly influenced by dilution (Cf. section **1**).

Perhaps the most obvious objection to the hypothesis which I have advanced is that a form of ionization involving the breaking of a double bond between carbon and nitrogen is very unusual and in fact without precedent in other fields of chemistry. It must be remembered, however, that as the experiments of Gomberg (7) have so beautifully and decisively shown, the precise point within a molecule at which ionization may occur is determined by the strains to which the molecule is subjected, and that when the strain is unusually great the break involved in ionization may occur at points which resist the tension due to strains of normal magnitude. Thus ethane, H_3C-CH_3, is a substance of remarkable stability, and not only ethane, but tetraphenyl ethane, $(C_6H_5)_2HC-CH(C_6H_5)_2$, and pentaphenyl ethane $(C_6H_5)_3C-CH(C_6H_5)_2$, are stable substances. Yet all attempts to prepare hexaphenylethane have failed for the reason that the compound breaks in the middle, the bond between the carbons, which we are accustomed to regard as one of the most stable types of union, being ruptured by the strain imposed upon it by the great weight of three phenyl groups at either end of the molecule. The rupture of this bond is all the more remarkable

because it involves the creation of a trivalent carbon atom and the independent existence of an unsaturated radical. Free radicals containing trivalent carbon have been prepared from compounds belonging to a wide variety of types, and, more recently the existence of divalent nitrogen has also been recognized. With facts such as these in mind the customary stability of any linkage in the simpler types of organic compounds no longer suffices as à priori evidence of the impossibility of its rupture in compounds of such a type as to expose it to strains of very unusual magnitude. Now the protein molecule weighing no less than from 10,000 to 20,000 and consisting of a large number of amino-acids linked together in long chains must evidently be subject to exceptional strains. Just as a long thin bar of material, no matter how great its strength, will, ultimately, if sufficiently long and unsupported, break of its own weight, so in the long chain of atoms composing a protein molecule, rupture of linkages may occur which are sufficiently strong to resist all the strains to which shorter or more symmetrical molecules are commonly subjected. It may of course be urged that this argument proves too much, since, by parity of reasoning, the split of the linkage between carbon and nitrogen should occur at all times and not be dependent upon salt-formation, the additional strain imposed by the weight of a molecule of hydrochloric acid or sodium hydroxide being negligible in comparison with the total strain to which the molecule is subjected. It must be remembered, however, that the additional strains which a molecule of acid or base introduces into the molecule are not merely those commensurate with and attributable to its weight, but also strains of electrostatic origin, since the salt which is formed unquestionably undergoes ionization. It may very possibly be true that the first step in salt formation consists in the neutralization of end $-NH_2$ or $-COOH$ groups, but that the ionization of the compound formed, leading to the development of electrostatic tension at the very places at which it must exert the greatest strain, namely the extremities of the molecule, results in the splitting of the otherwise stable linkage $-\overset{|}{C}=N-$ and the redistribution of the components of the molecule and the strains to which it is subjected.

6. Biological Applications; the "Selective" Action of Living Tissues. — The non-dissociable character of the inorganic con-

stituent of the protein salts affords, I believe, an explanation of an important physiological phenomenon; I refer to the well-known power which living tissues possess of "selecting" or storing up certain inorganic constituents in a concentration greater than that in which these substances are found in the surrounding liquid medium (23) (25) (26). Thus the skeletal muscles and the red blood corpuscles contain a marked excess of potassium over sodium, while the plasma which bathes them contains much less potassium than sodium. Again, although in fresh-water streams the concentration of potassium salts is often very low, the plants which live in them are capable of storing up a comparatively large amount of potassium in their tissues.

If we place within a dialyser an excess of diffusible potassium salts over diffusible sodium salts and dialyse against a solution containing excess of diffusible sodium salts, the proportions of sodium to potassium within and without the dialyser sooner or later readjust themselves, approaching equality. Hence the above-mentioned phenomenon, which is met with in living tissues, admits, as Loeb has pointed out (14) of only one explanation, — the inorganic constituents of a tissue which are found therein in excess of their concentration in the fluids which bathe it must exist within the tissue in the form of non-dissociated non-diffusible compounds. "If a tissue utilizes one kind of metal in this way, for example K, while another metal, for example Na, is chiefly used for the formation of dissociable compounds with Na as the free ion, the consequence will be that the ashes of the tissue contain K and Na in altogether different proportions from those in which they are contained in the surrounding solution. I think we may take it for granted that, at least, potassium forms a non-dissociable constituent of the protoplasm of a number of tissues of animals and plants" (Loeb. loc. cit.).

If we admit that, as many investigators now believe (13) (14) (32) (26), the inorganic constituents of living tissues are partly united with proteins, the fact that such unions dissociate only into protein and not into protein and inorganic ions affords a sufficient explanation of the above phenomenon.

Loeb (13) (14) and W. A. Osborne (21) have advanced an analogous explanation of the "oligodynamic" (16) (19) action of many highly toxic heavy metals.

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CHAPTER IX

THE COMBINING CAPACITY OF THE PROTEINS

1. The Electrochemical Determination of the Combining Capacity of the Proteins. — The potentiometric method was employed by Bugarszky and Liebermann (5) for the purpose of demonstrating that proteins possess a true combining capacity for acids and bases. I have utilized this method in determining the relationship of the combining capacity of the proteins to the alkalinity or acidity as well as the absolute concentration of their solutions (54) (59) (60). In brief, the principles upon which the method depends are as follows:*

When a metal or hydrogen is brought into contact with a liquid a certain amount of the metal tends to pass over into the liquid and it strives to do so with a certain measurable and characteristic force, analogous to gas- or osmotic-pressure, which Nernst (47) (48) has termed the solution pressure. The particles which this pressure tends to bring into solution are all, in the case of the metals and hydrogen, charged electropositively. The number of these particles which actually pass into solution, provided no current is allowed to traverse the liquid and the metal, and no *chemical* work is performed, must be very small and, in proportion to weighable qualities, quite evanescent; for as the positively charged particles pass out into the liquid an electrostatic tension is necessarily developed. The film of liquid which is in immediate contact with the metal becomes positively charged; the metal, having lost positive charges, acquires a corresponding negative charge, and the force driving fresh metal or hydrogen ions into the liquid becomes balanced by the electrostatic repulsion of the positive ions, from the film of liquid which is in contact with the metal into the surface of the metal again. Hence there is quickly developed a certain constant difference of electric potential be-

* For a fuller discussion of these principles and of the forms of apparatus used the reader is referred to standard works of general physical chemistry (21) (41) (64) (67).

tween the liquid (charged positively) and the metal (charged negatively) which is characteristic for each metal and each liquid.

The force which tends to drive the metal ions back into the metal again is the osmotic pressure of the dissolved ions. If, through the introduction of a dissociable salt of the metal, the concentration (osmotic pressure) of the metal ions in the liquid is increased, this may equal or even exceed the solution pressure of the metal itself. In the former case the potential difference between the metal and the solution is abolished; in the latter it is renewed again, but in the opposite sense, the metal being now charged positively and the solution negatively.

If, now, we build up a galvanic element as follows: Metallic silver in contact with dilute $AgNO_3$, in conducting communication with strong $AgNO_3$, the latter solution being again in contact with metallic silver, it is evident that there must be a difference in potential between the two portions of metallic silver, a potential which will be greater the greater the difference between the concentrations of the two silver nitrate solutions. Provided the potential between the two silver nitrate solutions themselves (due to the unequal migration-velocities of silver and NO_3 ions) can be neglected or made to vanish (and we will shortly explain how this may be approximately achieved), it has been shown by Nernst that the potential of this "concentration chain" can be expressed by the formula:

$$\pi = \frac{RT}{F\eta} \log_{\text{nat.}} \frac{C_2}{C_1}$$

where R is the gas constant, T the absolute temperature, F the Faraday constant, η the valency of the ion (in this case 1), C_2 the concentration of the stronger solution of silver nitrate, C_1 that of the weaker and π the potential in volts.

In this way the absolute concentrations of two different solutions of an ion can be accurately computed, provided only a minute amount of current be allowed to pass during the potential determination, without any significant alteration in the composition of the solutions. The measurement is, in other words, static; and static methods of measurement must be employed in determining the combining capacities of proteins because the combining capacity of proteins is not possessed of one fixed value but of fixed *minimum* and *maximum* values and, between these

intermediate values which are determined by the alkalinity or acidity (H^+ concentration) of their solutions.

In applying this method to the comparison of the acidity or alkalinity (H^+ concentration) of solutions the terminal electrodes must be hydrogen. A hydrogen electrode can be obtained by employing a saturated solution of hydrogen in a metal, e.g., platinum. In order to obtain a large surface, platinum gauze coated with platinum black is usually employed and a stream of pure hydrogen is passed over or through the gauze. The potential between the two acid or alkaline solutions themselves (as distinguished from that between the hydrogen electrodes and these solutions) may be abolished or at any rate greatly diminished by connecting them by means of a U-tube filled with agar saturated with KCl (3) (1).

I have utilized this method in determining the combining capacities of casein and of ovomucoid for acids or bases in solutions of varying acidity and alkalinity (59) (60). For further details regarding the technique employed and the precautions to be taken in carrying out such determinations in solutions which contain proteins, the reader is referred to the appendix.

In the following tables, which enumerate the results of these experiments, the symbols which are employed have the following significance:

$b_1 \equiv$ The concentration of a solution of base in which protein is dissolved.

$a_1 \equiv$ The concentration of a solution of acid in which protein is dissolved.

$\pi \equiv$ The potential of the chain:

$$H_2 \left| \begin{array}{c} \text{acid or base} \\ \text{plus protein} \end{array} \right| \begin{array}{c} \text{acid or base} \\ \text{alone} \end{array} \left| H_2 \right.$$

in volts. At 30 degrees, with monacid bases or monobasic acids

$$= 0.0601 \log_{10} \frac{b_1}{b} \quad \text{or} \quad 0.0601 \log_{10} \frac{a_1}{a}.$$

$b \equiv$ The hydroxyl concentration (unneutralized base) in the solution of base containing protein.

$a \equiv$ The hydrogen concentration (unneutralized acid) in the solution of acid containing protein.

$m \equiv b_1 - b$ or $a_1 - a =$ the concentration of base or acid neutralized by the protein.

To all the solutions employed a small amount of KCl was added. This is necessitated in the preparation of the casein solutions by technical difficulties of which an account will be found in the appendix, but it serves also to confer upon the solutions a higher conductivity and thus to render more easy the detection of the zero point upon the potentiometer bridge wire by means of a galvanometer. So far as the solutions of casein in KOH are concerned, the presence of KCl in the concentrations employed evidently did not appreciably affect the combining capacity of the protein for the base, since, as the tables reveal, in solutions of very different KCl-content (0.015 N to 0.030 N) the combining capacity at absolute neutrality proved to be the same. In the tables the total amount of potassium present in the solvent as KOH or KCl, is designated by the symbol r.

All of these determinations were made at 30° C.

0.5 PER CENT CASEIN DISSOLVED IN KOH SOLUTIONS
(r = 0.020)

b_1	π	b	m
0.02000	0.0160	1.083×10^{-2}	0.00917
0.01000	0.0462	1.701×10^{-3}	0.00830
0.00750	0.0708	4.987×10^{-4}	0.00700
0.00500	0.1181	5.429×10^{-5}	0.00495
0.00250	0.2600	1.181×10^{-7}	0.00250
0.00150	0.2710	4.648×10^{-8}	0.00150

1.0 PER CENT CASEIN IN KOH SOLUTIONS (FIRST SERIES)

b_1	π	b	m	r	Remarks
0.03028	0.0239	1.213×10^{-2}	0.01815	0.03028	
0.02907	0.0273	1.021×10^{-2}	0.01886	0.03028	
0.02422	0.0320	7.111×10^{-3}	0.01711	0.02422	
0.01817	0.0538	2.314×10^{-3}	0.01586	0.01817	
0.01212	0.0937	3.351×10^{-4}	0.01178	0.01817	
0.01091	0.1042	2.015×10^{-4}	0.01071	0.01817	
0.00970	0.1332	5.907×10^{-5}	0.00964	0.01817	
0.00849	0.1736	1.096×10^{-5}	0.00848	0.01817	
0.00728	0.2276	1.191×10^{-6}	0.00728	0.01817	
0.00607	0.2542	3.583×10^{-7}	0.00607	0.01817	
0.00486	0.2758	1.251×10^{-7}	0.00486	0.01817	Av. value of
0.00486	0.2753	1.276×10^{-7}	0.00486	0.01817	π=0.2756
0.00365	0.3034	3.265×10^{-8}	0.00365	0.01817	
0.00244	0.3130	1.514×10^{-8}	0.00244	0.01817	

1.0 PER CENT CASEIN KOH SOLUTIONS (SECOND SERIES)
($r = 0.030$)

b_1	x	b	m
0.03000	0.0231	1.237×10^{-2}	0.01763
0.02500	0 0313	7.523×10^{-3}	0.01748
0.02000	0 0471	3.292×10^{-3}	0.01671
0 01750	0 0603	1.736×10^{-3}	0.01576
0 01500	0.0741	8.782×10^{-4}	0.01412
0.01250	0 0954	$3.233\times1[illegible]^{-4}$	0.01218
0.01000	0.1299	6.892×10^{-5}	0.00993
0 00750	0.2265	1.276×10^{-6}	0.00750
0 00500	0.2596	2.398×10^{-7}	0.00500
0.00250	0 2903	3.699×10^{-8}	0.00250

1.5 PER CENT CASEIN IN KOH SOLUTIONS ($r = 0.030$)

b_1	x	b	m
0 03000	0.0439	5.578×10^{-3}	0.02442
0 02000	0.0881	6.842×10^{-4}	0 01932
0 01500	0.1280	1.113×10^{-4}	0.01489
0.01000	0.2523	6.337×10^{-7}	0.01000
0.00750	0.2897	1.136×10^{-7}	0.00750
0.00500	0.3128	3.119×10^{-8}	0.00500

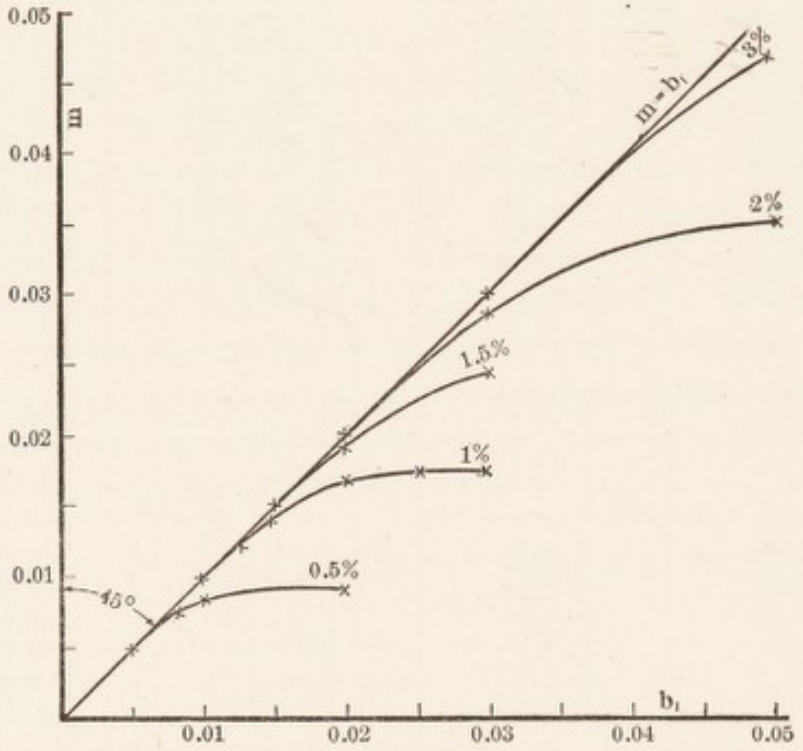
2.0 PER CENT CASEIN IN KOH SOLUTIONS

b_1	x	b	m	r
0.05000	0.0311	1.522×10^{-2}	0.03478	0.050
0.03000	0.0778	1.525×10^{-3}	0.02847	0.030
0.02000	0.1343	1.167×10^{-4}	0.01988	0.040
0.01500	0.2425	1.383×10^{-6}	0.01500	0.040
0.01000	0.3000	1.020×10^{-7}	0.01000	0.040
0 00500	0.3152	2.846×10^{-8}	0.00500	0.040

3.0 PER CENT CASEIN IN KOH SOLUTIONS

b_1	x	b	m	r	
0.05000	0.0733	3.011×10^{-3}	0.04699	0.050	Av.value of
0.05000	0.0739	2.943×10^{-3}	0.04706	0.050	$m=0.04703$
0.03000	0.1438	1.217×10^{-4}	0.02988	0.040	
0.02500	0.2343	3.156×10^{-6}	0.02500	0.040	
0.02000	0.2823	4.017×10^{-7}	0.02000	0.040	
0 01500	0.3172	7.920×10^{-8}	0.01500	0.040	
0 01000	0.3413	2.096×10^{-8}	0.01000	0.040	

The relation between m, the amount of alkali neutralized by the casein, and b_1, the alkalinity of the solution in which the casein was dissolved, is shown graphically, for all of the concentrations of casein employed, in the accompanying figure:



The ordinates = m = the concentration of KOH neutralized by 0.5%, 1%, 1.5%, 2.0% and 3% casein.
The abscissæ = b_1 = the concentration of the KOH solution in which the casein was dissolved.

It will be understood, of course, that the curves only represent this relation for the alkalinities of the original solutions in excess of that necessary to dissolve all of the casein. For an ordinary acid, forming only one salt with the base, which did not undergo hydrolytic dissociation, the curve would, of course, be a straight line parallel with the horizontal axis. It will be seen that as the proportion of base to casein declines, the combining capacity of the casein tends to become directly proportional to the concentration of the base, but that as the proportion of base to casein (and the excess of unneutralized base) becomes large, the combining capacity of the casein tends towards constancy, i.e., in comparatively strongly alkaline solutions the behavior of casein approximates more and more to that of an ordinary acid.

These results are to be interpreted as follows: When just enough alkali ($= 11.4 \times 10^{-5}$ equivalents per gram, Cf. Chap. V)

has reacted with casein to dissolve it, we may suppose that one $-N.HOC-$ group (or two such groups, if dicarboxylic radicals are involved) in each molecule of casein has been opened up, in accordance with the equation:

$$-N.HOC- + KOH = - \overset{H}{\underset{OH}{\overset{|}{\underset{|}{\diagdown N}}}} + \overset{++}{K}OC-$$

From the Guldberg and Waage mass law we know that if this equation represents the true nature of the reaction which occurs, then the mass of caseinate formed must be dependent (the temperature and other conditions of the reaction being constant) only upon the reacting masses of free casein and KOH and *not upon the total dilution of the system.* At the completion of the reaction these masses are both very small, since free casein is inappreciably soluble in water and the solution of this salt of casein is *acid* in reaction (Cf. Chap. V). Upon further addition of alkali, however, another $-N.HOC-$ group (or pair of groups) is opened up and when another 11.4×10^{-5} equivalents of alkali have been added per gram of casein we have now four ions of caseinate instead of two. Whether these ions are derived from one, or from two, molecules of caseinate these data do not enable us to decide. As the tables show, the solution of this salt is also acid to litmus. Upon still further addition of alkali yet another $-N.HOC-$ group (or pair of groups) in each casein molecule is opened up, and when 22.8×10^{-5} more equivalents of KOH ($= 45.6 \times 10^{-5}$ per gram in all) have been added all of the ions of caseinate have again been split in half. The solution of this salt is, as we shall see, almost exactly neutral to litmus. Again doubling the alkali-content the ions will be split again, yielding a salt containing 91.2×10^{-5} equivalents of KOH per gram, the solution of which, as the above tables reveal, is alkaline to phenolphthalein (62) and which, therefore, presumably, requires the presence of a slight excess of KOH to maintain its stability, i.e., to push the equilibrium in the above reaction sufficiently over to the right. We may suppose that this type of reaction is repeated until the last $-N.HOC-$ group that is dissociable by alkali has been opened up, when the combining capacity of the casein reaches, as we have seen, a constant maximal value. This maximum value of the combining capacity must necessarily, if

the above picture of the chain of events is correct, be an *even* multiple (1, 2, 4, 8, 16, etc.) of the quantity of alkali which is just sufficient to form the first salt, i.e., to carry the casein into solution. By interpolation from the above tables and by continuing the curves it is seen that *for all of the percentages of casein employed* the constant maximal value of the combining capacity for KOH is, as nearly as can be estimated, 180×10^{-5} equivalents per gram. Now $\frac{180}{11.4} = 15.8$ or, within the error of estimation, 16. The experimental results are therefore in good agreement with the theory and we may conclude that casein forms the following salts with KOH:

I	containing	11.4×10^{-5}	equivalents	of	KOH	per gram of casein
II	"	22.8×10^{-5}	"	"	"	"
III	"	45.6×10^{-5}	"	"	"	"
IV	"	91.2×10^{-5}	"	"	"	"
V	"	182.4×10^{-5}	"	"	"	"

In forming the last of these salts 16 $-$N.HOC$-$ groups (or a multiple of this number) have been opened up.

Similar experiments were carried out upon solutions of ovomucoid in dilute acid and alkali; they are tabulated below:

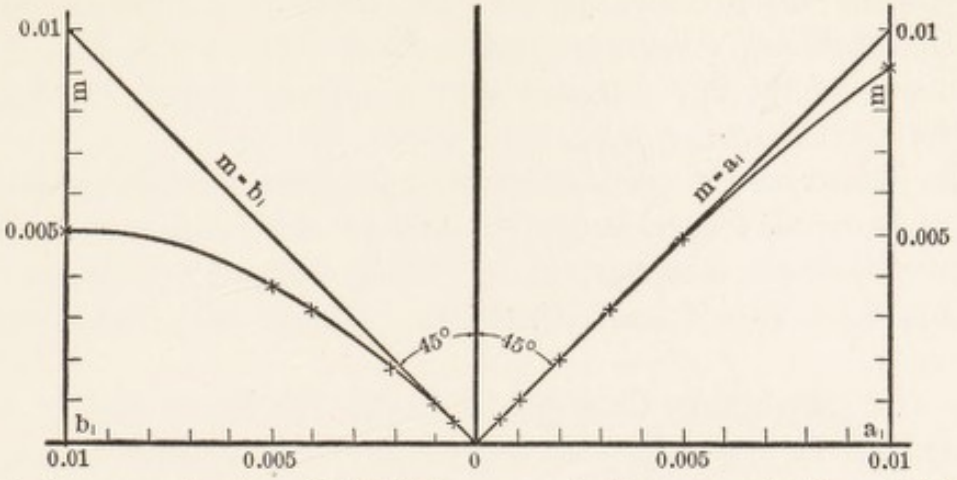
1 PER CENT OVOMUCOID IN KOH SOLUTIONS
(KCl in each solution = 0.01 N)

b_1	π	b	m
0.0005	0.0755	2.77×10^{-5}	0.00047
0.0010	0.0475	1.62×10^{-4}	0.00084
0.0020	0.0500	2.95×10^{-4}	0.00171
0.0030	0.0367	7.34×10^{-4}	0.00227
0.0050	0.0356	1.28×10^{-3}	0.00372
0.0100	0.0185	4.92×10^{-3}	0.00508

1 PER CENT OVOMUCOID IN HCL SOLUTIONS
(KCl in each solution = 0.01 N)

a_1	π	a	m
6.95×10^{-6}	0.1087	1.08×10^{-7}	6.84×10^{-6}
0.0005	0.2261	8.65×10^{-8}	0.00050
0.0010	0.2107	3.12×10^{-7}	0.00100
0.0020	0.1726	2.69×10^{-6}	0.00198
0.0030	0.1492	9.88×10^{-6}	0.00299
0.0050	0.1190	5.24×10^{-5}	0.00495
0.0100	0.0610	9.66×10^{-4}	0.00903

The results are shown graphically in the accompanying figure:



b_1 is the concentration of KOH in which 1 per cent ovomucoid is dissolved; m is the number of gram-equivalents of KOH which is neutralized per litre.

a_1 is the concentration of HCl in which 1 per cent ovomucoid is dissolved; m is the number of gram-equivalents of HCl which is neutralized per litre.

It is obvious that the relationship between the amount of acid or base neutralized by the ovomucoid and the total amount of base present is of very much the same general nature as the corresponding relationship for solutions of casein in KOH— solutions. Ovomucoid, however, differs very strikingly from casein in the fact that it is predominantly basic, while casein is predominantly acid. The maximum combining capacity of ovomucoid for KOH is obviously about 50×10^{-5} equivalents per gram, but its maximum combining capacity for acids was not attained in any of the solutions investigated and must be at least 100×10^{-5} equivalents per gram. Since free ovomucoid, unlike free casein, is soluble in water the quantity of a base or acid which enters into one —N.HOC— group cannot be estimated from these data alone, but it is evident that it cannot be in excess of 50×10^{-5} per gram of protein.

The combining capacities of serum albumin, gelatin and deaminized gelatin for acids have been determined electrometrically by Pauli and Hirschfeld (49). They find that, as in the case of casein, the *maximum* combining capacities of these proteins are independent of their dilution and, moreover, that in isohydric solutions acetic acid, despite the fact that it yields a much inferior proportion of hydrogen ions, is bound by proteins in greater proportion than hydrochloric acid, a fact which in

itself speaks very strongly in favor of the view that the protein compounds which are formed do not yield, by electrolytic dissociation, the corresponding acid anions. They, moreover, find that the combining capacity of deaminized gelatin is hardly inferior to that of normal gelatin.

The electrometric method of measuring the combining capacities of proteins for acids and bases has also been employed by D'Agostino and Quagliariello (2), Rohonyi (61), Manabe and Matula (38), Blasel and Matula (4), Ringer (52) and Schmidt (63) (64).

2. The Combining Capacity of Casein for Bases and of Ovomucoid for HCl at Absolute Neutrality. — A number of investigators have shown (66) (34) (71), by direct titration to neutrality to litmus, that the combining capacity of casein at absolute neutrality is constant, that is, independent of the total dilution of the system. The potentiometric determinations, cited in the above tables, enable us to confirm these observations. It will be recollected that the OH′ concentration in the solutions containing casein was determined by measuring the potential between two hydrogen electrodes, the one dipped in a solution containing a given concentration of casein, the other in an exactly similar solution to which no casein had been added. Plotting a curve in which the reaction of the solutions containing no casein form the abscissæ and the potentials between the two solutions the ordinates, the reactions ($= x$) of the solution to which the given concentration of casein had to be added in order to procure an exactly neutral solution * is given by the intersection of this curve with the curve defined by the formula:

$$y = 0.4107 - 0.0601 \log_{10} x.$$

The points of intersection of these curves may be found, with sufficient approach to accuracy, in the following way: The values of y in the above curve corresponding to x (alkalinity of the solution containing no casein) $= 0.0025$; 0.005; 0.0075; 0.010; 0.015 and 0.020 are computed and these points are marked upon accurately ruled "squared paper" and joined by straight lines. Experimental values of π lying upon each side of its value at neutrality, in each solution, are then also marked off upon the

* Taking the H^+ concentration at absolute neutrality at 30 degrees as 1.47×10^{-7}; cf. Kohlrauch and Heydweiller (33).

paper and joined by straight lines. The abscissa of the point of intersection of the two broken curves thus drawn yields the number of gram equivalents of KOH which are bound by the given percentage of casein in the production of an absolutely neutral solution.* Dividing the number of gram equivalents of KOH neutralized by the casein by the percentage concentration of the casein we obtain the number of gram equivalents of KOH neutralized by 1 gram of casein at absolute neutrality. The following are the results computed from the data tabulated above:

Concentration of casein, per cent	Gram equiv. of KOH neutralized by 1 gram of casein at absolute neutrality at 30 degrees
0.5	52×10^{-5}
1.0 (first series)	50×10^{-5}
1.0 (second series)	43×10^{-5}
1.5	53×10^{-5}
2.0	54×10^{-5}
3.0	56×10^{-5}
	Average 51×10^{-5}

The values of the combining capacities of the casein at neutrality to litmus computed in this manner are seen to be appreciably constant. The average value is 51×10^{-5} equivalents per gram of casein, which is in excellent agreement with that obtained by titration, namely, 50×10^{-5} equivalent gram molecules per gram.

Solutions of ovomucoid require the addition of a small quantity of *acid* to obtain an absolutely neutral solution.† The amount

* It was at first thought necessary to pass the curve $y = ax^3 + bx^2 + cx + d$, through four of the points of the experimental curve $\pi = f(b_1)$ and to determine algebraically the point of intersection of this curve with the curve $y = 4107 - 0.060 \log_{10} x$; this was, however, found to be an unnecessary refinement, as the results obtained did not differ appreciably from those obtained by the simpler method described above.

† In this connection attention may be called to the fact, which is revealed in the above tables, that when 1 per cent ovomucoid is dissolved in 6.95×10^{-6} N acid, although the ovomucoid neutralizes a little of this acid, yet this solution is actually *more* acid than the solution which is obtained by dissolving 1 per cent ovomucoid in 0.0005 N HCl. A similar phenomenon was observed by W. B. Hardy in solutions of serum globulin (22). It is possible that this indicates a slight decomposition of the salt which was present in these solutions by the protein, or, more probably that it is due to a small proportion of the *free* protein ionizing according to the equation $R.COOH = RCOO' + H^+$.

of HCl bound by ovomucoid at absolute neutrality, estimated in the above manner, is 7×10^{-5} equivalents per gram.

3. The Non-Dependence of the Composition of the Compounds of Protein with Acids and Bases upon the Dilution of their Solutions. — The view would appear to be very generally held (35) (9) (51) that the salts which proteins form with acids and bases are subject in a very high measure to hydrolytic dissociation. This view is nevertheless erroneous. When a base unites with an organic acid to form a salt by the neutralization of a $-COOH$ group the reaction may be expressed as follows:

$$R.COOH + KOH \rightleftarrows RCOOK + H.OH.$$

If the acid is a very strong one then the reaction will proceed completely from left to right and the salt will not be appreciably decomposed by dilution. If, however, the acid is tolerably weak (e.g., acetic acid) so that the water itself, acting as an acid, is able, if present in large amounts, to displace it partially from its combination with the base, then the reaction will not proceed completely from left to right but will pause, in accordance with the mass law, in a condition of balance, which is shifted towards the right by the addition of excess of base and towards the left by dilution. On adding excess of base to a dilute solution of an acid of this type, therefore, more of the base will be observed to be neutralized, while the addition of water will result in partial decomposition of the salt. Similar considerations hold good, of course, for the salts of weak bases. When one examines the evidence which has been brought forward by different observers in support of the thesis that protein salts are subject to hydrolytic dissociation one finds that it is all of the first kind, that is, consists in the fact that upon the addition of more acid or base to the protein solution, or of more protein, more of the acid or base is bound. From this the somewhat illogical assumption has been made that the protein salts must exhibit the other characteristic property of salts of weak acids and bases, namely, decomposability by water. We have, however, seen that the fact that protein will bind more of an acid or a base in the presence of an

This mode of dissociation would, of course, be prevented by dissociation of the molecule through its $-N.HOC-$ groups, and, since this is brought about by the addition of acid, the addition of small quantities of acid might well *decrease* the acidity (H^+ concentration) of the solution.

excess of these reagents admits of a very different explanation, and the data cited above also enable us to conclude that water is not able to appreciably decompose the potassium salt of casein.

We have seen that whether the percentage of casein in solution is 0.5 or 3.0 or intermediate between these, the quantity of potassium hydrate which is bound by a given weight of casein at absolute neutrality is the same. The dilution (relative mass of water to salt) may vary 600 per cent and yet the salt remains unaltered; it is not decomposed by the water in any preceptible degree. This result has also been obtained for other bases, including comparatively weak bases such as $Ca(OH)_2$, by the observers, cited in the previous section, who have employed the method of direct titration and these observers have also shown that at neutrality to phenolphthalein the composition of the casein salt ($= 80 \times 10^{-5}$ equivalents of base per gram) is always the same no matter what the dilution of the system. Moreover, as the curves in the figure on p. 200 clearly reveal, the composition of the casein salt, when the casein is exerting its maximum combining-capacity, is the same for very different concentrations of this salt. That this should be so in alkaline solution is not surprising, since the excess of alkali might be expected to drive the reaction:

$$R.COOH + KOH \rightleftarrows RCOOK + H.OH$$

over towards the right; but that it is so in absolutely neutral solution, when the concentration of free KOH is evanescently small, is an extremely striking fact.

But, not only is the composition of the caseinate of potassium independent of its dilution in *neutral* solution, it is also independent of its dilution in a solution which is pronouncedly acid in reaction. It will be shown in the succeeding chapter that the amount of a base which is bound by casein at "saturation" of the base with casein, that is, when there is just sufficient base to hold the casein in solution is also independent of the dilution of the system. Now in this solution the acidity is about 10^{-5} H^+ (cf. the table of reactions to indicators on p. 91). A glance at the formula

$$R.COOH + KOH \rightleftarrows R.COOK + H.OH$$

suffices to show that in acid solutions, when the KOH concentration is excessively small and the H^+ concentration large the

tendency to hydrolysis of a salt of the type R.COOK must be exceptionally great, yet potassium caseinate does not reveal this tendency, and the conclusion is forced upon us that potassium caseinate is not formed in accordance with an equation of the above type.

Reverting, now, to the hypothesis which I have advanced regarding the mode of formation of protein salts, we shall see that in the equation:

$$-\mathrm{N.HOC}- + \mathrm{KOH} \rightleftarrows \begin{matrix} \mathrm{H} \\ | \\ \mathrm{N} \\ | \\ \mathrm{OH} \end{matrix} \!\!\diagup\!\!\!\diagup - + \overset{++}{\mathrm{K}}\mathrm{OC}-$$

no water is involved, and, consequently, *the composition of the salt must be dependent only upon the relative concentrations of protein and base and not upon the total dilution.* Similarly in the formation of salts with acids:

$$-\mathrm{N.HOC}- + \mathrm{HCl} \rightleftarrows -\begin{matrix} \mathrm{H} \\ | \\ \mathrm{N} \\ | \\ \mathrm{Cl} \end{matrix} \!\!\diagup\!\!\!\diagup + \overset{++}{\mathrm{H}}\mathrm{OC}-$$

no water enters into the reaction. Hence both the dependence of the composition of the salt upon the excess of acid or base and its non-dependence upon dilution receive a simple interpretation in the light of this hypothesis.

It will I think be clear from the foregoing discussion, that the non-dependence of the composition of protein salts upon their dilution is one of the most emphatic proofs we possess of the fact that terminal $-\mathrm{COOH}$ and $-\mathrm{NH_2}$ groups are not responsible for their formation.

An experiment which demonstrates in a very striking manner the fact that protein salts do not undergo an appreciable amount of hydrolytic dissociation in solution in water, nor split off the inorganic radical as an ion, is the following. It will be recollected that casein, deprived of its combined base or acid, is insoluble, and that if, to a solution of a caseinate of a base, exactly enough free acid be added (e.g., HCl) to completely neutralize the combined base the free casein is entirely precipitated. Now one gram of ovomucoid (60) combines with 45×10^{-5} equivalents of HCl to form

a compound such that less than 1 per cent of the acid remains uncombined (as estimated by gas-chain measurements). One gram of casein combines with 90×10^{-5} equivalents of KOH to form a compound such that less than $\frac{1}{2}$ per cent of the KOH remains uncombined (59). If, now, these salts were appreciably subject to hydrolytic dissociation, or even if they yielded Cl′ and H^+ ions respectively, then on mixing two volumes of a solution of the ovomucoid salt with one volume of a solution of the casein salt (each of the same percentage concentration) the K^+ provided by the caseinate would be exactly neutralized by the Cl′ provided by the ovomucoid salt and it might be anticipated that free uncombined casein would be precipitated. Nothing of the sort occurs, however. If to 25 cc. of a 2 per cent solution of the casein salt are added 50 cc. of a 2 per cent solution of the ovomucoid salt the mixture is no more opalescent than its constituent parts and *the conductivity of the mixture is the sum of the separate conductivities of the two protein salts.** If the mixture be allowed to stand in the presence of toluol for a considerable period at 36 degrees, however, after 24 to 45 hours a marked increase in its opalescence is observed; after two or three days traces of casein begin to be deposited, and after three to four days all of the casein is found to have been precipitated. The precipitation of the casein is accompanied by a marked increase in the conductivity of the mixture, attributable to the setting free of KCl. It is therefore evident that at the beginning the mixture must contain only minute traces of K^+ and Cl′ ions and that the protein salts only yield up these ions with extreme slowness.

4. The "Isoelectric" Condition of Proteins at Certain H^+ and OH′ Concentrations. — If we assume that the proteins dissociate H^+ and OH′ ions according to the formula:

$$R\begin{cases}NH_3OH\\COOH\end{cases} \rightleftarrows R\begin{cases}NH_3OH\\COO'\end{cases} + H^+$$

and

$$R\begin{cases}NH_3OH\\COOH\end{cases} \rightleftarrows R\begin{cases}NH_3^+\\COOH\end{cases} + OH'$$

* Conductivity of a 0.5 per cent solution of potassium caseinate containing 90×10^{-5} equivalents of KOH per gram at 30 degrees equals 38.0×10^{-5} reciprocal ohms. Conductivity of a 1 per cent solution of ovomucoid chloride

then the dissociation of H^+ ions must be inhibited and that of OH' ions encouraged by the presence of an excess of H^+ ions, while that of OH' must be inhibited and of H^+ ions encouraged by an excess of OH' ions, and a reaction must exist at which H^+ and OH' are split off in exactly equal quantities so that the salt is "isoelectric," i.e., wanders equally in both directions in an electric field. A simple calculation suffices (53) to show that if the mode of dissociation of the protein is actually that represented by the above formulæ, then the number of protein ions in a solution of proteins must attain a minimum in solutions in which it is "isoelectric."

A number of observations, to which more detailed reference will be made in a later chapter, tend to establish the fact that the viscosity of protein solutions is in the greater proportion attributable to its ions, and that, consequently, when the ionization of a protein is at a minimum the viscosity of its solution will tend to a minimum also.

Applying these considerations, and also employing direct observation of the movement of the protein in an electric field, Michaelis and co-workers (40) (41) (42) (43) (44) (45) have endeavored to ascertain the hydrogen ion concentration of the solution in which egg-albumin is isoelectric.

In the light of the view which I have developed concerning the mode of formation and dissociation of the protein salts a somewhat different interpretation must be placed upon these results. A glance at the figure on p. 185 will show that if the protein is soluble in the free condition and uncombined with bases or acids it must, if it is ionized at all, migrate equally in both directions through the solution under the influence of an electric current, reacting at the electrodes as follows:

$$\text{(Anode)}\ 2\,\text{HOOC.R.}\underset{\displaystyle \text{OH}}{\overset{\displaystyle \text{H}}{\text{N}}}\!\!\diagdown = 2\,\text{HOOC.R.NH}_2 + \text{O}_2$$

$$\text{(Cathode)}\ \text{H}_2\text{N.R.}\overset{++}{\text{C}}\text{OH} + \text{H}_2\text{O} = \text{H}_2\text{N.R.COOH} + \text{H}_2$$

containing 45×10^{-5} equivalents of HCl per gram at 30 degrees equals 80.1×10^{-5} reciprocal ohms. Sum of these conductivities equals 118.1×10^{-5} reciprocal ohms. Conductivity of a mixture containing the two salts in the above concentrations at 30 degrees equals 108.5×10^{-5} reciprocal ohms.

and regenerating the free protein to again participate in carrying the current both ways. Provided, therefore, the two ions are of nearly equal weight (and we have seen that in the case of the ions of potassium caseinate the ions are nearly equal in weight, cf. previous chapter) no noticeable change in the distribution of the protein in the electric field will be observed. If the ions should be of unequal weight, then a trace of acid or alkali combined with the protein might suffice to neutralize this inequality and render the protein apparently "isoelectric." The "isoelectric" protein of Michaelis is, therefore, in all probability, merely free protein uncombined with acids or bases. This view of Michaelis' result is supported by Pauli and Wagner (50) who point out that the egg-albumin employed by Michaelis is, in reality, an albuminate of a base, which therefore requires the addition of some acid to set the protein free. The free protein is sparingly ionized and hence the viscosity of its solution is low.

5. Biological Applications; the Neutrality of the Tissues and of the Tissue Fluids. — The power which the protein salts possess of neutralizing additional equivalents of acid or base by the opening up of fresh $-N.HOC-$ bonds confers upon them, since the reaction of free protein is usually nearly neutral, a remarkable power of maintaining the neutrality of their solutions, and it appears probable that this characteristic of the protein salts plays an important part in maintaining the neutrality of the tissues and a not insignificant part in maintaining the neutrality of the tissue fluids.

The older statements which are found in physiological and medical literature concerning the reactions of the blood are totally unreliable, since they were based upon the erroneous belief that it is possible to ascertain the reaction of a fluid such as the blood by titration. The determinations of Hoeber (31), Farkas (10) (11) (12) (13), Fraenkel (18), Szily (69) (70), Hasselbalch (23) (24) and de Corral (8) carried out by the potentiometric method, have shown that the H^+ and OH' ion concentrations of normal blood are very close to those at neutrality (neutral point = 8×10^{-8} H^+ and OH'), the alkalinity first found by Hoeber having been traced to the fact that the stream of hydrogen, used to impregnate the platinum electrode of the gas-chain, washed the CO_2 out of the blood. On eliminating this source of error the blood is found to be almost absolutely neutral, the exact OH'

concentration in normal blood at physiological CO_2 pressures (0.028 to 0.054 atmosphere) being, according to recent determinations, about 0.37×10^{-7}.

Friedenthal (19) and von Szily (69), employing a somewhat different method of investigation, have reached the same conclusion as the above quoted observers. They utilized the indicator-method of Friedenthal, Fels and Salm. Mixtures of acid and basic phosphates of known H^+ and OH' content (determined by Salm, using the potentiometric method) were tinged with a number of different indicators and the samples of the body-fluid under examination were tinged with equal quantities of the same indicators — the colors of the two series of fluids were then compared. The phosphate-mixture which approximated most closely in its indicator reactions to the normal blood-serum had an acidity corresponding to 6.5×10^{-8} H^+, absolute neutrality being 8×10^{-8} H^+.

Moreover the investigations of Friedenthal (19) (20) and of Foa (15) (16) (17) have shown that not only is the blood almost exactly neutral but that almost all of the tissue-fluids are also approximately neutral. Thus, the pancreatic juice of a dog, one of the most alkaline of the body-fluids, contained 5×10^{-9} H^+, corresponding to an alkalinity of 13×10^{-7} OH'. Hitherto, according to Friedenthal (20), no animal fluid has been found containing less than 10^{-10} N H^+, that is, more than about 6×10^{-5} N OH'. The gastric juice of a dog was, however, found to be 1000 times more acid than the pancreatic juice is alkaline, that is, it possessed the acidity of, approximately, a hundredth normal solution of HCl.

With few exceptions, therefore, the tissue fluids are practically neutral in reaction. It is a significant fact also (Friedenthal (20), Loeb (37)) that the naturally occurring waters, in which the lives of aquatic animals are spent, are also very nearly neutral in reaction. "We may therefore draw the conclusion that life-phenomena occur in a neutral liquid" (Loeb, loc. cit.), and, moreover, save in certain special localities, such as the internal wall of the stomach, the life-process occurs exclusively in a neutral milieu; thus even an increase of 50 per cent in the minute H^+ concentration of normal blood induces profound toxic symptoms, and an increase to 1×10^{-7} N H^+ induces fatal acidosis.

In view of the fact that the products of metabolism include a

large proportion of acid substances, the mechanism which preserves the neutrality of the tissues and tissue-fluids despite the relatively enormous daily and hourly fluctuations in acid-production or consumption which necessarily occur within the body, is, in all probability, one of prime physiological importance.

The acid which is produced most copiously in the body is carbonic acid. The classic theory regarding the neutralization of this acid is that of Fernet (14), Heidenhain and Mayer (25) (26) and von Bunge (6) (7) who supposed that it is bound by the disodium phosphate in the blood, according to the balanced reaction:

$$CO_2 + H_2O + Na_2HPO_4 \rightleftarrows NaHCO_3 + NaH_2PO_4,$$

the reaction proceeding towards the right in the tissues, where the CO_2 tension is high, towards the left in the lungs, where the CO_2 tension (active mass of CO_2) is low. Sertoli (65), however, and Mroczkovsky (46) pointed out that the quantity of phosphoric acid in the blood of many animals, e.g., the ox and the pig,* is too small to play the important rôle assigned to it by these investigators.

We have seen that the alkali- and acid-equivalent of the proteins varies very markedly with the hydroxyl- or hydrogen-contents of their solutions. Thus one gram of serum-globulin (Cf. Chap. V) in solutions neutral, or approximately neutral, to litmus (absolute neutrality: $H^+ = OH' = 8 \times 10^{-8}$) neutralizes 10×10^{-5} equivalent gram molecules of a base, while in solutions neutral to phenolphthalein ($OH' = 20 \times 10^{-7}$) 1 gram neutralizes 20×10^{-5} equivalent gram molecules of a base. The significance of these figures may, perhaps, be more fully realized when stated thus: in one litre of NaOH solution containing 1 per cent of globulin and neutral to litmus, addition of 100×10^{-5} gram-equivalents of NaOH only raises the alkalinity (hydroxyl-content) of the solution by about 0.2×10^{-5}, that is, by two-tenths of a per cent of the change in the sodium content of the solution. Casein, at neutrality to litmus, neutralizes 50×10^{-5} gram-equivalents of base per gram, and at "saturation," i.e., at an acidity of about 50×10^{-7} H^+, it neutralizes only 11.4×10^{-5} gram-equivalents of base per gram. Hence to a litre of a 1 per cent solution of sodium caseinate, neutral to litmus, we should

* Cf. von Bunge (7).

have to add 400×10^{-5} equivalents of HCl to reach an acidity of 0.5×10^{-5} equivalents — just over one-tenth of a per cent of the acid added. Were the casein present in a concentration of 8 per cent (the *percentage* concentration of the proteins in the blood), 3200×10^{-5} gram equivalents of HCl, or 320 cc. of a tenth normal solution, would have to be added to a litre of the solution to attain an acidity of 0.5×10^{-5} equivalents. One *twentieth* of a cubic centimeter of tenth normal HCl, added to a litre of pure distilled water, would produce the same acidity. The power of the proteins to maintain the neutrality of the fluids in which they occur is therefore very remarkable, and Loeb (36), myself (55) (56) (57) (58) and others have suggested that they play an important part in regulating the neutrality of the tissues and tissue-fluids, while Hoppe-Seyler (32), Sertoli (65), Zuntz (72) (73) and myself (55) (57) have advanced the view that the liberation of CO_2 from the blood in the lungs is accompanied by a transport of sodium from the carbonic acid to the proteins of the plasma.

Friedenthal (19) (20), Spiro (68), Loeb (36) and Henderson (27) (28) (29) (30) have pointed out that the bicarbonates of the blood and other media in which organisms live must also be of importance in preserving their neutrality. Henderson points out that the rate of change in the alkalinity or acidity of a solution of an acid is a minimum when the dissociation constant of the acid is equal to the hydrogen ion concentration (8×10^{-8} N) at neutrality. He illustrates this by the following table, showing the amount of tenth normal alkali required to secure a definite but arbitrarily chosen change in alkalinity when added to equal amounts of the undermentioned acids.

Acid	Dissociation-constant $\times 10^{-7}$	Cubic centimeters of alkali required
Phenol	0.0013	0.01
Boric acid	0.017	0.08
Hydrogen sulphide	0.57	1.10
Mono-sodium phosphate	2.0	1.00
Carbonic acid	3.0	0.72
Picolinic acid	18.0	0.10
Acetic acid	180.0	0.03

Henderson believes that the neutrality of the tissue fluids is chiefly maintained by the bicarbonates which they contain

and in a minor degree by the phosphates and by the proteins. He illustrates his hypothesis by reference to a system tenth-molecular in total carbonic acid and equally concentrated in total phosphoric acid, combined or uncombined with sodium, and he shows that in order to appreciably change the acidity of this system a quantity of acid comparable with the total amount of sodium which is present at neutrality must be added. He estimates, from known quantitative data, the quantity of acid which would be neutralized by the bicarbonates of the blood in passing from the reaction of normal blood ($= 0.37 \times 10^{-7}$ H^+ at 38 degrees) to that of blood in advanced acid intoxication (about 1.00×10^{-7} H^+ at 38 degrees). At the same time, from data derived from experiments in which he employed indicators to determine the change in the reaction of solutions of the serum-proteins to which varying amounts of acid and alkali had been added, he estimated the amount of acid which is neutralized by the proteins of the blood, and their salts, as the reaction of their solutions changes by the same amount, and he concludes that it cannot be more than one-fifth of the amount of acid which would be neutralized by the bicarbonates.

Experimental results which involve the use of indicators to determine such slight changes in reaction as these afford an unsatisfactory basis for so important a theoretical conclusion. Accordingly, employing the potentiometric method, I carried out a series of determinations of the reactions of solutions of the proteins of serum, of the concentration in which they occur in serum, to which varying amounts of acid or alkali had been added (58). The results of these experiments entirely confirm Henderson's estimate of the neutralizing power of the proteins of blood-serum between the reactions mentioned above.

The proteins of ox-blood serum were coagulated by alcohol, carefully washed with alcohol and ether and dried. They were then dissolved in 0.01 *N* KCl containing varying concentrations of KOH or HCl, each solution containing 8 per cent of the proteins (= percentage concentration in blood). The following were the results obtained.*

* The value of K, the constant ionic product for water at 34 degrees, was taken as $(1.47 \times 10^{-7})^2 = 2.16 \times 10^{-14}$ (Cf. Kohlrausch and Heydweiller (33)). The experimental error in the determination of C_{H^+} in the "unknown" is not more than 6 per cent of C_{H^+}.

Known element of chain	"Unknown" element of chain	Potential of chain, volt	C_{H^+} in "unknown" element	$\text{Log}_{10} C_{H^+}$ in "unknown" element
0.01 *N* KOH	0.01 *N* KOH+8 per cent serum proteins.	0.0920	7.35×10^{-11}	−10.13
0.01 *N* KOH	0.005 *N* KOH+8 per cent serum proteins.	0.1186	2.03×10^{-10}	− 9.79
0.01 *N* KOH	8 per cent serum proteins.	0.1710	1.52×10^{-9}	− 8.82
0.01 *N* KOH	0.005 *N* HCl+8 per cent serum proteins.	0.2277	1.33×10^{-8}	− 7.88
0.01 *N* HCl	0.01 *N* HCl+8 per cent serum proteins.	0.2968	1.15×10^{-7}	− 6.94
0.01 *N* HCl	0.02 *N* HCl+8 per cent serum proteins.	0.2198	2.20×10^{-6}	− 5.66

If, taking the solution of 8 per cent serum-proteins in neutral 0.01 *N* KCl as the zero-point on the axis of the abscissæ, we measure off to the right of this point the concentrations of acid in which the proteins were dissolved, and to the left of this point the concentrations of alkali in which they were dissolved and plot the values of $\log C_{H^+}$ given in the above table, we obtain a curve,* by interpolation from which it is readily found that in passing from the reaction 0.37×10^{-7} H^+ to the reaction 1.00×10^{-7} H^+, 100 cc. of an 8 per cent solution of the serum protein neutralizes 2.25 cc. of *N*/10 HCl, that is 22.5 cc., or about one-fifth of its own volume of *N*/100 HCl. According to Henderson, in passing through the same range of H^+ concentrations the bicarbonates in 100 cc. of blood will neutralize the equivalent of 100 cc. of *N*/100 HCl. Hence we must conclude that, between the reactions mentioned, the proteins of the serum are only one-fifth as efficient in maintaining its neutrality as the bicarbonates. To the neutralizing power of the serum proteins must be added, in circulating plasma, that of the fibrinogen. This is, however, probably very slight, since only a very small percentage of fibrinogen is contained in the blood.

* For the form of the curve see my original communication (58).

It has been shown by Marshall (39) that the major part of the neutralizing power of saliva is likewise attributable to its inorganic constituents.

It would not be safe, however, to conclude from these results that the part played by the proteins in maintaining the neutrality of the *tissues* is not, possibly, equal in magnitude to, or even greater than, that played by the bicarbonates and acid phosphates. Thus, referring to the tables in section **2** it will be seen that 100 cc. of a 1.5 per cent solution of potassium caseinate, in passing from the reaction 0.34×10^{-7} H^+ to the reaction 1.14×10^{-7} H^+ neutralizes the equivalent of 25 cc. of $N/10$ HCl; a 3 per cent solution (the concentration of casein in cow's milk) would therefore neutralize, in passing from the former to the latter of the above reactions, the equivalent of one-half its volume of $N/100$ HCl. The part played by the casein of milk in maintaining the neutrality of this tissue-fluid must therefore be of very considerable importance. In the tissues, not only are the proteins more concentrated than they are in the tissue-fluids, but, owing to the predominance of nucleo-proteins which have a high combining-capacity for bases, their power of maintaining the neutrality of the solutions is not improbably even higher than that of the majority of the proteins which occur in the tissue-fluids. We are not, at present, in the possession of any data which would render an estimate of the relative importance of these factors, for the maintenance of the neutrality of the tissues themselves, in the slightest degree reliable.

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CHAPTER X

THE ELECTRICAL CONDUCTIVITY OF SOLUTIONS OF PROTEIN SALTS

1. The Influence of Dilution upon the Conductivity of Solutions of Protein Salts. — The Ostwald dilution-law for a binary electrolyte is usually formulated as follows:

$$K = \frac{\left(\frac{\mu_v}{\mu_\infty}\right)^2}{\left(1 - \frac{\mu_v}{\mu_\infty}\right) V},$$

where K is the dissociation-constant, μ_v is the molecular conductivity at dilution V and μ_∞ is the molecular conductivity at infinite dilution, that is: $96.44\ (u + v)$ where u and v are average equivalent migration velocities in centimetres per second under unit potential gradient, of the cations and anions respectively.

Now $\mu_v = \frac{x}{m}$, where x is the specific conductivity in reciprocal ohms, and m is the equivalent concentration, i.e., $\frac{1}{V}$; hence from the above equation we have

$$K\left(1 - \frac{1.037 \times 10^{-2}}{m\,(u+v)}\,x\right) = m\left(\frac{1.037 \times 10^{-2}}{m\,(u+v)}\,x\right)^2$$

which reduces to

$$m = \frac{1.037 \times 10^{-2}}{u+v}\,x + \frac{1.075 \times 10^{-4}}{K(u+v)^2}\,x^2. \qquad \text{(i)}$$

The same formula can be derived as follows (8); let c be the equivalent concentration of the ions, m and K having the same significance as before; then, applying the mass law, we have

$$c^2 = k\ (m - c).$$

Now $1.037 \times 10^{-2}\,x = (u + v)\,c$; hence

$$c = \frac{1.037 \times 10^{-2}}{u+v};$$

substituting in the above equation we regain equation (i).

From this mode of deriving the formula it is clear that the total equivalent concentration of the electrolyte may be expressed in terms of the electric conductivity of its solution as the sum of two factors, the first, directly proportional to the conductivity, being the equivalent concentration of the dissociated part of the electrolyte and the second, directly proportional to an exponent of the electrical conductivity (in this instance 2), being the undissociated part of the electrolyte.

The salts which the proteins form with inorganic bases and acids obey the Ostwald dilution-law for a binary electrolyte (7) (8) (10) (12) (15) (16) (17) (19), that is, the relationship between the equivalent concentration of the protein salt and the conductivity (in reciprocal ohms per cubic centimetre) is that indicated by formula (i). It is to be observed, however, that without additional assumptions we do not know what is the *equivalent concentration* of a protein salt. If we assume, however, that m, the equivalent concentration of the base or acid which is neutralized by the protein, bears a constant proportion to the *true equivalent concentration* of the protein salt, then (i) becomes

$$m = \frac{1.037 \times 10^{-2}}{\rho\,(u+v)}x + \frac{1.075 \times 10^{-4}}{K\rho\,(u+v)^2}x^2, \qquad \text{(ii)}$$

in which m is now the known equivalent concentration of the base or acid which is bound by the protein and ρ *is the number of equivalents of protein salt to which each equivalent of neutralized acid or base gives rise.* This equation may be written:

$$m = Ax + Bx^2, \qquad \text{(iii)}$$

in which A and B are constants, respectively equal to

$$\frac{1.037 \times 10^{-2}}{\rho\,(u+v)} \quad \text{and} \quad \frac{1.075 \times 10^{-4}}{K\rho\,(u+v)^2}.$$

The fact that the dependence of the conductivity of solutions of protein salts upon their dilution obeys the formula (iii), therefore, shows that for a given salt ρ is constant; in other words, for a given combination of acid or base with protein, containing a given proportion of the acid or base, the number of equivalents of protein salt to which one equivalent of neutralized acid or base gives rise is independent of the dilution.

The validity of equation (iii) as applied to solutions of protein salts may be gathered from the accompanying tables. In each case the most probable values of the constants A and B are com-

puted from all of the experimental data by Gauss' method of least squares. Inserting these values of the constants and the experimental values of x in equation (iii), the "calculated" values of m are computed. In the third column of the tables is given the "Degree of Dissociation" of the salt calculated from the ratio of Ax in equation 3 to the "calculated" value of m.

The conductivity of the distilled water (usually not in excess of 4×10^{-6}) is subtracted from each of the observed conductivities.

TABLE I

Sodium Caseinate. Approx. neutral to Litmus. Approx. 50×10^{-5} Equivalents NaOH per gram. Temperature 25 degrees

$A = 19.51 \quad B = 9611$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation (per cent)
200	201	73
100	98	83
68	66	88
50	51	89
40	42	90
28	29	94
20	22	93
10	17	98

TABLE II *

Ammonium Caseinate. Approx. neutral to Litmus. Approx. 50×10^{-5} Equivalents NH_4OH per gram. Temperature 25 degrees

$A = 16.27 \quad B = 6188$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
200	201	74
100	99	84
68	67	88
50	50	90
40	40	93
28	30	93
20	24	96
10	11	99

* T. Brailsford Robertson (8). The exact proportion of base to casein in these experiments is not certain, since the original solution which was diluted to yield the remainder was made up by shaking up an 0.02 N solution of the base with excess of casein under the erroneous belief (Cf. T. Brailsford Robertson (6) (8) (11)) that the maximum amount of casein which a given concentration of a base would dissolve, when shaken up with excess of casein, was that with which it will unite to form the "neutral" caseinates; i.e., containing 50×10^{-5} equivalents of base per gram. Since, however, casein dissolves rapidly up to this point and very slowly afterwards the composition of the salt formed was probably very nearly that indicated at the head of the tables. Moreover the solutions were tested and found to be neutral to litmus. The last observation in Table I and the last but one in Table II are omitted in computing the values of the constants.

TABLE III*

Sodium Caseinate. Neutral to Phenolphthalein. 80×10^{-5} Equivalents NaOH per gram. Temperature 25 degrees

$A = 15.76$ $B = 4979$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
250	250	73
125	132	77
63	62	89
31	33	94
16	18	96

TABLE IV*

Ammonium Caseinate. Neutral to Phenolphthalein. 80×10^{-5} Equivalents NH_4OH per gram. Temperature 25 degrees

$A = 12.60$ $B = 3978$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
180	181	75
90	88	84
45	44	91
23	23	96
11	11	100

* The calculations in Tables III and IV are based upon the experimental data of Sackur (21). Cf. T. Brailsford Robertson (10).

TABLE V

Potassium Caseinate. Neutral to Phenolphthalein. 80×10^{-5} Equivalents KOH per gram. Temperature 30 degrees

$A = 12.41$ $B = 1666$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
250	250	85
125	124	89
63	64	94
31	33	97
16	17	100

TABLE VI

Calcium Caseinate. Neutral to Phenolphthalein. 80×10^{-5} Equivalents $Ca(OH)_2$ per gram. Temperature 30 degrees

$A = 27.89$ $B = 26{,}700$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
320	320	26
240	243	29
160	151	35
120	115	39
80	81	44
60	66	48
40	47	53
20	24	67

TABLE VII

Strontium Caseinate. Neutral to Phenolphthalein. 80×10^{-5} Equivalents $Sr(OH)_2$ per gram. Temperature 30 degrees

$A = 32.65$ $B = 4930$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
224	226	61
168	167	66
112	106	74
84	86	77
56	58	83
42	47	85

TABLE VIII*

Barium Caseinate. Neutral to Phenolphthalein. 80×10^{-5} Equivalents $Ba(OH)_2$ per gram. Temperature 30 degrees

$A = 23.73$ $B = 23{,}400$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
320	326	24
160	147	33
80	76	42
40	41	54
20	22	64

* For data used in Tables V, VI, VII and VIII, Cf. T. Brailsford Robertson (15) (17).

TABLE IX*

Potassium Caseinate. 90×10^{-5} Equivalents KOH per gram. Temperature 30 degrees

$A = 11.59$ $B = 1739$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
180	180	83
135	135	87
90	90	90
45	46	96
23	25	96

* The conductivity of the trace of free KOH was in no case more than 2 per cent of the observed conductivity; the observed conductivities were not corrected for this (18).

TABLE X*

Potassium Caseinate. 100×10^{-5} Equivalents KOH per gram. Temperature 30 degrees

$A = 9.57$ $B = 1242$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
300	300	76
200	201	82
150	149	85
100	100	89
50	53	93

* Calculated for solutions containing small amounts of KCl according to the method described in the previous chapter (section **1**) and, therefore, corrected for the conductivity of the free KOH as determined by gas-chain measurements. T. Brailsford Robertson (14).

TABLE XI*

Potassium Paranucleinate. Neutral to Phenolphthalein. 36×10^{-5} Equivalents KOH per gram. Temperature 25 degrees

A = 12.38 B = 3784

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
92	93	85
46	46	93
23	23	96
12	13	100
6	7	100

* T. Brailsford Robertson (12).

TABLE XII*

Sodium Serum-globulinate. Neutral to Litmus. 9.75×10^{-5} Equivalents NaOH per gram. Temperature 18 degrees

$A = 17.65$ $B = 36,200$

m (experimental)	m (calculated)	Degree of dissociation, per cent
657	657	66
329	302	78
164	155	87
82	83	92
41	44	96
21	29	97

* Calculated from experimental data obtained by W. B. Hardy (2).

The constants determined by least squares ($A = 22.23$; $B = 17,391$) do not give, for the smaller values of m, so good an agreement as the above. From the evidence afforded by the numerous data set forth in these tables there is strong presumptive reason for believing that, notwithstanding this lack of agreement, the dilution-law holds good in this case also, and that experimental errors are responsible for the deviations. A very probable source of error in these determinations is, as Hardy points out, the conductivity of the undissolved suspension of globulin, a quantity of very uncertain magnitude and meaning. This conductivity was very appreciable with the globulin employed by Hardy (precipitated by acetic acid), while it was unappreciable with the globulin employed in my experiments (precipitated by CO_2). Since the method of least squares fails to yield constants which satisfy the experimental results, I have taken A for this salt to be the same as A for the salt containing 18×10^{-5} equivalents NaOH per gram. B is estimated from the first observation. It is evident that the order of agreement between the calculated and observed values is tolerably good.

TABLE XIII*

Sodium Serum-globulinate. Neutral to Phenolphthalein. 18×10^{-5} Equivalents NaOH per gram. Temperature 18 degrees

$A = 17.65$ $B = 11,500$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
313	310	59
156	156	71
78	77	81
39	38	88
10	10	95

* Calculated from the experimental data of W. B. Hardy (2).

TABLE XIV*

Serum-globulin Chloride. 9.318×10^{-5} Equivalents HCl per gram. Temperature 18 degrees

$A = 7.42$ $B = 36,964$

m (experimental)	m (calculated)	Degree of dissociation, per cent
933	926	33
461	484	42
231	219	55
115	105	68
58	54	78
29	30	83

* Calculated from the experimental data of W. B. Hardy (2).

TABLE XV

Potassium Serum-globulinate. Neutral to Phenolphthalein. 20×10^{-5} Equivalents KOH per gram. Temperature 30 degrees

$A = 19.62$ $B = 51,900$

$m \times 10^5$ (experimental)	$m \times 10^5$ (calculated)	Degree of dissociation, per cent
296	297	77
148	147	85
74	70	92
37	37	95
18	18	98

TABLE XVI*

Calcium Serum-globulinate. Neutral to Phenolphthalein. 20×10^{-5} Equivalents $Ca(OH)_2$ per gram. Temperature 30 degrees

$A = 43.25$ $B = 502,000$

$m \times 10^5$ (experimental)	$m \times 10^5$ (calculated)	Degree of dissociation, per cent
296	296	66
148	146	77
74	76	85
37	43	91
18	18	96

* Fourth observation omitted in computing the constants.

TABLE XVII

Strontium Serum-globulinate. Neutral to Phenolphthalein. 20×10^{-5} Equivalents $Sr(OH)_2$ per gram. Temperature 30 degrees

$A = 32.85$ $B = 628{,}000$

$m \times 10^5$ (experimental)	$m \times 10^5$ (calculated)	Degree of dissociation, per cent
74	74	76
37	36	82
18	19	87

TABLE XVIII*

Barium Serum-globulinate. Neutral to Phenolphthalein. 20×10^{-5} Equivalents $Ba(OH)_2$ per gram. Temperature 30 degrees

$A = 42.61$ $B = 369{,}000$

$m \times 10^5$ (experimental)	$m \times 10^5$ (calculated)	Degree of dissociation, per cent
296	297	70
140	142	81
74	78	87
37	41	93
18	18	94

* For the data utilized in Tables XV, XVI, XVII, XVIII, Cf. T. Brailsford Robertson (17).

TABLE XIX*

Ovomucoid Chloride. 45×10^{-5} Equivalents HCl per gram. Temperature 30 degrees

$A = 5.103$ $B = 525$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
180	181	78
90	89	87
45	44	93
23	22	96
11	11	97

* Free HCl = less than 0.5×10^{-4} for the 1 per cent solution; observed conductivity not corrected for this. Data utilized in this and the following table from T. Brailsford Robertson (16).

TABLE XX

Ovomucoid Sulphate. 45×10^{-5} Equivalents H_2SO_4 per gram. Temperature 30 degrees

$A = 5.488$ $B = 986$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
180	180	71
90	89	81
45	45	89
23	23	96
11	12	96

TABLE XXI

Protamin (Salmin) Sulphate. 424×10^{-5} Equivalents H_2SO_4 per gram. Temperature 30 degrees

$A = 9.09$ $B = 6408$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
105.3	105.7	65
84.2	83.4	69
52.6	52.7	76
26.3	26.7	85
13.2	13.7	94
6.6	7.1	100
3.3	3.5	100

TABLE XXII

Protamin (Salmin) Chloride. 424×10^{-5} Equivalents HCl per gram. Temperature 30 degrees

$A = 6.92$ $B = 1781$

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
55.8	55.9	85
27.9	27.6	92
13.9	14.1	95
7.0	7.1	97
3.5	3.6	100

It will be observed that as a rule the agreement between the calculated and the observed values of m is excellent and we may therefore conclude that equation (ii) (or (iii)) represents the dependence of conductivity upon dilution, for the salts of the proteins, with very satisfactory fidelity. Recollecting the mode of derivation of equation (ii) this fact might be held to prove that the protein salts, in all of the solutions investigated, dissociate into *two ions*. This deduction is not altogether a safe one, however. The *generalized* form of equation (iii) for electrolytes which dissociate into n ions is:*

$$m = Ax + Bx^n \qquad \text{(iv)}$$

and, provided the departure of equation (iv) from the linear form is small, the factor Bx^n may be represented, within the experimental error, by Bx^2 even if n be in reality a higher exponent than 2, while vice versa, Bx^2 may be approximated by Bx^n, where n is a higher exponent than 2. For example, in the following table the values of m for calcium caseinate solutions (Cf. Table VI), calculated from formula (iii) and from the formula

$$m = A_1x + B_1x^3, \qquad \text{(v)}$$

are compared.†

TABLE XXIII

$m \times 10^3$ (experimental)	$m \times 10^3$ (calculated from formula (iii))	$m \times 10^3$ (calculated from formula (v))
32	32	33
24	24	23
16	15	14
12	12	11
8	8	8
6	7	7
4	5	5
2	2	3
	$\Sigma\Delta = +1$	$\Sigma\Delta = \pm 0$

* If the electrolyte dissociates partly into 2, partly into 3, partly into 4, etc., ions the equation becomes:

$$m = Ax + Bx^2 + Cx^3 \cdots + Nx^n.$$

† Sutherland (22) (23), urging the applicability of his theory of ionization to the salts of proteins, has pointed out that the results of Hardy (cited above, Table XIV) obtained with HCl-globulin containing 9.32×10^{-5} equivalents of acid per gram of protein may be expressed by the equation:

$$\frac{1}{\mu} = 0.00026 + 0.009\, v^{-\frac{1}{2}},$$

in which μ is the molecular conductivity of the salt, calculated in terms of HCl-concentrations, and v is the volume of solution containing one equivalent of Cl.

There is little to choose between the two formulæ, although, on the whole, the agreement between the values calculated from formula (iii) and the experimental values is somewhat closer than that between the values calculated from formula (v) and the experimental values. As we shall see, however, the internal evidence which is afforded by the numerical values of the constants demonstrates that the true relationship between dilution and conductivity for solutions of the salts which proteins form with bases is that which is expressed by equation (iii); namely that which is characteristic for an electrolyte which yields two ions per molecule.

As regards the salts which proteins form with acids, the binary formula expressed by equation (iii) yields demonstrably better agreement with the experimental data obtained with salmin sulphate and chloride than the ternary formula expressed by equation (v). The salts which protamins form with acids would therefore appear to yield only two ions, irrespective of the valency of the acid anion with which the protein is combined. On the other hand, the relationship of conductivity to dilution which is displayed by the salts which ovomucoid forms with hydrochloric acid is more adequately expressed by the ternary formula.

It will be recollected that the constants A and B in equation (iii) represent, respectively, the quantities

$$\frac{1.037 \times 10^{-2}}{\rho\,(u+v)} \quad \text{and} \quad \frac{1.075 \times 10^{4}}{K\rho\,(u+v)^2},$$

where $u + v$ is the sum of the *equivalent* migration velocities of the ions in centimeters per second under a potential gradient of 1 volt per centimeter, ρ is the number of equivalents of the protein salt which is yielded by the neutralization of one equivalent of inorganic base or acid, and K is the dissociation constant of the salt. It will be observed that $\frac{A}{B} = \frac{K}{\rho}$. We can, therefore, from the data enumerated in the above tables, compute $\rho\,(u + v)$ and $\frac{K}{\rho}$ for each of the salts investigated. The values of these constants follow.

TABLE XXIV. CASEINATES OF INORGANIC BASES

Caseinates of monacid bases

Nature of the base and proportion of base to 1 gram protein	Temp., degrees	$\rho(u+v)$	$\frac{K}{\rho}$
50×10^{-5} NaOH per gram	25	53×10^{-5}	40×10^{-3}
80×10^{-5} NaOH per gram	25	66×10^{-5}	50×10^{-3}
50×10^{-5} NH_4OH per gram	25	63×10^{-5}	44×10^{-3}
80×10^{-5} NH_4OH per gram	25	82×10^{-5}	40×10^{-3}
80×10^{-5} KOH per gram	30	84×10^{-5}	91×10^{-3}
90×10^{-5} KOH per gram	30	89×10^{-5}	77×10^{-3}
100×10^{-5} KOH per gram	30	108×10^{-5}	74×10^{-3}
Caseinates of diacid bases			
80×10^{-5} Ca(OH) per gram	30	37×10^{-5}	2.9×10^{-3}
80×10^{-5} $Sr(OH)_2$ per gram	30	32×10^{-5}	21.6×10^{-3}
80×10^{-5} $Ba(OH)_2$ per gram	30	44×10^{-5}	2.4×10^{-3}

TABLE XXV. SERUM GLOBULINATES OF INORGANIC BASES

Globulinates of monacid bases

Nature of the base and the proportion of base to 1 gram protein	Temp., degrees	$\rho(u+v)$	$\frac{K}{\rho}$
9.75×10^{-5} NaOH per gram	18	59×10^{-5}	8.6×10^{-3}
18.0×10^{-5} NaOH per gram	18	59×10^{-5}	27.0×10^{-3}
20.0×10^{-5} NaOH per gram	30	53×10^{-5}	7.4×10^{-3}
Globulinates of diacid bases			
20×10^{-5} $Ca(OH)_2$ per gram	30	24×10^{-5}	3.7×10^{-3}
20×10^{-5} $Sr(OH)_2$ per gram	30	32×10^{-5}	1.7×10^{-3}
20×10^{-5} $Ba(OH)_2$ per gram	30	24×10^{-5}	4.9×10^{-3}

TABLE XXVI. COMPOUNDS OF PROTEINS WITH ACIDS

Monobasic acids

Nature of the acid and protein and the proportion of acid to 1 gram protein	Temp., degrees	$\rho(u+v)$	$\frac{K}{\rho}$
9.318×10^{-5} HCl per gram serum globulin	18	140×10^{-5}	1.5×10^{-3}
45×10^{-5} HCl per gram ovomucoid	30	203×10^{-5}	50×10^{-3}
442×10^{-5} HCl per gram salmin	30	150×10^{-5}	26.9×10^{-3}
Dibasic acids			
45×10^{-5} H_2SO_4 per gram ovomucoid	30	189×10^{-5}	31×10^{-3}
442×10^{-5} H_2SO_4 per gram salmin	30	114×10^{-5}	12.9×10^{-3}

For potassium paranucleinate, containing 36×10^{-5} equivalents of KOH per gram, the value of $\rho(u+v)$ is 84×10^{-5} and that of $\frac{K}{\rho}$ is 41×10^{-3}.

On surveying these tables several striking facts are immediately revealed. In the first place the ratio of the value of $\rho\ (u + v)$ for monacid bases to its value for diacid bases is, in every case (Cf. Tables XXIV and XXV) exceedingly close to the ratio 2 : 1. This may be interpreted in either of two ways; either the equivalent velocity of the protein ions which are yielded by the salts of monacid bases is twice as great as that of the ions which are yielded by the salts of diacid bases, or else one equivalent of a monacid base gives rise to twice as many equivalents of protein salt as one equivalent of a diacid base. We have seen that the protein salts dissociate into two heavy protein ions and, as Bredig has shown (1), the equivalent velocities of heavy ions approach a constant minimum. The former of the above alternatives may therefore be dismissed, and we must conclude that ρ, the number of equivalents of protein salts to which one equivalent of base gives rise, *is twice as great for the monacid as for the diacid bases.*

Reverting to the hypothesis developed in the preceding chapters, it appears that the monacid bases form salts with proteins in the following way:

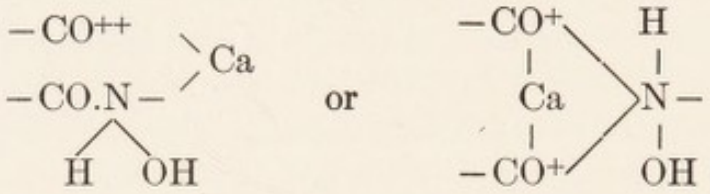
$$-\mathrm{COH.N}- + \mathrm{NaOH} = -\mathrm{CO}\overset{++}{\mathrm{Na}} + \searrow\overset{\overset{\mathrm{H}}{|}}{\underset{\underset{\mathrm{OH}}{|}}{\mathrm{N}}}-$$

from which it is evident that for the salts of the monacid bases ρ must be 2, i.e., one equivalent of a monacid base, when united with protein, yields 2 equivalents of the protein salt. We must conclude, then, that one equivalent of diacid base gives rise to only *one* equivalent of protein salt. The most natural assumption regarding the mode of combination of the proteins with diacid bases, pursuing the above hypothesis, would be to suppose that it takes place in accordance with the equation:

$$2-\mathrm{COH.N}- + \mathrm{Ca(OH)_2} = \begin{matrix}-\mathrm{CO}^{++}\diagdown\\ -\mathrm{CO}^{++}\diagup\end{matrix}\mathrm{Ca} + 2\searrow\overset{\overset{\mathrm{H}}{|}}{\underset{\underset{\mathrm{OH}}{|}}{\mathrm{N}}}-$$

upon which supposition each equivalent of the diacid base would give rise to just the same number of equivalents of protein salt as an equivalent of a monacid base, namely 2. This is evidently

not the case, and we must assume that two of the positive valencies of the cation are neutralized and at the same time that two of the negative valencies which the anions supply are also neutralized. This immediately suggests that one of the two anions neutralizes two of the valencies of the cation and that the true constitution of the cation is either



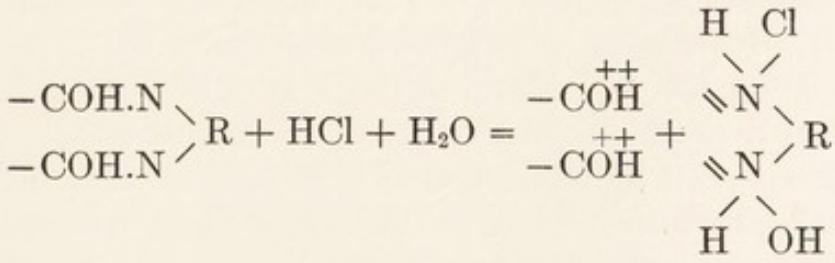
or either of these formulæ less the elements of water which are attached to the nitrogen atom. If this be so then we must conclude that each *molecule* of a diacid base gives rise to exactly the same number of ions (apart from the question of the *degree of dissociation* of the salt) and equivalents of protein salt as a molecule of a monacid base, and that *the molecule of a protein salt of a diacid base must be twice as heavy as a molecule of a protein salt of a monacid base.* In this connection the following differences between the physical behavior of solutions of the caseinates of the alkalies and those of the caseinates of the alkaline earths may be recalled to the reader: The aqueous solutions of the "neutral" or "basic" caseinates of the alkalies and ammonium are clear, do not (except lithium caseinate) show any increase in turbidity on warming, and are not precipitated by the addition of finely divided insoluble substances or by passing through a clay filter, while the aqueous solutions of the caseinates of the alkaline earths are opalescent, show a marked turbidity on heating their solutions to 35–45° C., which disappears on cooling, and are precipitated from their solutions by the addition of finely divided insoluble substances or by passage through a clay filter (5). Equally concentrated solutions of the hydroxides of the alkalies and ammonium dissolve casein at approximately the same rate. Solutions of the hydroxides of the alkaline earths dissolve casein much more slowly (9) (13).

The salts which casein forms with monacid bases at "saturation" of the base with casein, that is, when the proportion of base to casein is 11.4×10^{-5} equivalents per gram, are soluble in water, while the corresponding salts which casein forms with

diacid bases are insoluble in water (11) (25). All of these differences, it will be observed, suggest more or less strongly that the molecules of the caseinates of the alkaline earths are of greater size and more ponderous than those of the caseinates of the alkalies.

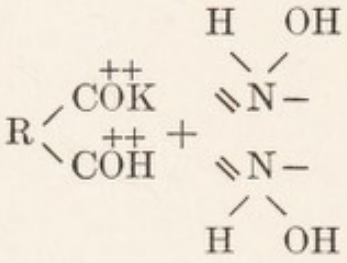
Assuming, therefore, that $\rho = 2$ for the salts of the alkalies and 1 for the salts of the alkaline earths we find that the numerical values of $\rho\,(u + v)$ in Tables XXIV and XXV accord very well with our knowledge of the mobilities of heavy ions. The average value of $u + v$ at 30 degrees for potassium caseinate containing 80×10^{-5} equivalents of KOH per gram is 42×10^{-5} cm. per sec., while for the salts of the alkaline earths at the same temperature the average value of $u + v$ is 38×10^{-5}. Assuming the migration velocities of the anions and cations to be equal (since, as we have seen, their masses are nearly equal) the equivalent velocity of a protein ion at 30 degrees would appear to be about 20×10^{-5} cm. per sec. under a potential gradient of 1 volt per centimeter. Now Bredig (1) has shown that the equivalent velocities of heavy ions tend to attain, with increasing weight, a constant minimum value of about 15×10^{-5} cm. per sec. at 15 degrees. At 30 degrees this velocity would be increased by about 30 per cent, i.e., would become 20×10^{-5} cm. per sec.

Referring to Table XXVI it is evident that $\rho\,(u + v)$ is much larger for the salts which proteins form with acids than it is for the salts which they form with bases. It is about twice as large, in the case of serum globulin, as it is for the salts of diacid bases. We can hardly assume that the migration-velocity of protein ions is twice as great in acid as in alkaline solution and we must therefore adopt the alternative assumption that ρ is twice as great in acid as in alkaline solutions. This might arise by the splitting of *double* $-\mathrm{COH.N}-$ bonds, thus:

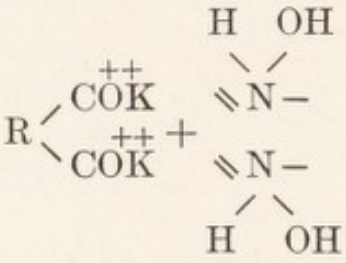


which indicates that, as Kossel has suggested (3) the active agents in bringing about the neutralization of acids by proteins

are the *diamino* radicals (hexone bases) which are contained in the protein molecule. As we shall see in the succeeding section of this chapter, the neutralization of bases by the proteins is analogously accomplished, at any rate for the greater part, by the dicarboxylic radicals which they contain; only the evidence which we have so far discussed does not reveal this fact because salts of the type



do not appear to exist, but only salts of the type



in which the ratio of the valencies of the cation to the number of equivalents of base included in the molecule of the protein salt is 2; the same as it would be for a salt formed by the opening up of a single $-COH.N-$ bond.

The results obtained with solutions of ovomucoid in dilute acids are complicated by the possible presence of free ovomucoid which is itself ionized and therefore contributes to the conductivity of these solutions. It is evident, however (Cf. Tables XXI and XXII), that the conductivity of a solution of ovomucoid sulphate is practically identical with that of a solution of ovomucoid chloride containing the same equivalent concentration (45×10^{-5} equivalents per gram) of neutralized HCl. Hence it follows that in the formation of these salts each equivalent of a dibasic acid must give rise to the same number of equivalents of protein salt that an equivalent of a monobasic acid produces. This conclusion also applies to the salts which the protamin, salmin, forms with acids (19).

2. The Depression of the Freezing-point of Water which is Caused by Dissolved Protein Salts, and the Stoichiometry of

Protein Salts. — We have hitherto found it convenient in discussing the mode of formation of the salts which the proteins form with bases, only to consider the consequences which arise out of the opening up of a single $-COH.N-$ bond, without involving a consideration of the question whether this $-COH.N-$ bond is supplied by a dicarboxylic acid group (such as glutamic acid), in which case *two* such bonds must be opened up before actual electrolytic dissociation can occur, or whether it is supplied by a mono-carboxylic acid group (such as glycocoll) in which case dissociation could occur directly one such group was opened up. We have been able to do this because, in the formation of salts with bases, all of the $-COH.N-$ groups which are opened up suffer the introduction of the basic radical and the equivalence between the protein salt and the base which gives rise to it is the same as that which would subsist were the salt formed by the opening up of only one $-COH.N-$ group. All of the preceding considerations and theoretical deductions, therefore, remain equally valid whether the $-COH.N-$ groups under consideration in each separate case are derived from a dicarboxylic acid radical or from a mono-carboxylic acid radical. Only, so far as our experimental knowledge goes, when we investigate the freezing-point depression (i.e., the osmotic pressure) of solutions of protein salts do we encounter facts which are definitely irreconcilable with the view that the protein salts with bases are formed through the agency of mono-carboxylic acid radicals and which point unmistakably to the *dicarboxylic acid radicals* as the active agents in accomplishing these unions.

It has been shown by Robertson and Burnett (20) that whether the caseinate formed is neutral to litmus and contains 50×10^{-5} equivalents of base per gram, or neutral to phenolphthalein and containing 80×10^{-5} equivalents of base per gram, *the freezing-point depression which is brought about by the dissolved protein salt bears the same proportion to the concentration of the neutralized base.*

The significance of this observation has been alluded to in the latter part of Chap. I. If we prepare a number of solutions all containing the same amount of a base and add to these varying amounts of a polybasic acid $R(COOH)_n$, such that in the first solution only one, in the second two, etc., $-COOH$ groups are neutralized, then, if all these salts are highly dissociated, calling the osmotic pressure of the first 1, that of the second

will be $\frac{3}{4}$, that of the third $\frac{2}{3}$, etc. But if, to solutions which contain the same amount of a base we add varying amounts of casein, the osmotic pressures of all of these solutions, as indicated by their freezing-points are the same. Evidently, therefore, a given quantity of base always gives rise to the same number of protein ions, whether the base is combined with more or with less protein. This obviously corresponds with the view that each equivalent of base opens up a given number of $-COH.N-$ groups, and not at all with the view that it neutralizes terminal $-COOH$ groups.

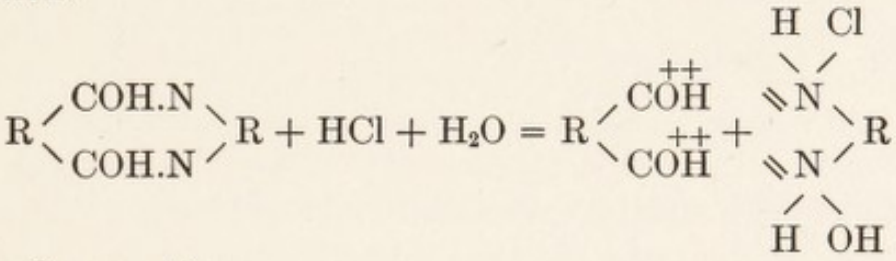
The experimental data which were obtained by Robertson and Burnett are enumerated in Tables I and II in Chap. XIII (p. 333). On referring to the results which are therein cited it will be observed that the depression of the freezing-point which is brought about by the dissolved caseinate of the alkalies is in every case very nearly that which would be observed in a solution of the same *molecular* concentration as that of the alkali which is neutralized by the casein. Now these solutions are quite extensively (over 80 per cent, Cf. Tables, section 1) dissociated and it therefore follows that each *molecule* of alkali gives rise to one *ion* of caseinate. That the same is true for the alkaline earths is readily seen when allowance is made for their comparatively slight degree of dissociation (Cf. Tables VI, VII and VIII). Now in the formation of a salt by the splitting of a single $-COH.N-$ bond each molecule of neutralized base would give rise to *two* ions of caseinate, while in the formation of a salt by the splitting of a double $-COH.N-$ bond one molecule of neutralized base might give rise to only *one* ion of caseinate. This will be clear from the following equations:

$$-COH.N- + NaOH = -CO\overset{++}{N}a + \diagdown N- \begin{matrix} H \\ | \\ OH \end{matrix}$$

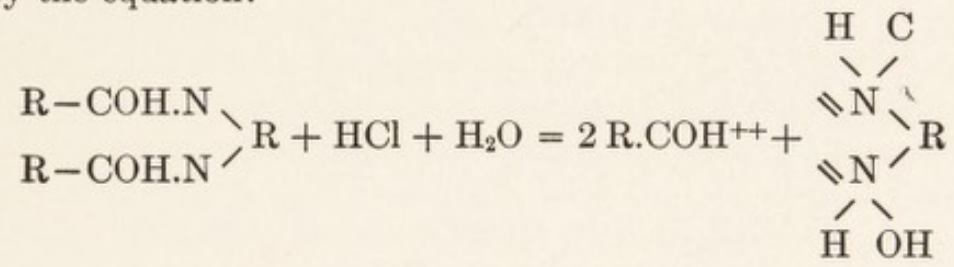
$$R\begin{matrix} \diagup COH.N \\ \diagdown COH.N \end{matrix}\diagup R + 2\,NaOH = R\begin{matrix} \diagup CO\overset{++}{N}a \\ \diagdown CO\overset{++}{N}a \end{matrix} + \begin{matrix} H\ OH \\ \diagdown\ \diagup \\ \diagdown N \\ \diagup\ \diagdown \\ H\ OH \end{matrix} R$$

Evidently, therefore, it is the latter alternative which represents the true mode of formation of these salts, and we must conclude that in the neutralization of bases the dicarboxylic radicals play a leading part, just as, in the neutralization of acids, the diamino radicals play a leading part.

We have seen that in the formation of ovomucoid chloride, containing 45×10^{-5} equivalents of acid per gram, only *one* of the $-COH.N-$ bonds which is opened up suffers the introduction of an HCl molecule. In this case, therefore, if the above reasoning has been correct, the freezing-point depression due to the dissolved salt should be either *twice* or *three times* that of a solution of the molecular concentration of the neutralized acid according as to whether the formation of the salt is represented by the equation:



or by the equation:



that is, according as to whether the diamino acid radicals in the ovomucoid molecule are directly united with both carboxyls of dicarboxylic acid radicals, or with only one or with monocarboxylic acid radicals. The experimental fact is that the freezing-point-depression of a 0.018 m. solution of HCl containing 4 per cent of ovomucoid (1 gram per 45×10^{-5} equivalents) is 0.09 ± 0.005 degree, corresponding to a 0.055 m. solution, or almost exactly *three* times the molecular concentration of the neutralized acid.

Hence we may conclude that the formation of this salt is represented by the latter of the above two equations and that in ovomucoid *the diamino radicals are not directly attached to both carboxyls of dicarboxylic radicals.*

Continuing the addition of acid (HCl) to the above solution of ovomucoid a remarkable phenomenon is observed, namely, that *doubling the amount of acid in the solution does not appreciably alter its freezing-point* * and, consequently does not alter the total number of ions per cubic centimeter of the solution. Evidently, upon further addition of acid, the remaining nitrogen atom of the diamino radical becomes neutralized and the ion

is formed.

The form of the dilution-law which we should apply to these solutions is therefore (Cf. equation (v)):

$$m = \frac{1.037 \times 10^{-2}}{\rho\,(u+v)}\,x + \frac{1.115 \times 10^{-6}}{\rho K\,(u+v)^3}\,x^3.$$

Applying this equation to the results enumerated in Table XIX we obtain $m = 5.63\,x + 117{,}000\,x^3$.

In the accompanying table the experimental and calculated values of m are compared. In the third column is given the degree of dissociation of the salt, estimated as the ratio of the calculated value of $5.63\,x$ to the calculated value of m.

TABLE XXVII. OVOMUCOID CHLORIDE

(45×10^{-5} equivalents HCl per gram)

$m \times 10^4$ (experimental)	$m \times 10^4$ (calculated)	Degree of dissociation, per cent
180	180	86
90	89	96
45	46	98
23	24	100
11	12	100

The same equation applies so nearly to the experimental results obtained with ovomucoid sulphate (Table XX) that it is not necessary to recompute the constants for this salt.

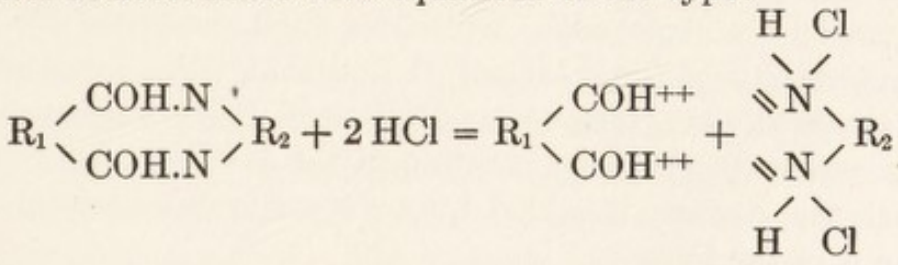
* The freezing-point-depression due to *free* ovomucoid is only *one-third* of that of the above solution, namely, 0.03 ± 0.005 degree for a 4 per cent solution.

From the value of $\frac{1.037 \times 10^{-2}}{\rho\,(u+v)}$ for the ovomucoid chloride we obtain:

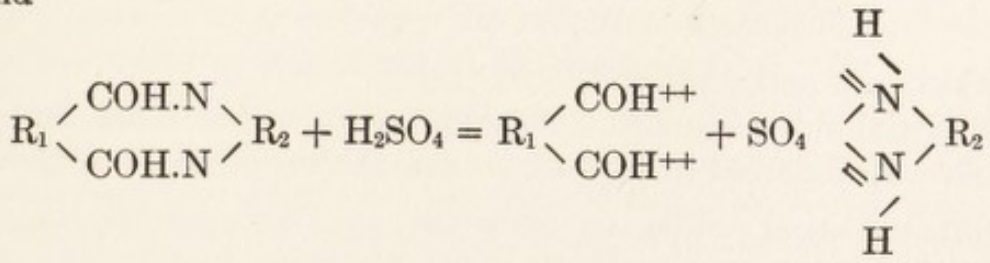
$$\rho\,(u+v) = 184 \times 10^{-5},$$

whence, if $\rho = 4$, $u + v = 46 \times 10^{-5}$, which agrees very well with the values obtained with other protein salts for this constant (Tables XXIV and XXV; Cf. also p. 235).

A one-half per cent solution of salmin hydrochloride freezes at a temperature of 0.04 degrees lower than distilled water. This corresponds to the freezing-point depression of a solution in which the molecular + ionic concentration is $\frac{m}{46}$. Now the equivalent concentration of HCl neutralized by salmin in a $\frac{1}{2}$ per cent solution of salmin hydrochloride is $\frac{m}{45}$. Each molecule of combined hydrochloric acid yields, therefore, one molecule or one ion of salmin hydrochloride. Salmin hydrochloride in $\frac{1}{2}$ per cent solution is very highly ionized, since dilution only increases its equivalent conductivity to a small extent and we must infer, therefore, since the salt exists in solution largely in the form of ions, that each molecule of combined acid yields one ion of the protein salt. One molecule of salmin hydrochloride contains at least four molecules of combined hydrochloric acid (3) (24), hence one molecule of salmin hydrochloride must yield four ions or a multiple thereof. From the value of the constant $\rho\,(u+v)$ in Table XXVI we must infer that the valency of each of these ions is 4. These phenomena obviously correspond with those which would be exhibited by a salt which forms and dissociates in accordance with equations of the type:



and



which are in satisfactory accord with the high diamino-acid content of the salmin molecule (4).

Summing up these results, therefore, we see that *in the formation of the salts of proteins with acids diamino radicals are primarily concerned, while in the formation of the salts of protein with bases dicarboxylic radicals are primarily concerned.* The correspondence between the diamino-acid content of a protein and its binding capacity for acids, to which Kossel has drawn attention (loc. cit.), and that between the dicarboxylic acid content and the combining capacity for bases to which I have drawn attention in the latter part of the first chapter is therefore seen to be not accidental but an expression of an essential feature of the union between proteins and inorganic acids and bases.

3. The Dependence of the Electrical Conductivity of Solutions of Protein Salts upon the Proportion of Inorganic Acid or Base which the Salt Contains. — In preceding paragraphs we have discussed the effects of *dilution* upon the conductivities of solutions of protein salts; that is, of altering the ratio $\frac{\text{water}}{\text{salt}}$ while keeping the composition of the salt; i.e., the proportion of protein to base or acid constant. We have found that these effects admit of interpretation in the light of the well-known laws of electrolytic dissociation and of the view that the dissociation of protein salts is accomplished by the splitting of $-\text{COH.N}-$ bonds. We must now take up the consideration of the effects of altering, not only the total concentration of the system, but also the proportion of protein to base or acid.

If we add protein to dilute solutions of an acid or base there usually results a depression, which we shall designate by λ, of the conductivity of the solution. I find that when 1 per cent of casein is added to varying concentrations of KOH this depression is connected with the concentration of the KOH solution ($= b_1$) to which the protein is added by a very simple relation. This relation is of the form

$$\lambda \times 10^{-5} = \alpha b_1 - \beta b_1^2 - \gamma \qquad \text{(vi)}$$

in which α, β and γ are constants (14).

Applying this formula to observations in which 1 per cent of casein was dissolved in varying concentrations of KOH * and the

* These solutions were the same as those employed in the gas-chain measurements cited in the previous chapter (1 per cent casein, second series), and,

conductivities of the solvent and of the solution were determined at 30 degrees, and computing the most probable values of the constants α, β and γ from all of the observations by the method of least squares, we obtain:

$$\lambda \times 10^5 = 26880\, b_1 - 475800\, b_1^2 - 28.98.$$

In the accompanying table (Table XXVIII) the experimental values of $\lambda \times 10^{-5}$ for 1 per cent solutions of casein in KOH of various concentrations ($= b_1$) and those calculated from the above formula are compared. In the first column are given the alkalinities of the solutions to which casein was added ($= b_1$); in the second are given the values of $\lambda \times 10^5$ experimentally ascertained; in the third the calculated values of $\lambda \times 10^5$; in the fourth the difference ($= \Delta$) between the experimental and the calculated values of $\lambda \times 10^5$; and in the fifth the possible metrical error ($= \epsilon$) in the experimental determination of $\lambda \times 10^5$.

TABLE XXVIII

b_1	$\lambda\times10^5$ (experimental)	$\lambda\times10^5$ (calculated)	Δ	ϵ
0.03000	348.8	349.3	+0.5	±8.0
0.02500	343.0	345.6	+2.6	±6.5
0.02000	322.1	318.3	−3.8	±5.5
0.01750	299.7	295.9	−3.8	±5.1
0.01500	268.2	267.2	−1.0	±4.7
0.01250	230.1	232.8	+2.7	±4.4
0.01000	186.8	192.3	+5.5	±4.1
0.00750	141.6	145.8	+4.2	±4.0
0.00500	92.1	93.5	+1.4	±3.8
0.00250	43.9	35.4	−8.5	±3.7
			$\Sigma\Delta = -0.2$	

It will be seen that the deviations of the calculated from the experimental values of $\lambda \times 10^5$ are nearly always less than the possible error, due to instrumental sources alone, in the experimental determination of $\lambda \times 10^5$, while the algebraic sum of these deviations is negligible. The formula therefore represents, in a highly satisfactory manner, the relation between b_1 and λ for 1 per cent solutions of casein in KOH-solutions.

therefore, contained varying amounts of KCl (Cf. Appendix). The fact that the irregularity in KCl-content does not disturb the regularity of the relation between λ and b_1 is further proof (Cf. Chap. VIII, **1**), that KCl, in moderate concentrations, does not appreciably influence the conductivity of potassium caseinate solutions.

Investigating the similar relations which obtain in solutions containing different percentages of casein we find that the relation between λ and b_1 for *all* of the concentrations of casein investigated can be represented by the more general formula:

$$\lambda \times 10^5 = \alpha b_1 - \frac{\beta}{C} b_1^2 - \gamma C, \qquad \text{(vii)}$$

where C is the percentage of casein and α, β and γ are constants, the values of which we have already determined for 1 per cent casein. For a 0.5 per cent solution of casein this equation, therefore becomes:

$$\lambda \times 10^5 = 26{,}880\, b_1 - 951{,}600\, b_1^2 - 14.49.$$

In the following table the experimental and calculated values of $\lambda \times 10^5$ are compared — the symbols have the same meaning as in Table XXVIII.

TABLE XXIX

b_1	$\lambda\times10^5$ (experimental)	$\lambda\times10^5$ (calculated)	Δ	ϵ
0.01000	149.9	159.2	+ 9.3	±2.3
0.00750	123.5	133.6	+10.1	±2.0
0.00500	88.2	96.1	+ 7.9	±1.8
0.00250	40.6	37.6	− 3.0	±1.8
0.00150	23.3	24.2	+ 0.9	±1.8

For a 1.5 per cent solution of casein equation (vii) becomes:

$$\lambda \times 10^5 = 26{,}880\, b_1 - 317{,}200\, b_1^2 - 43.47.$$

In the following table the experimental and calculated values of $\lambda \times 10^5$ are compared:

TABLE XXX

b_1	$\lambda\times10^5$ (experimental)	$\lambda\times10^5$ (calculated)	Δ	ϵ
0.03000	476.4	477.5	+ 1.1	±7.0
0.02000	366.5	367.3	+ 0.8	±5.1
0.01500	283.5	288.4	+ 4.9	±4.5
0.01000	188.5	193.6	+ 5.1	±4.1
0.00750	145.0	140.4	− 4.6	±3.9
0.00500	95.4	83.0	−12.4	±3.8

For a 2.0 per cent solution of casein equation (vii) becomes:

$$\lambda \times 10^5 = 26{,}880\, b_1 - 237{,}900\, b_1^2 - 57.96.$$

In the following table the experimental and calculated values of $\lambda \times 10^5$ are compared:

TABLE XXXI

b_1	$\lambda\times10^5$ (experimental)	$\lambda\times10^5$ (calculated)	Δ	ϵ
0.05000	661.6	691.4	+29.8	±18.5
0.03000	539.3	534.4	− 4.9	±11.5
0.02000	380.5	384.5	+ 4.0	± 7.6
0.01500	286.2	291.7	+ 5.5	± 7.1
0.01000	189.3	187.1	− 2.2	± 6.7
0.00500	95.7	70.5	−25.2	± 6.2

For a 3 per cent solution of casein equation (vii) becomes:

$$\lambda \times 10^5 = 26{,}880\, b_1 - 158{,}600\, b_1^2 - 86.94.$$

In the following table the experimental and calculated values of $\lambda \times 10^5$ are compared:

TABLE XXXII

b_1	$\lambda\times10^5$ (experimental)	$\lambda\times10^5$ (calculated)	Δ	ϵ
0.05000	873.2	860.7	−12.5	±16.4
0.03000	579.7	576.8	− 2.9	± 9.0
0.02500	488.9	486.0	− 2.9	± 8.4
0.02000	391.7	387.3	− 4.4	± 7.6
0.01500	296.9	280.6	−16.3	± 7.2
0.01000	206.6	166.1	−40.5	± 6.8

The same relation holds good for 1.5 per cent solutions of casein in $Ca(OH)_2$ solutions of varying concentration (equivalent concentration = b_1) (17).

To 50 cc. portions of a 3 per cent solution of casein in 0.024 N $Ca(OH)_2$ (neutral to phenolphthalein) were added 0, 2, 4, 6, or 12 cc. of 0.048 N $Ca(OH)_2$ and each mixture was diluted to 100 cc. Exactly similar solutions were made up, containing however, no casein. The conductivities of each of these solutions were then determined at 30 degrees. Applying equation

(vii) to these results and computing the constants from the first, fourth and sixth observations,* we obtain:

$$\lambda \times 10^5 = 30{,}830\, b_1 - 488{,}000\, b_1^2 - 53.4.$$

In the following table the experimental and calculated values of $\lambda \times 10^5$ are compared:

TABLE XXXIII

b_1	$\lambda \times 10^5$ (experimental)	$\lambda \times 10^5$ (calculated)
0.01200	246.3	246.3
0.01296	266.9	264.2
0.01392	281.8	281.1
0.01488	297.5	297.5
0.01584	312.6	312.5
0.01776	340.4	340.4

Formula (vii) also applies to 1 per cent solutions of ovomucoid in varying concentrations of KOH and HCl, only in the *acid* solutions we must replace b_1 by a_1, the acidity of the solution to which the ovomucoid is added. For 1 per cent ovomucoid in KOH solutions, computing the constants by the method of least squares, we obtain:

$$\lambda \times 10^5 = 20{,}850\, b_1 - 1{,}250{,}000\, b_1^2 - 35.6.$$

In the following table the experimental and calculated values of $\lambda \times 10^5$ are compared:

TABLE XXXIV

b_1	$\lambda \times 10^5$ (experimental)	$\lambda \times 10^5$ (calculated)
0.0005	−26.8	−25.5
0.0010	−16.0	−16.0
0.0020	+ 1.1	+ 1.1
0.0030	+15.7	+15.7
0.0050	+38.2	+38.2

For 1 per cent ovomucoid in HCl solutions, computing the constants by the method of least squares, we obtain:

$$\lambda \times 10^5 = 41{,}990\, a_1 - 852{,}700\, a_1^2 - 41.4.$$

* These results do not lend themselves to determination of the constants by the method of least squares, since the normal equations are nearly identical, the factors differing only by magnitudes commensurate with the experimental error.

In the following table the experimental and calculated values of $\lambda \times 10^5$ are compared:

TABLE XXXV

a_1	$\lambda \times 10^5$ (experimental)	$\lambda \times 10^5$ (calculated)
0.0005	− 17.7	− 20.6
0.0010	+ 2.0	− 0.3
0.0020	+ 37.7	+ 39.2
0.0030	+ 74.5	+ 76.9
0.0040	+111.1	+113.0
0.0050	+146.2	+147.2
0.0075	+228.9	+225.5
0.0100	+291.8	+293.2

The relation does not, however, hold good for solutions of casein in HCl. Doubtless it would hold good for these solutions also were it possible to obtain solutions of casein in dilute HCl, without at the same time introducing the chloride of the base employed to *dissolve* the dry casein. But, as I have explained in Chap. V, dry casein is not readily dissolved by dilute acids, and in order to secure a solution of casein in acid it is necessary to first dissolve the casein in dilute alkali, and then add acid in excess of that necessary to neutralize the base and sufficient to redissolve the wet, freshly precipitated casein. Now casein, dissolved in *acids* is very markedly influenced in its solubility by the presence of salts; the departure of the conductivities of these solutions, therefore, from the regularity indicated in equation (vii) is sufficiently explicable.

The physico-chemical significance of this relation is, at the present stage of our knowledge, not at all clear. It is possible that equation (vii) is merely an interpolation-equation and that the relation is of purely empirical significance.* It is chiefly of use, at present, as we shall see, in throwing light upon questions

* It should here be noted that any relationship of this type must involve the mutual dependence of the following factors:

(i) The variation of m, the amount of acid or base bound by the protein with variation in a_1 or b_1, the concentration of acid or alkali in which the protein is dissolved.

(ii) The variation of K (the dissociation-constant of the salt) and possibly of $(u + v)$ with the quantity of acid or alkali bound by the protein.

(iii) The effect of dilution upon the conductivities of the protein salts.

involving the minimal combining capacity of proteins, such as casein, which are insoluble in the free condition.

4. The Solubility and Minimal Combining Capacity of Casein and of Serum Globulin in Solutions of Bases. — We have seen that the relation between b_1, the concentration of a KOH-solution in which casein is dissolved, and λ, the depression of its conductivity which is caused by the casein, are connected by the relation

$$\lambda \times 10^5 = 26{,}880\, b_1 - \frac{475{,}800}{C} b_1^2 - 28.98\, C;$$

putting λ equal to zero we obtain

$$b_1 = 0.00114\, C \text{ or } 0.05536\, C.$$

Considering, for the present, only the smaller value of b_1, we see that when $\lambda = 0$, that is, when the change in the conductivity of an alkaline solution which is brought about by dissolving a given percentage of casein is zero, then the proportion of alkali to casein is such that one gram of casein is combined with 11.4×10^{-5} equivalents of alkali. *This is precisely the combining capacity of casein at "saturation" of the base with casein, that is, when the base has dissolved the maximum amount of casein which it will dissolve.*

The exact coincidence of the two numerical values, especially when we consider that the above is computed by least squares from a number of determinations which are apparently not connected with estimates of the *solubility* of casein, is surprising, and leaves no room for doubt that the magnitude of the two quantities is determined by identical factors. This result is probably to be interpreted as follows: The conductivity ($= x_1$) of the solution of base to which casein is added is μb_1, where μ is the equivalent molecular conductivity of the base and b_1 the equivalent concentration of the base. The conductivity ($= x$) of the solution containing casein is $\mu b + 96.43\,(u + v)\, z$, where b is the equivalent concentration of the unneutralized base, $u + v$ is the sum of the equivalent mobilities of the ions and z is the equivalent concentration of protein ions. Assuming that at the concentrations employed the casein salt is tolerably completely dissociated $z = \rho m$, where m is the equivalent concentration of base neutralized by the protein and ρ the number

of equivalents of protein salt to which one equivalent of base gives rise. Hence:

$$\lambda = x_1 - x = \mu m - 96.43\,\rho\,(u + v)\,m.$$

When $m = 0$, therefore, that is, when the quantity of base is insufficient to combine with the given mass of casein, λ must be 0. Hence the value of b_1 when $\lambda = 0$ is a true measure of the minimum combining capacity of casein.

For 1.5 per cent solutions of casein in calcium hydrate solutions we found that

$$\lambda \times 10^{-5} = 30{,}830\,b_1 - 488{,}000\,b_1^2 - 53.4;$$

putting $\lambda = 0$ and estimating b_1 we find that

$$b_1 = 0.001782;$$

dividing this by 1.5, the percentage of casein, we find that $\lambda = 0$ when the equivalence between the casein and the calcium hydrate is

$$1 \text{ gram} = 11.9 \times 10^{-5} \text{ equivalents};$$

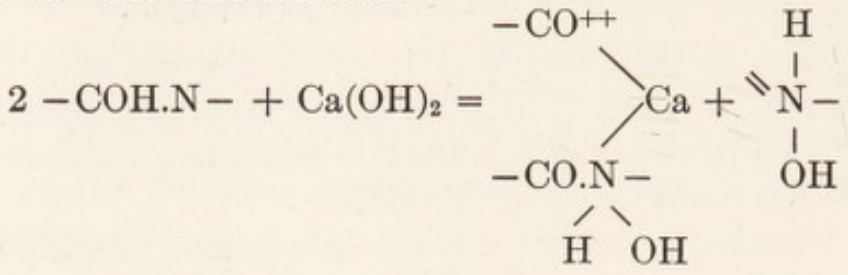
a value so near that (11.4×10^{-5}) obtained for the alkalies that they may be regarded as being, within the experimental error, identical.

In the previous (German) edition of this work I stated that we were justified in the light of these results in tentatively assuming, pending more direct evidence, that the various bases dissolve casein in equivalent-molecular proportions. Since then Van Slyke and Bosworth have shown (25), by direct analytical determinations, employing dialysis to remove the chloride formed on neutralizing the excess of base with hydrochloric acid, that the minimal proportion of a diacid base which will *dissolve* casein is about 23×10^{-5} equivalents but that an *insoluble* compound is formed containing 11.3 equivalents of the base. In other words the minimal equivalent combining capacity of casein for bases is the same for diacid as monacid bases.

We have seen (**1**) that each $-\text{COH.N}-$ bond of casein which is opened up by the entrance of a KOH molecule reacts with the base in accordance with the equation:

$$-\text{COH.N}- + \text{KOH} = \text{CO}\overset{++}{\text{K}} + \diagdown\!\!\!\!\diagup \overset{\overset{\text{H}}{|}}{\underset{\underset{\text{OH}}{|}}{\text{N}}}-$$

while in reacting with diacid bases two $-COH.N-$ groups are involved, part of the valencies of the ions which are formed suffering internal neutralization thus:



If this view be correct, then one molecule of calcium hydrate must obviously bind twice the weight of casein that is bound by one molecule of KOH. Hence di- and monacid bases unite with casein in equivalent-molecular proportions, but at "saturation" of the base with casein, when the mass of the protein ions is at its maximum, the cations formed as a result of the union with diacid bases, being much more bulky than those formed by the union with monacid bases, fail to pass into solution. Hardy (2) has found that bases dissolve serum-globulin, not in *equivalent molecular* but in *molecular* proportions. It is probable that in this case also an insoluble salt is formed with the minimal proportion of diacid base.

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CHAPTER XI

THE ELECTROCHEMISTRY OF COAGULATION

1. The Coagulation of the Caseinates by Alcohol. — If one-half a cubic centimeter of a 0.0125 *N* solution of KOH, neutralized either to phenolphthalein or to litmus by the addition of casein, be added to 10 cc., i.e., to 20 volumes of 99.8 per cent alcohol, no coagulation of the protein occurs, although the solution which is thus obtained is appreciably more opalescent than a solution of equal concentration in water containing no alcohol. Even if, instead of employing a solution of potassium caseinate in water, we employ a 0.0125 *N* solution of KOH in 75 per cent alcohol, containing 1.6 or 2.5 per cent of casein, adding $\frac{1}{2}$ cc. of this to 10 volumes of 99.8 per cent alcohol, still no coagulation of the caseinate occurs, although it is now dissolved or forms a stable suspension in a 98.6 per cent solution of alcohol. The caseinate can, however, be readily coagulated by adding to this mixture an equal volume of ether and allowing it to stand for a few hours.

Very different is the behavior of the caseinates of the alkaline earths. If to 10 cc. each of 60, 70, 75 per cent, etc., solutions of alcohol we add $\frac{1}{2}$ cc. of a 0.012 *N* solution of $Ca(OH)_2$, neutralized to phenolphthalein by casein, distinct coagulation of the caseinates occurs, on shaking, when the final concentration of alcohol in the mixture is about 55 per cent. The same is true for an equally concentrated solution of barium hydroxide neutralized to phenolphthalein by casein. For strontium caseinate the limiting concentration of alcohol at which coagulation occurs is much higher, about 70 per cent. In all cases coagulation is much accelerated by energetic shaking of the mixture.

It is of interest to compare the marked difference between the behavior of the caseinates of the alkalies and of the alkaline earths upon the addition of alcohol to their aqueous solutions, with the numerous other differences which subsist between the caseinates of the alkalies and those of the alkaline earths.

Thus the aqueous solutions of the "neutral" or "basic" casein-

ates of the alkalies and ammonium are clear, do not, except lithium caseinate, show any increase in turbidity on warming, and are not precipitated by the addition of finely divided insoluble substances or by passing through a clay filter; while the aqueous solutions of the caseinates of the alkaline earths are opalescent, show marked increase in turbidity on heating their solutions to 35–45° C. which disappears on cooling, and are precipitated from their solutions by the addition of finely divided insoluble substances or by passage through a clay filter (4).

The "neutral" or "basic" caseinates of the alkalies, upon solution in water, depress its freezing point and the amount of the depression is that of a solution of the same molecular concentration as the alkaline solution which is employed as solvent. The depression produced by calcium caseinate is much less, being less than half as great (10).

Equally concentrated solutions of the hydroxides of the alkalies and ammonium dissolve casein at approximately the same rate. Solutions of the hydroxides of the alkaline earths dissolve casein much more slowly (8). The rate of the solution of casein by solutions of the hydroxides of the alkalies is accelerated by raising the temperature above 36 degrees; the rate of the solution of casein by solutions of calcium hydroxide is materially diminished by a similar rise in temperature (6).

The caseinates of the alkaline earths are precipitated from their solutions by the addition of small concentrations of the chloride of the corresponding alkaline earth (7); the caseinates of the alkalies are not precipitated by the addition of similar quantities of the chlorides of the alkalies.

I have alluded to the fact that the limiting concentration of alcohol at which strontium caseinate is coagulated is considerably higher than that at which calcium and barium caseinates are coagulated. This is curious because we should expect, as a rule, to find the salts of strontium possessed of properties intermediate between those of calcium and barium. It is therefore the more interesting to observe that this is not the only respect in which the caseinates of strontium exhibit behavior differing from that of the caseinates of calcium and barium and approaching the behavior of the caseinates of the alkalies; for casein dissolves much more rapidly in solutions of strontium hydroxide than in solutions of other alkaline earth hydroxides (8); the

solutions of strontium caseinate are less opalescent than equally concentrated solutions of calcium or barium caseinate, and the dissociation-constant of "basic" strontium caseinate is much larger than those of barium or calcium caseinate, and is intermediate in magnitude between these and the dissociation constants of the "basic" caseinates of sodium and ammonium. This, however, is not a general phenomenon where protein salts of the alkaline earths are concerned, because the dissociation constant for the strontium salt of "insoluble" serum globulin is not very appreciably different from those of the calcium and barium salts (Cf. previous chapter).

The caseinates of the alkalies and alkaline earths dissolved in various concentrations of alcohol afford, therefore, very favorable material for the investigation of the intimate nature of the process of coagulation, since different caseinates, in the presence of the same quantity of alcohol, may be, to all appearance completely soluble, or completely coagulated, and as we shall see, a comparison of the behavior of these different caseinates towards the presence of varying percentages of alcohol in their solutions leads to a very considerable insight into the chemical mechanics of protein-coagulation by alcohol.

2. The Applicability of the Ostwald Dilution-Law to Solutions of the Caseinates in Alcohol-Water Mixtures. — In the preceding chapters it has been shown that the Ostwald dilution-law for a binary electrolyte written in the form

$$m = \frac{1.037 \times 10^{-2}}{\rho\,(u+v)}x + \frac{1.075 \times 10^{-4}}{\rho K\,(u+v)^2}x^2 \qquad \text{(i)}$$

is applicable to many aqueous solutions of the salts which proteins form with inorganic acids and bases, m being the equivalent molecular concentration of the acid or base which is combined with the protein, ρ the number of equivalents of protein salt to which one equivalent of neutralized acid or base gives rise, x the conductivity of the solution in reciprocal ohms per cubic centimeter, K the dissociation constant of the salt and $u + v$ the sum of the equivalent specific migration-velocities of the ions at infinite dilution. The same law applies to solutions of potassium caseinate (80×10^{-5} equivalents KOH per gram) in alcohol-water mixtures which contain up to and less than 60 per cent alcohol, as the following experimental results show (9).

In five hundred cubic centimeters of $N/10$ KOH were dissolved 62.5 grams of casein, thus forming a solution of potassium caseinate, neutral to phenolphthalein. To 50 cc. portions of this solution were added, respectively, 0, 20, 40, etc., cubic centimeters of alcohol, and the mixtures were diluted to 200 cc. with water. Each of these solutions was then diluted, by the addition of alcohol of the same concentration, to the desired concentrations of casein. All alcohol percentages are percentages of 99.8 per cent alcohol by volume. The solutions in "75 per cent alcohol" were made up by adding 99.8 per cent alcohol to 50 cc. of the original solution of caseinate until the volume was 200 cc., and diluting with a 75 per cent by volume solution of 99.8 per cent alcohol. The conductivity determinations were made at 30° C. The conductivity of the distilled water ($= 4 \times 10^{-6}$) has been subtracted from each of the tabulated conductivities. The results are given in Table I on the following page.

Applying the above equation to these results and computing the constants $\frac{1.037 \times 10^{-2}}{\rho\,(u+v)}$ and $\frac{1.075 \times 10^{-5}}{\rho K\,(u+v)^2}$ for each concentration of alcohol from all of the results by the method of least squares, we obtain:

For the solutions containing 0 per cent of alcohol:

$$m = 12.41\,x + 1666\,x^2. \qquad \text{(ii)}$$

For the solutions containing 10 per cent of alcohol:

$$m = 15.97\,x + 3325\,x^2. \qquad \text{(iii)}$$

For the solutions containing 20 per cent of alcohol:

$$m = 21.14\,x + 5880\,x^2. \qquad \text{(iv)}$$

For the solutions containing 30 per cent of alcohol:

$$m = 27.23\,x + 10{,}858\,x^2. \qquad \text{(v)}$$

For the solutions containing 40 per cent of alcohol:

$$m = 34.63\,x + 18{,}931\,x^2. \qquad \text{(vi)}$$

For the solutions containing 50 per cent of alcohol:

$$m = 42.91\,x + 35{,}937x^2. \qquad \text{(vii)}$$

For the solutions containing 60 per cent of alcohol:

$$m = 53.49\,x + 78{,}335\,x^2. \qquad \text{(viii)}$$

For the solutions containing 75 per cent of alcohol:

$$m = 29.95\,x + 1{,}219{,}800\,x^2. \qquad \text{(ix)}$$

TABLE I

Alcohol = 0 per cent by volume		Alcohol = 10 per cent by volume		Alcohol = 20 per cent by volume		Alcohol = 30 per cent by volume	
Equivalent concentration of KOH neutralized by casein	x = conductivity in reciprocal ohms per cc. at 30 degrees	Equivalent concentration of KOH neutralized by casein	x = conductivity in reciprocal ohms per cc. at 30 degrees	Equivalent concentration of KOH neutralized by casein	x = conductivity in reciprocal ohms per cc. at 30 degrees	Equivalent concentration of KOH neutralized by casein	x = conductivity in reciprocal ohms per cc. at 30 degrees
0.0250	1648×10^{-6}	0.0250	1241×10^{-6}	0.0250	938×10^{-6}	0.0250	715×10^{-6}
0.0125	890×10^{-6}	0.0125	677×10^{-6}	0.0125	509×10^{-6}	0.0125	391×10^{-6}
0.0063	482×10^{-6}	0.0063	368×10^{-6}	0.0063	274×10^{-6}	0.0063	213×10^{-6}
0.0031	258×10^{-6}	0.0031	199×10^{-6}	0.0031	149×10^{-6}	0.0031	116×10^{-6}
0.0016	138×10^{-6}	0.0016	107×10^{-6}	0.0016	80×10^{-6}	0.0016	62×10^{-6}
Alcohol = 40 per cent by volume		Alcohol = 50 per cent by volume		Alcohol = 60 per cent by volume		Alcohol = 75 per cent by volume	
Equivalent concentration of KOH neutralized by casein	x = conductivity in reciprocal ohms per cc. at 30 degrees	Equivalent concentration of KOH neutralized by casein	x = conductivity in reciprocal ohms per cc. at 30 degrees	Equivalent concentration of KOH neutralized by casein	x = conductivity in reciprocal ohms per cc. at 30 degrees	Equivalent concentration of KOH neutralized by casein	x = conductivity in reciprocal ohms per cc. at 30 degrees
0.0250	554×10^{-6}	0.0250	429×10^{-6}	0.0250	319×10^{-6}	0.0250	130×10^{-6}
0.0125	305×10^{-6}	0.0125	240×10^{-6}	0.0125	182×10^{-6}	0.0125	94×10^{-6}
0.0063	171×10^{-6}	0.0063	131×10^{-6}	0.0063	101×10^{-6}	0.0063	59×10^{-6}
0.0031	92×10^{-6}	0.0031	74×10^{-6}	0.0031	58×10^{-6}	0.0031	37×10^{-6}
0.0016	49×10^{-6}	0.0016	39×10^{-6}	0.0016	30×10^{-6}	0.0016	20×10^{-6}

Inserting the observed values of x in equations (ii) to (ix), we can compute the "theoretical" values of m, that is, the equivalent concentration of KOH neutralized by the casein which should, provided the dilution-law holds good, correspond to the observed conductivities. In Table II, on the following page, the observed and calculated values of m are compared.

It is evident that the correspondence between the experimental results and those which are indicated by the Ostwald dilution-law for a binary electrolyte is all that could be desired for the solutions containing from 0 to 60 per cent of alcohol. For the solutions containing 75 per cent of alcohol the divergences between theory and experiment are, however, considerably greater than could be accounted for by experimental error and probably indicate that the law does not hold good for these solutions. The sudden alteration in the relative values of the constants which occurs when the percentage of alcohol attains this magnitude indicates, whether the Ostwald law be considered as holding good or not, a profound change in the molecular and ionic condition of the protein.

It would appear, however, that we are justified in concluding that potassium caseinate dissociates in alcohol-water mixtures containing from 0 to 60 per cent of alcohol in the same manner as it does in aqueous solutions.

3. The Influence of the Concentration of Alcohol in the Solvent upon the Conductivities of Solutions of Potassium Caseinate. — I find (9) that the conductivity ($\equiv x_y$) of a solution containing any given concentration of casein combined with KOH in the proportion requisite to secure neutrality to phenolphthalein, and dissolved in an alcohol-water mixture containing y per cent of alcohol by volume is connected with the conductivity ($\equiv x_0$) of a similar solution in water by the formula

$$x_y = \frac{x_0}{A^y} \qquad \text{(x)}$$

where A is a constant which varies but slightly, if at all, with the concentration of the caseinate. This constant is determined in the following manner: If the logarithms to base 10 of x corresponding to $y = 0$, 10, 20, 30, 40, 50, and 60 per cent be tabulated in that order and each successive value of $\log x$ be subtracted from the one lying immediately above it, the differences thus

TABLE II

Alcohol = 0 per cent by volume		Alcohol = 10 per cent by volume		Alcohol = 20 per cent by volume		Alcohol = 30 per cent by volume	
$m \times 10^4$ observed	$m \times 10^4$ calculated	$m \times 10^4$ observed	$m \times 10^4$ calculated	$m \times 10^4$ observed	$m \times 10^4$ calculated	$m \times 10^4$ observed	$m \times 10^4$ calculated
250	250 0	250	250 0	250	251 −1	250	250 0
125	124 −1	125	123 −2	125	123 −2	125	123 −2
63	64 +1	63	63 0	63	62 −1	63	63 0
31	33 +2	31	33 +2	31	33 +2	31	32 +1
16	16 0	16	17 +1	16	17 +1	16	17 +1
	$\Sigma\Delta = +2$		$\Sigma\Delta = +1$		$\Sigma\Delta = +1$		$\Sigma\Delta = \pm 0$
Alcohol = 40 per cent by volume		Alcohol = 50 per cent by volume		Alcohol = 60 per cent by volume		Alcohol = 75 per cent by volume	
$m \times 10^4$ observed	$m \times 10^4$ calculated	$m \times 10^4$ observed	$m \times 10^4$ calculated	$m \times 10^4$ observed	$m \times 10^4$ calculated	$m \times 10^4$ observed	$m \times 10^4$ calculated
250	250 0	250	250 0	250	251 +1	250	245 −5
125	123 −2	125	124 −1	125	124 −1	125	136 +11
63	63 0	63	62 −1	63	62 −1	63	62 −1
31	34 +3	31	34 +3	31	34 +3	31	28 −3
16	17 +1	16	17 +1	16	17 +1	16	11 −5
	$\Sigma\Delta = +2$		$\Sigma\Delta = +2$		$\Sigma\Delta = +3$		$\Sigma\Delta = -3$

computed are observed to be appreciably constant and equal to ten times the log to base 10 of A in the above equation. The average value of A for all of the determinations enumerated in Table I (omitting, in the computation, the solutions containing 75 per cent alcohol) is 1.0265. For the various concentrations of potassium caseinate the values of A are as follow:

TABLE III

Concentration of KOH neutralized by casein	A
0.0250	1.0279
0.0125	1.0270
0.0063	1.0265
0.0031	1.0252
0.0016	1.0257
	Average 1.0265

In the accompanying table (Table IV) the experimental values of x and those computed from the formula

$$\frac{x_y}{x_0} = \frac{1}{A^y}$$

are compared. Two "calculated" values of x_y are given, the first calculated value of x_y being computed from the average value of A for the given concentration of caseinate, the second being computed from the average value of A for all the determinations enumerated in Table I, with the omission, of course, of those for the solvent containing 75 per cent of alcohol.

The same relation also holds good for solutions of "neutral" potassium caseinate, that is, for solutions which contain 50×10^{-5} equivalents of base per gram and are neutral to litmus instead of, as in the experiments cited above, 80×10^{-5} equivalents of base per gram (neutral to phenolphthalein). Two and a half per cent solutions of casein in 0.0125 N KOH were prepared, containing 0, 10, 20, 50, and 75 per cent of alcohol respectively The relation as we shall see, does not hold good for the solution containing 75 per cent alcohol and so, in computing the value of A for these solutions, we must omit the determination for the solution containing 75 per cent alcohol. The average difference in $\log_{10} x_y$ for 10 per cent increase in y is computed by adding

TABLE IV

Concentration of KOH neutralized by casein			Concentration of KOH neutralized by casein			Concentration of KOH neutralized by casein			Concentration of KOH neutralized by casein			Concentration of KOH neutralized by casein		
0.0250			0.0125			0.0063			0.0031			0.0016		
y	$x \times 10^5$ exper.	$x \times 10^5$ calc.	y	$x \times 10^5$ exper.	$x \times 10^5$ calc.	y	$x \times 10^5$ exper.	$x \times 10^5$ calc.	y	$x \times 10^5$ exper.	$x \times 10^5$ calc.	y	$x \times 10^5$ exper.	$x \times 10^5$ calc.
0	165	165	0	89	89	0	48	48	0	25.8	25.8	0	13.8	13.8
10	124	125	10	68	68	10	37	37	10	19.9	20.1	10	10.7	10.7
20	94	95	20	51	52	20	27	28	20	14.9	15.7	20	8.0	8.3
30	72	72	30	39	40	30	21	22	30	11.6	12.2	30	6.2	6.4
40	55	55	40	31	31	40	17	17	40	9.2	9.5	40	4.9	5.0
50	43	42	50	24	23	50	13	13	50	7.4	7.4	50	3.9	3.9
60	32	32	60	18	18	60	10	10	60	5.8	5.8	60	3.0	3.0
75	13	21	75	9	12	75	6	7	75	3.7	4.0	75	2.0	2.1
		23			13			7			3.6			1.9

together the three observed differences (between x_y corresponding to $y = 0$ and 10 per cent; 10 and 20 per cent; 20 and 50 per cent) and dividing their sum by 5. In this way A was ascertained to possess, for these solutions (at 30 degrees) the average value 1.0248. In the following table the actual and computed values of x_y are compared:

TABLE V. CONCENTRATION OF KOH NEUTRALIZED BY CASEIN 0.0125 N

y	$x_y \times 10^5$ (observed)	$x_y \times 10^5$ (calculated)
0	87	87
10	66	68
20	51	53
50	25	25
75	8	14

It is evident that the law holds good for solutions containing from 0 to 60 per cent of alcohol inclusive, but that it does not hold good for solutions containing 75 per cent alcohol, since for these solutions the calculated and observed values of x_y are widely divergent. Moreover the law does *not* hold good for solutions of the caseinates of the alkaline earths in alcohol-water mixtures; this is evidenced by the lack of constancy in the first differences of $\log_{10} x$ corresponding to equal increments of y. In the accompanying table are given the values of $\log_{10} x$ corresponding to various values of y for solutions of calcium caseinate at 30 degrees, containing 1.5 per cent of casein combined with 80×10^{-5} equivalents of base per gram.

TABLE VI

y = per cent of alcohol by volume	$\log_{10} x \times 10^5$	Differences
0	1.24797	
		0.37291
10	0.87506	
		0.30686
20	0.56820	
		0.22578
30	0.34242	
		0.13830
40	0.20412	
		0.12494
50	0.07918	

In the following table are given the corresponding data for solutions of strontium caseinate at 30 degrees, containing 1.5 per

cent of casein combined with 80×10^{-5} equivalents of base per gram:

TABLE VII

y = per cent of alcohol by volume	$\log_{10} x \times 10^5$	Differences
0	1.39967	
		0.30625
10	1.09342	
		0.29408
20	0.79934	
		0.17609
30	0.62325	
		0.11810
40	0.50515	
		0.14342
50	0.36173	

It is perhaps not without significance that in the above table the first two differences are much more alike than the succeeding differences. In solutions containing 0 to 20 per cent of alcohol, strontium caseinate is further from coagulation than corresponding solutions of calcium caseinate and, as we have seen, A, in the equation $x_y = \frac{x_0}{A^y}$, approaches constancy. Having regard to the observed failure of the solutions of potassium caseinate in 75 per cent alcohol to conform to the law, and to the fact that at an alcohol-concentration lying between 60 and 75 per cent, solutions of potassium caseinate undergo a great and relatively sudden increase in opacity, we may, I think, conclude that *the coagulation of a caseinate by alcohol is heralded by a failure of the solution to conform to the law* $x_y = \frac{x_0}{A^y}$, *connecting the percentage of alcohol in the solution with its conductivity.*

4. The Interpretation of the Law $x_y = \frac{x_0}{A^y}$. — As we have observed (Table III) the value of A is not only constant for all dilutions of potassium caseinate, but also is very nearly constant for all of the proportions of alcohol to water employed as solvents. Provided A were rigorously constant we would obviously obtain the relation:

$$\frac{x_{H_2O}}{x_{\text{alc. } H_2O}} = \text{constant}$$

for any given proportion of alcohol to water. This is the relation, suggested by Cohen (2), connecting the conductivities of inorganic salts in mixtures of alcohol and water. Roth (12), however, found that this rule does not strictly hold good for

solutions of KCl in mixtures of alcohol and water, but that the quotient $\frac{x_{H_2O}}{x_{alc.\ H_2O}}$ decreases somewhat with increasing dilution. In the accompanying table (page 264) the values of $\frac{x_{H_2O}}{x_{alc.\ H_2O}}$ are given for solutions of potassium caseinate (containing 80×10^{-5} equivalents of base per gram, Cf. Table I) containing each of the proportions of alcohol to H_2O employed.

It will be observed that the values of $\frac{x_{H_2O}}{x_{alc.\ H_2O}}$ are appreciably constant, for each proportion of alcohol to water, for the solutions containing 0 to 60 per cent of alcohol, but that in the solutions containing 75 per cent of alcohol the value of this ratio does not even approximate to constancy but decreases rapidly. There is, it is true, a slight but regular diminution in the value of this ratio even in the solutions containing 0 to 60 per cent of alcohol, in passing from an equivalent concentration of neutralized KOH of 0.0250 to 0.0016, but, except in the solutions containing 75 per cent of alcohol, the diminution is not more than a few per cent.

We have seen (equations (ii) to (ix)) that the Ostwald dilution-law, in the form expressed in equation (i) holds good, at any rate for solutions of potassium caseinate in alcohol-water mixtures containing 0 to 60 per cent of alcohol.

From the symmetry of the equation:

$$m = \frac{1.037 \times 10^{-2}}{\rho\,(u+v)}\,x + \frac{1.075 \times 10^{-4}}{\rho K\,(u+v)^2}\,x^2,$$

it would follow, were A strictly constant for all dilutions, that the proportions of alcohol to water employed as solvent for the caseinate affects only $u + v$, i.e., the migration-velocities of the ions, and not the dissociation-constant ($= K$) or the number of equivalents of caseinate resulting from the neutralization of one molecule of KOH ($= \rho$).* Since the alteration in A with dilution of the potassium caseinate is so small for solvents containing

* For, since x appears in one term of the equation as the first power, and in the second as the square, if the ratio of $\frac{x_{H_2O}}{x_{alc.\ H_2O}}$ is constant any other factor in the equation which is affected by the presence of alcohol must also appear in the one term of the equation as the first power and in the other as the square.

TABLE VIII

Alcohol 10 per cent		Alcohol 20 per cent		Alcohol 30 per cent	
Equivalent concentration of KOH neutralized by casein	$\frac{x_{H_2O}}{x_{alc.\ H_2O}}$	Equivalent concentration of KOH neutralized by casein	$\frac{x_{H_2O}}{x_{alc.\ H_2O}}$	Equivalent concentration of KOH neutralized by casein	$\frac{x_{H_2O}}{x_{alc.\ H_2O}}$
0.0250	1.33	0.0250	1.76	0.0250	2.31
0.0125	1.31	0.0125	1.75	0.0125	2.28
0.0063	1.31	0.0063	1.76	0.0063	2.26
0.0031	1.30	0.0031	1.73	0.0031	2.22
0.0016	1.29	0.0016	1.73	0.0016	2.22

Alcohol 40 per cent		Alcohol 50 per cent		Alcohol 60 per cent		Alcohol 75 per cent	
Equivalent concentration of KOH neutralized by casein	$\frac{x_{H_2O}}{x_{alc.\ H_2O}}$	Equivalent concentration of KOH neutralized by casein	$\frac{x_{H_2O}}{x_{alc.\ H_2O}}$	Equivalent concentration of KOH neutralized by casein	$\frac{x_{H_2O}}{x_{alc.\ H_2O}}$	Equivalent concentration of KOH neutralized by casein	$\frac{x_{H_2O}}{x_{alc.\ H_2O}}$
0.0250	2.98	0.0250	3.84	0.0250	5.17	0.0250	12.70
0.0125	2.92	0.0125	3.71	0.0125	4.89	0.0125	9.47
0.0063	2.82	0.0063	3.68	0.0063	4.77	0.0063	8.19
0.0031	2.80	0.0031	3.49	0.0031	4.45	0.0031	6.98
0.0016	2.82	0.0016	3.54	0.0016	4.60	0.0016	6.90

0 to 60 per cent of alcohol, we may conclude that in these solutions it is for the greater part the migration-velocity of the caseinate ions which is affected by the percentage of alcohol in the solvent. From the values of the constants in equations (iii) to (ix) it is apparent that the migration-velocities of the casein-ions are progressively *diminished* by increasing alcohol-concentration from 0 to 60 per cent. From the facts that the law $x_v = \frac{x_0}{A_v}$ no longer holds good for the solutions containing 75 per cent alcohol and the ratio $\frac{x_{H_2O}}{x_{alc.H_2O}}$ is not constant we may conclude that in these solutions the degree of dissociation of the caseinate or the number of equivalents of casein produced by one equivalent of KOH or both undergo a profound modification. The values of the constants in equation (x) indicate an *increase* in the magnitude of $u + v$ and a very great *decrease* in the magnitude of the dissociation-constant. In the following table the values of $\rho\,(u + v)$ and of $\frac{K}{\rho}$ corresponding to the equations (iii) to (x) are enumerated; in the fourth column is given the percentage dissociation of the caseinate in the solutions containing 0.025 N KOH bound by casein (at 30 degrees).

TABLE IX

Potassium Caseinate. 80×10^{-5} Equivalents of KOH per gram

Per cent of alcohol by volume	$\rho\,(u + v)$	$\frac{K}{\rho}$	Per cent of caseinate dissociated in solutions containing 0.025 equivalent of potassium
0	83.6×10^{-5}	0.0923	82
10	64.9×10^{-5}	0.0767	80
20	49.1×10^{-5}	0.0760	79
30	38.0×10^{-5}	0.0682	78
40	29.9×10^{-5}	0.0633	77
50	24.2×10^{-5}	0.0513	74
60	19.4×10^{-5}	0.0365	68
75	34.6×10^{-5}	0.0007	17

The degree of dissociation of the caseinate in the solutions containing 60 *per cent and less than* 60 *per cent of alcohol is evidently but little affected by the percentage of alcohol in the solvent, the major part of the effect of the alcohol upon the conductivity being attributable to the decreased mobility of the caseinate ions.*

5. The Viscosities of Solutions of Potassium Caseinate in Alcohol-Water Mixtures. — The striking resemblance between the formula $\frac{x_v}{x_0} = \frac{1}{A_v}$ and the Arrhenius-Euler formula, $\frac{\eta_n}{\eta_0} = a^n$, for the dependence of the viscosity of a solution upon its concentration (1), where η_n is the viscosity of the solution, η_0 that of the solvent, n the concentration of the solution and a is constant, forcibly suggests that the decrease in the conductivity of potassium caseinate solutions due to the addition of alcohol between alcohol-percentages of 0 to 60 may be attributable for the greater part to the hampering of the protein ions by the increased internal friction of the solvent.*

Accordingly the following determinations were made:

31.25 grams of casein were dissolved in 250 cc. of $N/10$ KOH. To 25 cc. portions of this were added 0, 10, 20, etc. cc. of alcohol, the volume of each mixture being made up to 100 cc., the solution containing 75 per cent alcohol being made up by adding 99.8 per cent alcohol until the volume of the mixture was 100 cc.

A series of exactly similar solutions were made up in which, however, 25 cc. of $N/10$ KOH were employed instead of 25 cc. of caseinate solution.

The viscosities of these solutions were determined at 30 degrees in an Ostwald viscometer (5), for which the time of outflow, for water, was 90 seconds. The times of flow were read with a stop watch. The viscosities were calculated from the formula $\frac{\eta}{\eta_0} = \frac{st}{s_0 t_0}$, where η_0 is the viscosity of distilled water, η that of the solution under investigation, s_0 and t_0 are the density and the time of outflow, respectively, of distilled water and S and t are the density and time of outflow, respectively, of the solution. The densities of the solutions were determined by means of a normal hydrometer, reading the density to within 0.002.

Taking the viscosity of water at 30 degrees to be 0.00798 dyne per cubic centimeter (13) and its density as 0.996, the following were the results obtained:

* In this connection the work of H. C. Jones and collaborators on the conductivities of solutions of inorganic salts in alcohol-water and acetone-water mixtures should be consulted (3). Cf. also Walden (14).

TABLE X

Concentration of KOH neutralized to phenolphthalein by Casein = 0.0250 N

Per cent of alcohol by volume	η in dynes per cc. for solvent	η in dynes per cc. for solution	Difference due to caseinate
0	0.00816	0.01668	0.00852
10	0.01065	0.02049	0.00984
20	0.01376	0.02484	0.01108
30	0.01694	0.02814	0.01120
40	0.01913	0.03237	0.01324
50	0.02064	0.03400	0.01336
60	0.02011	0.03200	0.01189
75	0.01735	0.02345	0.00610

Walden (loc. cit.) found that for solutions of tetraethylammonium iodide in thirty organic solvents the product of the molecular conductivity of the salt at infinite dilution ($= u + v$ or, the sum of the migration-velocities of its ions) and the viscosity of its infinitely dilute solution is nearly constant, indicating an inverse proportionality between the viscosity of the solution (or what comes to the same thing at infinite dilution, the solvent) and the migration-velocities of the ions which it contains. In the accompanying table are given the values of $\rho\,(u + v) \times \eta$ solvent, for the various solutions investigated.

TABLE XI

Per cent of alcohol	$\rho\,(u + v).\eta$ solvent	Per cent of alcohol	$\rho\,(u + v).\eta$ solvent
0	0.68×10^{-5}	40	0.57×10^{-5}
10	0.69×10^{-5}	50	0.50×10^{-5}
20	0.68×10^{-5}	60	0.39×10^{-5}
30	0.64×10^{-5}	75	0.60×10^{-5}

It is evident that the product of the ionic mobility of potassium caseinate ions at infinite dilution and the viscosity of the solvent varies very much less than either of these quantities taken alone, indicating in a qualitative sense at all events, that *the ionic mobilities of the caseinate ions are in the main determined by the viscosity of the solvent.* The observed increase in the ionic mobility of the caseinate ions on increasing the alcohol content of the solvent from 60 to 75 per cent, in view of the fact that the viscosity of the solvents passes through a maximum at from 50

to 60 per cent alcohol and rapidly decreases between 60 and 75 per cent alcohol, is especially confirmatory of this view.

It will be noted that the viscosity of the solvent is notably increased by the addition of the concentrations of casein employed, and this effect, of course, must vary very considerably with the dilution; *yet the Ostwald law holds good, and despite the fact that the dissolved substance itself very materially affects the viscosity of the solutions, it is the viscosity of the solvent alone which determines the mobility of the protein ions.* We can only conclude from this that that portion of the viscosity of the solution which is attributable to the *caseinate* itself does not in any appreciable degree interfere with the mobility of the protein ions. The significance of this fact will be more fully discussed in Chap. XIII.

6. The Molecular Condition of Potassium Caseinate in 75 Per Cent Alcohol. — We have seen that the behavior, optical,* and electrical, of potassium caseinate dissolved in alcohol-water mixtures, undergoes an abrupt change in passing from 60 to 75 per cent alcohol-content. The degree of dissociation, which is but little affected by lower concentrations of alcohol, undergoes a profound diminution in 75 per cent alcohol, and the opacity of the solution undergoes a concurrent increase. The phenomena suggest the possibility that this concentration of alcohol leads not only to the formation of anhydrides of the protein and therefore, as explained in Chaps. I and VI, to a diminution of the degree of dissociation, but also to a polymerization of the protein, as outlined in equations (i) to (iv) in Chap. VI. This polymerization might, very conceivably, be accompanied by an increase in the weight of casein involved in the transport of one atomic charge. Accordingly, experiments were undertaken with a view to ascertaining the effect of increasing alcohol-content of the solvent upon the electrochemical equivalent of casein.

The experiments were carried out in the manner described in Chap. VIII, upon solutions containing 50 and 75 per cent of alcohol, made up in the manner described above. The alterations in the percentage casein-content of the solutions due to deposition of the casein upon the anode, was estimated from the

* Not only is the opalescence of the solution greatly increased but also the change in the refractive index of the solvent due to the introduction of one gram of casein per 100 c.c. is considerably diminished (Cf. Chap. XIV).

change in the refractive indices of the solutions, the proportion between the percentage of casein in these solvents and the change in the refractive index of the solvent, due to the protein, having been previously determined (Cf. Chap. XIV). The volume of the solution employed being always 25 cc., the actual amount of casein deposited by the current was obtained by dividing the alteration in percentage by 4.

The results obtained are tabulated below, the figures in the 6th column being obtained from those in the 5th column through multiplication by the Faraday constant.

TABLE XII*

50×10^{-5} Equivalents of KOH per Gram of Casein. Concentration of KOH neutralized by Casein = 0.015 *N*

Per cent of alcohol	Current in amperes	Time of passing	Grams casein lost from solution	Electrochemical equivalent in grams per coulomb	Grams of casein carrying one ionic charge
0	.....	.....	.....	.....	2336 ±183
50	6.88×10^{-4}	3 hrs. 25 min.	0.2550 ± 0.0170	0.0301 ± 0.0020	2906 ±193
75	1.94×10^{-4}	4 hrs. 0 min.	0.1260 ± 0.0200	0.0452 ± 0.0072	4363 ±695

* The weight of casein, in grams, which carries one atomic charge, in aqueous solution, is cited after the determinations enumerated in Chap. VIII. The amount of casein lost from the anode through resolution in electrolysis is not allowed for in the above estimates. In solutions containing alcohol it is probably very small, since the rate of solution of casein in alkaline solvents is much diminished by alcohol.

The smallness of the current employed in the electrolysis of the solution containing 75 per cent alcohol was due to the high resistance of the caseinate solution. The precipitate from the solution containing alcohol is slimy and not spongy as it is from aqueous solution. Nor, in 75 per cent alcohol, does it adhere well to the anode so that precipitation appears to take place in the body of the anodal portion of the fluid, and the solution had to be filtered to remove the casein precipitated by the electrolysis.

There is evidently a very marked increase in the weight, or decrease in the valency of casein ions in solution of potassium caseinate when the added alcohol attains 75 per cent, leading to a doubling of the weight of casein required to transport one atomic charge of electricity.

From these facts and from the failure of Ostwald's dilution-law to adequately represent the behavior towards dilution of solutions of potassium caseinate in 75 per cent alcohol, and the opalescence of these solutions, it appears probable that they partake rather of the character of suspensions than of true solutions, and that the transport of electricity by the caseinate in 75 per

cent alcohol is a phenomenon of "electric endosmose" rather than of true electrolytic conduction through the migration of ions.

7. The Chemical Mechanics of the Coagulation of Proteins by Alcohol. — We have seen that when potassium caseinate is dissolved in varying concentrations of alcohol, the opalescence of the solution undergoes an abrupt increase in the neighborhood of 70 per cent, indicating the beginning of coagulation, which, however, does not proceed far enough, even on further addition of alcohol, to lead to the actual separation of flocculi. We have also seen that for solutions containing 60 per cent and less of alcohol the law $\frac{x_{H_2O}}{x_{\text{alc. } H_2O}} = \text{constant}$ holds good and that this implies that almost the sole effect of the alcohol in these concentrations is to modify the ionic mobility of the casein ions, leaving the degree of dissociation of the caseinate comparatively unaffected. In solutions containing 75 per cent of alcohol, however, the degree of dissociation of the caseinate is profoundly diminished and, consequently, the law $\frac{x_{H_2O}}{x_{\text{alc. } H_2O}} = \text{constant}$ no longer holds good. On the basis of the hypothesis and experimental data outlined in preceding chapters this phenomenon can readily be interpreted. We have seen that the protein salts dissociate, not at terminal $-NH_2$ or $-COOH$ groups but at $-COH.N-$ groups within the molecule. If, however, the terminal $-NH_2$ and $-COOH$ groups of the molecule are bound together, as they are in anhydrides of the type $\underline{HN.RCOH.NR.CO}$, then *this dissociation can no longer occur*. We have already (Chap. VI) seen strong reason for concluding that the coagulation of proteins is accomplished by dehydration of the protein; the electrochemical behavior of caseinates dissolved in alcohol-water mixture is clearly in harmony with this view.

As explained in Chap. VI, section **6,** equations (i) to (iv), however, not merely "internal neutralization" of the protein molecule may result from the dehydration of its terminal $-NH_2$ and $-COOH$ groups, but also polymerization. The high electrochemical equivalent of potassium caseinate, dissolved in 75 per cent alcohol, and the opacity of its solution favor the view that this phenomenon also occurs in these solutions.

From the effects of alcohol upon the rate of solution of casein

by alkalies Robertson and Miyake (11) have also been led to infer that alcohol induces polymerization of the casein molecules even in solutions of sodium caseinate in 20 to 30 per cent alcohol. From the above results, however, we must infer that if in such solutions the mass of the casein ions is doubled their valency must also be doubled, for otherwise ρ would be halved and the observed constancy of the ratio $\frac{x_{H_2O}}{x_{H_2O.alc.}}$ in these solutions would not be obtained.

Incidentally, the profound diminution in the degree of dissociation of the caseinate which results when the alcohol-concentration attains a certain value explains, *independently of any theory as to the mode of dissociation of the protein salts in solution*, the observed fact that alcohol precipitates protein salts as such from their solutions (Cf. Chap. IV, **2**) and not the uncombined proteins, since, previously to their precipitation, the combined base or acid is bound up in an undissociated molecule.

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PART III

THE PHYSICAL PROPERTIES OF PROTEIN SYSTEMS

CHAPTER XII

THE PHENOMENA WHICH ACCOMPANY CHANGES IN THE STATE OF AGGREGATION OF PROTEINS

1. The Passage of Dry Protein into Solution. — The question whether or not the proteins as a class possess a definite solubility in water is one which has not so far admitted of any satisfactory solution. True, certain sparingly soluble salts of the proteins undoubtedly are soluble only to a definite extent in water, as for example the researches of Galeotti, cited in Chap. VI, reveal; when this limit of solubility is overstepped the protein salt is precipitated; then other forms of protein, coagulated proteins, "denatured" proteins, and so forth are definitely insoluble in water *under the conditions which accompany their formation.* But the very proteins which display these phenomena under certain conditions, under somewhat different conditions, either in the form of free protein or of a salt, appear to be indefinitely soluble in water; miscible with it in all proportions. The phenomena of "solution," chemical combination with water, and of the hygroscopic, possibly purely physical, retention of water, become, in these cases and so far as our knowledge at present extends, inextricably confused.

On the whole it appears very probable that such proteins and protein salts as gelatin and the caseinates are not only miscible with water in all proportions, but also capable of entering into chemical combination with water in many different proportions. To this view Pauli (72), in contradistinction to Hardy (34) (35), also inclines.

Base- and acid-free casein is definitely insoluble in distilled water; but in water containing a base its solubility would appear to be limited only by the quantity of base which the water contains and not by the volume of water. The casein continues to dissolve until, as explained in Chaps. V and X, 11.4×10^{-5} equivalents of base are combined with each gram of casein and solution of the casein stops merely because the power of the base to neutralize casein is exhausted, and not because the solvent

will hold no more of the caseinate. Addition of alkali to the mixture results in the passage of more casein into solution; addition of acid in the throwing of uncombined casein out of solution. Apparently caseinates, of the alkalies at all events, are indefinitely soluble in water, although in practice we are limited in the preparation of strong solutions, not only of the caseinates but of other proteins, by the viscous and glairy nature of such solutions, and the consequent difficulty of ensuring their homogeneous character. Corresponding to this fact we find that it is very difficult to prepare the caseinates of the alkalies in the form of dry powders. I have precipitated lithium caseinate from 90 per cent alcohol by the addition of ether and endeavored to dry this product by washing with ether and standing over sulphuric acid at 30 degrees. The result is merely a soft gelatinous mass; on prolonged standing over sulphuric acid (a matter of weeks) it becomes horny; at no definite time can we state that the product has become anhydrous; it simply exhibits a continual change in consistency with the continual withdrawal of water, just as gelatin does. On the other hand, proteins which are insoluble in water and certain other proteins and protein salts are readily rendered anhydrous by treatment with alcohol and can be prepared through this means in the form of dry powders (Cf. Chap. II). The affinity of a protein for water, therefore, is a function which varies, not only with the character of the protein, but with the character of any base or acid which may be combined with it. In all cases it would appear to be of considerable magnitude, however, the difficulty which histologists experience in thoroughly dehydrating tissues, for example, being eloquent testimony of this fact.

It has been shown by Noyes and Whitney (61) (10) that under conditions involving no chemical interaction (except the possible formation of "solvates" (Cf. Chap. VI)), the rate of solution of a crystalloid in water is at each moment proportional to the difference between its concentration at that moment and its concentration at saturation of the solvent. They conclude from this that at the boundary of the crystals and the solution there exists a film of solution, which is always saturated and, for a constant rate of stirring, of constant thickness. The velocity of solution is thus determined by the rate of diffusion of the

dissolved crystalloid out of this film into the body of the fluid. This leads to the equation:

$$\log \frac{a}{a-x} = skt, \qquad \text{(i)}$$

where a is the concentration of a saturated solution of the crystalloid, x the concentration of the solution at time t, s the area of the surface of the crystalloid which is exposed to the action of the solvent and k a constant which varies with the rate of stirring and with the rate of diffusion of the crystalloid (11), and, consequently, with the temperature.

On the other hand, Boguski (4) (5) and Veley (104) (105) have shown that the rate of solution of basic substances such as marble or zinc in acids, a process involving chemical interaction, is proportional at each instant to the concentration of the unneutralized acid and to the surface of the solid. Upon the supposition that the surface remains appreciably constant in area during the period occupied by the experiment (a readily realizable condition) this leads to the equation:

$$\log \frac{a}{a-x} = kst, \qquad \text{(ii)}$$

which is identical with the above, save that a is, in this case, *the initial concentration of the acid.* In this case also Nernst believes (60) the velocity of solution is determined by a diffusion-velocity, namely the velocity with which the acid diffuses into the film in contact with the solid. Naturally, were the velocity of the chemical interaction low in comparison with the velocity of diffusion of the acid (or any reagent playing a similar part) the determining velocity would be a chemical and not a *diffusion* velocity. An examination of this case in the light of the mass law shows, however, that the *form* of the relation between time and mass dissolved would be the same as that expressed in equation (ii).*

In the light of what has been said concerning the indefinite solubility of the caseinates in water, it might be anticipated that equation (i) would not apply to the dissolving of solid casein in alkaline water. It is quite imaginable, however, that equation (ii) might apply, a, in that equation, being the number of

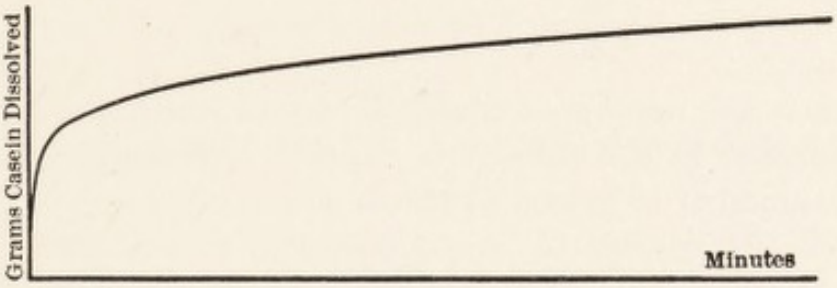
* Provided only one molecule of acid (or other *dissolved* reagent) took part in the reaction.

equivalents of base in the water employed to dissolve the casein. The experimental fact is, however, that *neither* of these equations expresses the relationship between the time for which a mixture of solid casein and alkaline water is stirred and the mass of casein dissolved (86).

In the experiments about to be described the casein which was employed was especially prepared so as to readily sink in water (Cf. Chap. II, **2**), that is, it was not absolutely anhydrous but contained a trace of water insufficient to disturb the accuracy of the results, but very material to the success of the experiments. Since the presence and amount of associated traces of water play such an important part in determining the readiness with which casein is wetted by a solvent, it proved to be necessary, in obtaining comparable experimental results, *always to employ the same preparation of casein*, since no two samples of incompletely anhydrous casein can be relied upon to contain exactly the same proportion of water.

A measured amount (usually 100 cc.) of the fluid employed as solvent was placed in a beaker of squat form and 400 cc. capacity and was agitated by a flattened glass rod which was bent at right angles, the plane of the horizontal arm being somewhat inclined to the vertical, so as to communicate an upward thrust to the rotating liquid. The horizontal arm of the stirrer was about $2\frac{1}{2}$ cm. long and as near as possible to the bottom of the beaker; this was rotated at an approximately constant rate of about 1600 revolutions per minute by a small motor. While stirring, a weighed amount of the casein was dropped into the fluid. At stated intervals samples of the mixture were almost instantaneously abstracted by means of a 10 cc. pipette which was provided with a rubber bulb. The samples were then very rapidly filtered through lightly packed glass wool. The time occupied in filtration was never more than 30 seconds for the 5 and 10 minute samples, or more than 1 minute for the later samples. The refractive index of the filtrate from each sample was then determined. Denoting the refractive index of any given sample by n and that of the pure solvent by n_1, the quotient $\frac{n - n_1}{0.00152}$ is the number of grams of casein dissolved in 100 cc. of the solvent at the moment when the sample was extracted (Cf. Chap. XIV).

The type of relation which was found to subsist between the time which elapsed after the introduction of the casein and the number of grams of casein dissolved in 100 cc. of solvent is shown diagrammatically in the accompanying figure, in which the abscissæ represent minutes and the ordinates the number of grams of casein dissolved in 100 cc. of solvent. It will be observed that the rate of solution is at first very great, but that it very rapidly falls off. It does not fall to zero, however, that is, the curve does not appear to approach an asymptote, but is rather of a parabolic form. Nor does this appear strange when we observe that although, after two hours of stirring, the



rate of solution of the casein is very small, yet the solvent is still very far from being "saturated" with casein. The alkali-equivalent of 1 gram of casein is, as we have seen (Chaps. V, IX and X), 11.4×10^{-5} equivalent gram molecules, but the proportion of the casein actually dissolved to the amount of base present in the solvent was always, even after two hours of stirring at room temperatures, very much less than this.*

* It might be imagined that the solutions of the caseinates which contain only 11.4×10^{-5} equivalents of base per gram of casein, and which are prepared by neutralizing the excess of base which is employed to dissolve the casein by the addition of a strong acid, are "supersaturated" with respect to casein. Two facts speak very strongly against this view, however. The first is that the electro-chemical behavior of casein in much more alkaline solutions already foreshadows the fact that the least proportion of alkali which will hold one gram of casein in solution is 11.4×10^{-5} equivalents (Cf. Chap. X). The second is that if a 3 per cent solution of casein in 0.0043 *N* KOH (1 gram of casein to 14×10^{-5} equivalents of base) be prepared by dissolving casein in excess of base and then neutralizing the excess with acid, it can be kept in a sealed glass vessel, in the presence of excess of toluol for 6 months without any deposition of casein occurring. At the end of from 10 months to a year a very bulky white precipitate is deposited leaving the

The relation between the time of stirring and the quantity of casein dissolved does not obey either of the above cited equations; it does not appear to obey any of the ordinary chemical reaction or solution velocity formulæ. The rate at which the velocity of solution falls off; the negative acceleration of the process; is far too great to permit of representation by either of the formulæ (i) and (ii). Nor is any better agreement obtained if we insert, for the value of a in these equations, the actual number of grams of casein present in the mixture, or if, allowing for the diminution in the surface of the casein exposed to the action of the solvent, as solution proceeds, we endeavor to apply the relation

$$\frac{dx}{dt} = K(A - x)\,(B - x), \qquad \text{(iii)*}$$

where A is the number of grams of casein which the amount of alkali present in the solvent is capable of holding in solution, B is the number of grams of casein actually present in the mixture, x is the amount of casein dissolved at any given moment and K is a constant. The relation between the time of stirring and the amount of casein dissolved, however, does obey, very accurately, the relation

$$x = Kt^m, \qquad \text{(iv)}$$

where x is the amount dissolved after time t, and K and m are constants which vary with the nature and concentration of the alkali solution employed as solvent and with the total mass of casein present in the mixture.

The following tables enumerate illustrative results.† The

supernatant fluid very nearly clear. It must be recollected, however, that when periods of time of the order of a year are concerned no protein solution can be regarded as being in equilibrium. Even in absolutely neutral solutions the H^+ and OH' ions of the water lead to a measure of "autohydrolysis" (Cf. Chap. XVI) and the above solution was distinctly acid (Cf. Chaps. V and IX). Hydrolysis of caseinates of the bases leads to the formation of paranucleins which are insoluble in neutral and faintly acid solutions, and to substances which bind bases and therefore tend to abstract them from the casein (Cf. Chap. XVI) and consequently, when the total amount of base present is so very little more than enough to hold the casein in solution, to indirectly precipitate the casein itself.

* In its integrated form: $\log \frac{B\,(A - x)}{A\,(B - x)} = Kt.$

† For additional experimental results the reader is referred to my original communication (86).

TABLE I

Solvent: 0.00870 *N* KOH. Temperature 18–20 degrees

$K = 1.48$ $m = 0.146$

Time in minutes	Grams casein dissolved in 100 cc. solvent		Δ
	Found	Calculated	
5	1.84	1.87	+0.03
10	2.11	2.07	−0.04
30	2.43	2.43	±0.00
60	2.70	2.69	−0.01
120	2.96	2.98	+0.02
			$\Sigma\Delta = \pm 0.00$

TABLE II

Solvent: 0.00870 *N* NaOH. Temperature 22–23 degrees

$K = 1.33$ $m = 0.163$

Time in minutes	Grams casein dissolved in 100 cc. solvent		Δ
	Found	Calculated	
5	1.71	1.73	+0.02
10	1.97	1.94	−0.03
30	2.30	2.32	+0.02
60	2.63	2.60	−0.03
120	2.89	2.91	+0.02
			$\Sigma\Delta = \pm 0.00$

TABLE III

Solvent: 0.01740 *N* $Ba(OH)_2$. Temperature 20 degrees

$K = 1.34$ $m = 0.194$

Time in minutes	Grams casein dissolved in 100 cc. solvent		Δ
	Found	Calculated	
5	1.85	1.84	−0.01
10	2.05	2.10	+0.05
30	2.64	2.60	−0.04
60	3.03	2.98	−0.05
120	3.36	3.40	+0.04
			$\Sigma\Delta = -0.01$

quantity of solvent employed was 100 cc. and the number of grams of casein initially added to it was 5. For a reason which will shortly appear no especial effort was made to maintain a constant temperature during the progress of an experiment, but at the head of each table are given the temperatures of the mixture at the beginning and at the end of the experiment. The temperature at the beginning of the experiment is placed first. In the column headed "calculated" are given the values of x calculated from the above formula, the constants K and m being determined from all of the observations by the method of least squares, employing for this purpose the form:

$$\log_{10} x = m \log_{10} t + \log_{10} K.$$

The possible experimental error in the determination of the concentration of a casein solution by means of its refractive index is always ± 0.07 gram per 100 cc. It will be seen that the differences ($= \Delta$) between the observed and calculated values of x are usually considerably less than the possible error in the determination of the concentration of the casein in the filtrates.

Equally concentrated solutions of KOH, NaOH, and NH_4OH dissolve casein with about equal rapidity, while solutions of the hydroxides of the alkaline earths dissolve casein much more slowly, $Sr(OH)_2$ dissolving the casein most rapidly, $Ca(OH)_2$ more slowly and $Ba(OH)_2$ more slowly still. As I have pointed out in Chaps. X and XI this fact is of significance when viewed in the light of the facts that solutions of the caseinates of the alkaline earths become opalescent on heating, while those of the caseinates of the alkalies and ammonium do not (63), that the caseinates of the alkaline earths will not pass through the pores of a clay filter, while those of the alkalies and ammonium readily do so (63), and that in these and in other ways the caseinates of the alkaline earths give evidence of being present, in their solutions, in the form of more bulky molecules than those of the caseinates of the alkalies and of ammonium under equivalent conditions. It appears probable that when casein is suspended in solutions of the hydroxides of the alkaline earths the soluble caseinate is just as readily formed as it is in solutions of the alkalies, but that it is hindered in passing out of the casein particles, through their capillary pores, into the solution, just as it is hindered in passing through the pores of a clay filter. We

have had occasion to comment upon the fact (Cf. Chap. V) that dry casein will not pass into solution in dilute acids, although wet, flocculent, freshly precipitated casein will do so; nevertheless, acid is taken up from the solution and bound by the casein (103). It appears probable, as I indicated in Chap. V, that in this case *soluble* casein salts are formed but that they are prevented, by the grossness of their molecules, from passing out through the pores of the casein particles into the body of the solvent.

The influence of neutral salts upon the rate of solution of casein by dilute sodium hydroxide has been investigated by Robertson and Miyake (89) who find that even in the presence of high concentrations of these salts the relationship between the time and the amount of casein dissolved is expressed by the relation $x = Kt^m$. The presence of the salts retards the solution of the casein, however, the retardation due to alkaline earth chlorides being about one hundred times as great as that which is brought about by chlorides of the alkalies. The degree of retardation increases with increase of the concentration of salt employed.

The amount of casein which is dissolved, in a given period of time, by a solution of KOH is, within the limits of accuracy of the determinations, *directly proportional to the concentration of the* KOH *solution.* This is very clearly shown in Table IV, in which r denotes the ratio of the number of grams of casein dissolved to the number of equivalent gram molecules (multiplied by 100) of KOH present in 100 cc. of solvent employed. It will

TABLE IV

Time in minutes	Concentration of the KOH solution employed as solvent							
	0.00218 N r	0.00435 N r	0.00653 N r	0.00870 N r*	0.01088 N r	0.01305 N r	0.01523 N r	0.01740 N r
5	21	18	16	20	19	21	20	19
10	23	21	18	23	23	24	22	21
30	29	26	26	28	27	28	26	26
60	33	29	29	31	30	31	28	28
120	33	31	31	34	33	33	30	.....

* Average of two determinations at 26.5 degrees and 18 to 20 degrees, respectively.

be observed that this ratio, for any of the given periods of time, is very nearly constant.*

The temperature-coefficient of the velocity of solution is very small. The difference between the amounts of casein dissolved, after a given time, at 18–20 degrees and at 26 degrees are only slightly greater than or equal to the possible error of the determinations. So far as the accuracy of the method employed enables us to decide, the temperature-coefficient of the rate of solution, between the temperatures of 20 and 36 degrees, is practically zero.† At higher temperatures the rate of solution in solutions of the hydroxides of the alkalies is increased and the rate of solution in solutions of the hydroxides of the alkaline earths is very materially diminished. At these temperatures, also, solutions of the caseinates of the alkaline earths, which are neutral to phenolphthalein, become alkaline to phenolphthalein, while solutions of the caseinates of the alkalies which are neutral to phenolphthalein do not (63). I have sought to account for these facts by supposing that temperatures above 36 degrees lead to a polymerization of the protein moiety of the caseinates of the alkaline earths (83).

The low temperature-coefficient would in itself lead us to suspect that the process which determines the *rate* of solution of casein in solutions of bases is not chemical in nature.

That the rate of solution of the casein is not determined by the velocity of a chemical reaction occurring exclusively in the *liquid* phase is also shown by the fact that the rate of solution of the casein is dependent upon the mass of casein initially introduced into the mixture. Were the rate of solution of the casein dependent solely upon the velocity of a reaction between casein and the alkali, taking place in the liquid phase, then since, in the presence of undissolved casein, the liquid would always be saturated with casein, the rate of solution should be independent of the mass of undissolved casein. We are led to conclude, therefore, that the processes which determine the velocity of solution occur, in part at all events, either *within* or at the *surfaces* of the suspended particles of undissolved casein.

* At "saturation" of the alkali with casein the numerical value of this ratio would be 91.

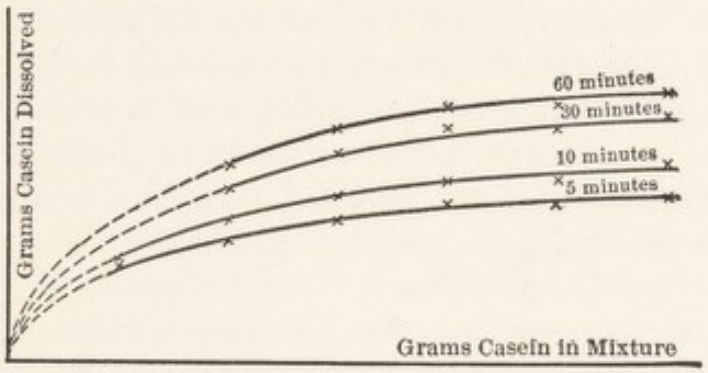
† It was for this reason, of course, that no special effort was made to maintain a constant temperature during the progress of the experiments.

TABLE V

Grams casein added to 200 cc. of solvent (0.010 *N* KOH)	2.5	5.0	7.5	10.0	12.5	15.0
Time in minutes	Grams dissolved	Grams dissolved	Grams dissolved	Grams dissolved	Grams dissolved	Grams dissolved
5	2.24	2.76	3.28	3.68	3.68	3.82
10	2.36	3.28	3.82	4.22	4.22	4.60
30	(2.50)*	3.96	4.86	5.40	5.40	5.66
60	....	4.60	5.40	5.92	5.92	6.18

* That is to say, at some undetermined time previous to this all of the casein introduced into the solvent had been dissolved.

The relation between the amount of casein dissolved in a given time and the mass of casein initially added to the solvent is shown in Table V and graphically in accompanying figure. The temperature of the mixtures was, in all of these experiments,



20 degrees. It will be seen that the rate of solution increases, at first somewhat rapidly with the mass of casein added to the solvent, later more slowly.

Reverting to equation (iv), the relation $x = Kt^m$ is, it is of interest to observe, the same as that found by Cameron and Bell (14) (15) and later confirmed by Ostwald from the investigations of Goppelsroeder (66), to subsist between the amount of fluid absorbed by a column of sand or a strip of filter-paper and the time during which the fluid has remained in contact with a portion of its surface.*

The values of the constants are also of the same order of magnitude as those found in these investigations. It is possible, therefore, that the *rate* of solution of the casein is primarily

* According to Cameron and Bell (14), this formula can be derived from the formula of Poiseuille for the flow of liquids through capillary spaces.

determined by the rate at which the particles are penetrated and wetted by the solvent, the process of chemical reaction between the alkali and the casein taking place at a relatively great velocity.

It is, perhaps, not surprising that the factor which determines the rate of solution of casein should be the velocity with which it is wetted by the solvent, while that which determines the rate of solution for a crystalloid is the velocity with which the dissolved substance diffuses out of a thin layer of saturated solution in immediate contact with the surfaces of the crystals. A crystal is only wetted upon its external surface, and the wetting, naturally, takes place instantly. A particle of casein (or, in general, of the solid phase of any colloid) is, however, comparable in structure with a sponge; the surface which may be wetted by the solvent is, per unit volume, very much larger than that of a crystal, and the solvent must, in wetting this surface, traverse a relatively immense network of minute capillary pores. Under such conditions the time occupied in wetting the surfaces of the particles may well be great compared with the time required for the dissolved substance to diffuse from these surfaces into the solvent, or with the time required for the accomplishment of the union between the protein and the alkali in the solvent.

As might be expected, similar relationships are encountered in the extraction of a protein from desiccated and finely divided fragments of tissue. The rate of extraction of the protamin salmin by dilute hydrochloric acid from the dried spermatozoa of the salmon is determined primarily by capillary forces (87) (88). The accompanying chemical phenomena (decomposition of compounds of salmin within the tissue, formation of salmin hydrochloride, etc.) occur at a relatively very great velocity and hence do not affect the rate of extraction.

The rate of extraction or passage of a protein from a colloidal phase into a surrounding solvent may be determined either by the rate of passage of the soluble protein compound from within the colloid particles into the surrounding solvent, or by the rate of penetration of the solvent into the colloid particles, or by both of these processes. That the latter process, namely the *penetration* of the colloid, is of very great importance in determining the observed time-relations may be inferred from the fact that the absorption of acid from dilute acid solutions by

suspended particles of casein takes place in accordance with the equation $x = Kt^m$, although in this case either no soluble compound of casein is formed or, which is more probable, the soluble compound which is formed is unable to issue forth from the colloid particles within which it arises (88) (89).

Differentiating the equation:

$$x = Kt^m$$

we find

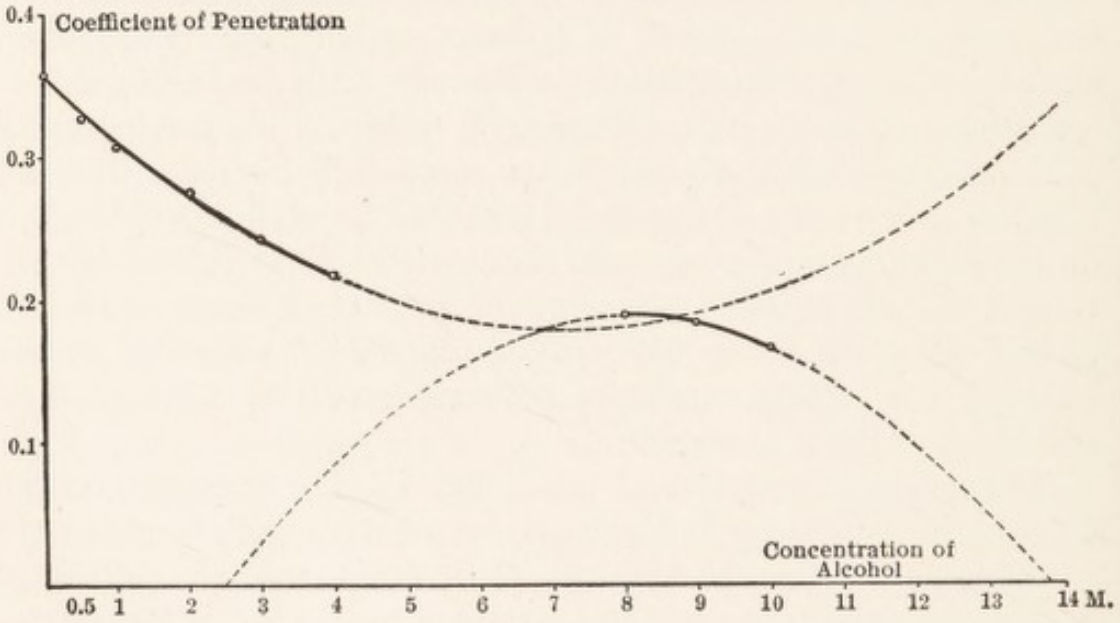
$$\frac{dx}{dt} = Kmt^{m-1};$$

in other words, the product Km, which has been termed by Robertson and Miyake the *coefficient of penetration*, expresses the constant proportionality between the velocity of solution and an exponent (peculiar to each solvent) of the time during which the protein has been exposed to the action of the solvent. The coefficient of penetration is, in the case of the solution of casein by dilute sodium hydroxide, very characteristically affected by the presence in the solvent of inorganic salts. The magnitude of the coefficient of penetration decreases with increasing concentrations of NaCl, KCl, $CaCl_2$, $SrCl_2$ or $BaCl_2$, the acceleration of the decrease being positive in the cases of NaCl and KCl and negative in the cases of $CaCl_2$, $SrCl_2$ or $BaCl_2$. Lithium chloride, on the other hand, *increases* the value of the coefficient of penetration with a negative acceleration, so that at concentrations of this salt lying above 0.33 normal the algebraic sum of these two opposite effects results in a decrease of the value of the coefficient of penetration (89).

If the rate of solution of casein by alkalies were primarily determined by the rate of penetration of the capillary pores in the suspended colloid particles by the solvent, then the addition to the solvent of any substance which markedly reduces the tension of a solid-water interface should retard the rate of penetration and, consequently, the rate of solution of the casein. It has been found by Robertson and Miyake (90) that alcohol and glycerol, both of which reduce the tension of a solid-water interface, also retard the solution of casein by dilute sodium hydroxide. The penetration-formula $x = Kt^m$ expresses the relationship between the quantity of casein dissolved and the time of stirring of the mixture in all mixtures of glycerol and water

and in alcohol-water mixtures which contain less than 4.5 mol. or more than 7 mol. of alcohol. The formula most decidedly fails to apply, however, to the rate of solution of casein in dilute alkali containing between 4 and 8 mol. of alcohol, for in these mixtures the extremely curious phenomenon is observed of partial re-precipitation, on continued stirring, of the casein which is initially dissolved, so that after 2 hours of stirring, the casein dissolved in these mixtures may actually be considerably less than after only ½ hour of stirring. The penetration-formula obviously implies a continuous and irreversible process, while in the presence of the above-mentioned concentrations of alcohol the process of solution is evidently partially reversible. Above and below this critical zone of concentrations the relation between the time of stirring and the concentration of casein dissolved is adequately expressed by the penetration-formula.

On examining the effect of alcohol upon the magnitude of the coefficient of penetration it becomes evident that in the zone of



concentrations lying between 4 and 8 mol. the character of the relationship between the concentration of alcohol and the magnitude of the coefficient of penetration is undergoing a transition. For concentrations of alcohol lying below 4.5 mol., the coefficient of penetration decreases with negative acceleration as the concentration of alcohol increases. In mixtures containing over 7 mol. alcohol, however, the coefficient of penetration decreases with positive acceleration, as the concentration of alcohol in-

creases. In mixtures containing concentrations of alcohol lying between 4.5 and 7 mol. inclusive, the character of the relationship is indeterminate. These relationships are illustrated by the accompanying figure in which the broken lines indicate the calculated continuation of the continuous lines drawn through the experimentally determined points, the formula used for the calculated exterpolations being:

$$K_1M_1 - KM = \alpha c + \beta c^2,$$

in which $K_1M_1 - KM$ represents the decrease in the coefficient of penetration, c represents the concentration of alcohol, α is a constant which is positive when c lies below 4.5 mol. and negative when c lies above 8 mol., and β is a constant which is negative when c lies below 4.5 mol. and positive when c lies above 8 mol.

The only explanation of these curious phenomena which offers itself lies in the fact that alcohol is a coagulant of proteins, and therefore brings about dehydration and consequent polymerization of protein molecules. At low concentrations of alcohol we have to deal with the rate of solution of "single" molecules of sodium caseinate, at higher concentrations with the rate of solution of polymerized molecules which we may for the sake of brevity term "double" molecules, while at intermediate concentrations we have to deal with a rate of solution which is compounded of the separate rates of solution of the two types of molecules. With increasing concentration of alcohol the rate of solution of the single molecules is progressively diminished, but that of the double molecules is increased, owing to the fact that the proportion of double to single molecules coming off from the internal surfaces of the casein particles is being increased sufficiently rapidly to more than compensate for the retardation of the total rate of solution by the alcohol. At 8 mol. concentration, so large a proportion of the molecules coming off are of the double type that thereafter the retarding effects of increasing concentration of alcohol upon the total rate of solution or penetration more than compensates for any further increase in the proportion of double molecules.

At intermediate concentrations the quantity of casein dissolved at first increases and thereafter diminishes with time, casein which is initially dissolved being later reprecipitated.

This can only be interpreted by supposing that the condition of the bulk of the solution outside the casein particles differs, at any rate for some portion of the time occupied in solution, from the condition of that portion of the solvent which has actually penetrated the protein particles. We know that any substance such as alcohol, which reduces the tension of a solid-water interface, tends to become concentrated at such an interface (102). Hence at the surfaces of the casein particles the concentration of alcohol will be greater than in the bulk of the fluid. Suppose that the concentration of alcohol in the bulk of the fluid be such that at equilibrium the proportion of double molecules is 50 per cent. If, now, the concentration of alcohol at the surface of the casein particles be such as to lead to the formation of 60 per cent of double molecules, then out of every thirty molecules coming off from the interior surfaces of the casein particles eighteen will be of the double type and only twelve of the single type. As these come out into the bulk of the liquid, equilibrium will tend to be re-established, resulting in the formation of sixteen double and sixteen single molecules, with an increase in the total number of molecules to thirty-two. This equilibrium may be supposed to be slowly established, and meanwhile the rate of solution of the casein has fallen very low; that is, the fall in concentration or concentration gradient from within the casein particles to the bulk of the fluid outside is very small. By this time, however, as a result of depolymerization, the molecular concentration of casein in the outer fluid has actually become greater than that in the saturated solution which fills the interior spaces of the casein particles. Thus the concentration gradient has become negative, and as the more concentrated casein solution diffuses back into the less concentrated but nevertheless saturated solution filling the interspaces of the sponge-like particles of casein, the excess of casein must be reprecipitated. When the concentration of alcohol is sufficiently great, so that the molecular condition of the casein is practically the same within and without the particles, a negative concentration gradient can never arise and the curve of solution reassumes the normal form, representing now, however, the curve of solution of double molecules instead of single molecules as at lower concentrations of alcohol.

This interpretation of the above facts finds further confirma-

tion in the effect of glycerol upon the rate of solution of casein in dilute alkali. In glycerol we have a substance which, like alcohol, reduces the tension of a solid-water interface but which, unlike alcohol, does not coagulate (polymerize) proteins in solution. Accordingly we find that glycerol decreases the rate of solution of casein progressively as its concentration increases. No sign of reprecipitation of dissolved casein is observed in any of the mixtures. The penetration formula $x = Kt^m$ applies to the rate of solution in all of the mixtures employed and the value of the coefficient of penetration progressively decreases in a smooth curve with decreasing acceleration as the concentration of glycerol increases.

These results illustrate the part which may be played by capillary phenomena in *heterogeneous* systems which contain proteins, the importance of which has, of recent years, been especially insisted upon by, among others, Wo. Ostwald and H. Freundlich (67).

It is of interest to estimate, by exterpolation from the penetration formula, the amount of time which would be required to "saturate" an alkaline solution with casein by stirring up excess of undissolved casein in it. We have seen that the relation between the percentage ($= x$) of casein dissolved in a given solution of alkali and the time ($= t$) of stirring is expressed by the equation $x = Kt^m$. For a mixture of 5 grams of solid casein with 100 cc. of 0.00870 N KOH the values of K and m at 18–20 degrees are, respectively, 1.48 and 0.146. The quantity of casein required to "saturate" 100 cc. of this solution would be 7.63 grams. Calculating the value of t corresponding to this value of x we find that it would take no less than *thirty-one years* to fully "saturate" the solution with casein, at the rate of stirring employed.* This very clearly indicates the great importance which the time factor may assume in heterogeneous systems which contain proteins.

* For more dilute solutions of alkali mixed with the same number (5) of grams of casein per 100 cc. the time required for "saturation" would appear to be shorter, thus for 0.00435 N KOH, calculating as above, it is only about 17 days. This is because not only the concentration of alkali but also the mass of casein, as we have seen, plays a part in determining the rate of solution. The ratio of casein to alkali was of course greater in the more dilute alkaline solutions in inverse proportion to the concentration of the alkali.

2. The Swelling of Protein Jellies. — A phenomenon which is doubtless very closely allied to that of the solution of solid proteins is the *swelling* or taking up of water which protein jellies undergo when immersed in water or in certain watery solutions.

When a plate of water-poor gelatin is immersed in water, especially if the water contains a little added acid or alkali, the plate takes up a very considerable quantity of water, many times its own weight, and at the same time swells to relatively enormous dimensions.

It was first pointed out by Quincke (80) that the swelling of gelatin is accompanied by a volume contraction, that is, the volume of the swollen jelly is less than the sum of the volumes of the original unswollen jelly and the absorbed water, and it was further pointed out by Wiedemann and Ludeking (107) that this process is accompanied by a disengagement of heat. Careful measurements of the heat liberated and the volume contraction during the swelling of casein in water have been made by Katz (46) who finds that the relationship between the heat liberated ($= W$) and the amount of water ($= i$ grams) taken up by one gram of protein is expressed by the equation:

$$W = \frac{Ai}{B + i},$$

in which A and B are constants. This implies that at the initial moment of the swelling process, the heat liberated by one gram of protein per gram of water absorbed will be equal to the ratio $\frac{A}{B}$, which in this case equalled 265 Cal. The volume-contraction follows an analogous relationship:

$$C = \frac{fi}{g + i},$$

in which f and g are constants and we may similarly calculate, from the ratio $\frac{f}{g}$ the value of C_0, or the volume contraction per gram of protein per gram of water absorbed at the moment of initiation of the process, and this calculation yields the value 0.3 cc.

The time-relations and equilibria in the process of the swelling of gelatin have been especially studied by Hofmeister (40), Pauli

(70) (71) (72), Wo. Ostwald (64) (65), Chiari (16), Ehrenberg (23), Procter (74) (75) (76) (77) (78), and Lenk (50).

Hofmeister found that the swelling of gelatin plates proceeds, at first rapidly and then more slowly, until it attains a maximum which is a function of the thickness and weight of the plate. After the attainment of this maximum the superficial layers of the plate tend to go into solution, especially if the water be acid or alkaline, and the plate, in consequence *loses* weight. Designating by the symbol W the weight of water which unit weight of the gelatin absorbs from water or a watery solution in t minutes, Hofmeister found that the empirical equation:

$$W = P\left(1 - \frac{1}{1 + \frac{c}{d}t}\right) \qquad \text{(v)}$$

applies with tolerable accuracy, P being the maximum amount of water which unit weight of the gelatin plate will imbibe (the swelling-maximum), c a constant and d the thickness in millimeters of the plate at its maximal degree of swelling. This leads to the conclusion that the initial velocity of swelling is proportional to the amount of swelling which the plate is able to undergo and that it therefore decreases regularly as the degree of swelling approaches more and more nearly the maximum.

Pauli assumed that each particle of the gelatin takes up water from every neighboring, more fully swollen particle at a velocity proportional to the difference between their water contents. This leads to the equation:

$$K = \frac{1}{t - t_1} \log \frac{M - Q}{M - Q_1}, \qquad \text{(vi)}$$

where Q is the quantity of water taken up by unit weight of gelatin in time t, M is the maximum degree of swelling which the gelatin attains, Q_1 is the quantity of water taken up by unit weight of gelatin in time t_1 and K is a constant which varies inversely with the thickness of the plate.

Hofmeister's formula leads to the conclusion that the velocity of swelling is at every instant proportional to the square of the swelling which the plate has yet to undergo, Pauli's to the conclusion that the velocity of swelling is at every instant proportional to the first power of the same quantity (termed by Pauli the "swelling deficit"). Both formulæ lead to the conclusion

that the velocity of swelling is at a maximum at the instant of immersion and therefore decreases with time. This corresponds to the experimental facts.

As we shall see in the next chapter, the proteins exert a small, but definite, osmotic pressure. They are at the same time not diffusible through colloids, or only very slightly so. Any crystalloids which may be present in the external fluid which bathes the gelatin can penetrate the gelatin albeit, possibly, more slowly than the water. The gelatin plate, therefore, acts like an osmometer which provides its own membrane which is permeable to crystalloids and not to colloids. Hence osmotic forces must play a part in the taking up of water by protein jellies. A phenomenon in the domain of crystalloids which presents some analogies to this aspect of the swelling of colloids is the following:* If we place at the bottom of a column of distilled water a layer of phenol and introduce below this a layer of a saturated solution of KCl in water and now allow the system to stand at constant temperature the layer of phenol gradually moves up the column of water; in other words the layer of solution below the phenol "swells." The solvent, water, being soluble in phenol, the phenol is permeable to it, while the KCl being insoluble in phenol, cannot pass through the layer of phenol.

Not only osmotic, but also chemical phenomena must, however, play a part in the swelling of protein jellies. As we have seen in Chaps. VI and XI the passage of a protein into solution involves the addition of the elements of water to terminal $-NH_2$ and $-COOH$ groups and also, possibly, to internal $-N.HOC-$ groups, resulting in the depolymerization of the protein. Not only osmotic phenomena but hydration of the gelatin must therefore occur in the process of swelling. In evidence of the correctness of this view Pauli points to the fact that, according to Weidemann and Lüdeking (loc. cit.), the swelling of gelatin is accompanied by a disengagement of heat, while the *solution* of gelatin is accompanied by an absorption of heat. Evidently the processes of solution and swelling are each composed of two factors, one leading to a disengagement and the other to the absorption of heat. The former process is, Pauli believes, the chemical binding of water by the protein, the latter the passage of the hydrated

* To which my attention was drawn, in this connection, by my colleague Dr. F. G. Cottrell.

protein into solution (or, in swelling, the osmotic inbibition of water). In swelling the chemical heat-effect predominates; in the dissolving of the gelatin, the heat-effect of solution.

That a part, at least, of the water in swollen gelatin is chemically bound by the protein is shown by the following experiment (74). If two gelatin plates be brought to the same degree of swelling, the one in neutral and the other in acid water, and if they be then immersed in absolute alcohol, the alcohol will abstract all of the water from the plate which has been swollen in neutral water, but not from the plate which has been swollen in acid water. Now, as we have seen in Chap. V, the fact that a constituent of a system can be completely removed from it by washing out with an appropriate solvent affords no valid proof that the constituent in question did not exist, within the system, in a state of chemical combination. But if, on the contrary, it should prove impossible or exceptionally difficult to remove the constituent by washing with a fluid in which it is very soluble, then the prima facie evidence that it exists in the system in a condition of chemical union is very strong. We may conclude, therefore, that when gelatin is swollen in acid water a part of the water which is taken up by the gelatin is chemically bound by it; and we have no valid reason for supposing that the water taken up from neutral solutions is not similarly bound although less firmly.

The equilibria attained in the swelling of gelatin in solutions of acids have recently been very thoroughly investigated by Procter. This investigator has found that gelatin absorbs both acid and water from acid solutions, but absorbs the acid in excess, so that the proportion of acid in the surrounding fluid diminishes. If the initial concentration of acid in the external fluid lies between 0.01 and 0.25 N, then assuming that at the end of the process (attainment of maximal swelling) the concentration of free acid is the same within and without the jelly, the amount of acid which is "bound" by the gelatin is 0.7 to 0.8 $\times 10^{-3}$ ($= 70$ to 80×10^{-5}) equivalents per gram. The equivalence, at the attainment of maximal swelling, is the same for all strong acids but falls below this value for weak acids. While the proportion of acid which is "bound" by the gelatin varies but slightly with the concentration of the acid in the surrounding fluid, this is not true of the degree of swelling attained, which in strongly acid

solutions attains its maximum at a dilution below that required for complete fixation of the acid by the gelatin, and then falls in a continuous curve with increasing concentration of the external acid solution. Procter interprets this by supposing that the swelling of the gelatin is determined by two opposing forces, the osmotic pressure of the acid-gelatin compound imprisoned by its indiffusibility within the jelly being balanced by the osmotic pressure of the acid outside. That the acid in the outer fluid does exert an osmotic pressure at the surface of the jelly Procter infers from the fact that concentrated neutral salts contract the jelly to a horny consistency and expel the associated (imbibed or combined) water without affecting the acid-gelatin compound. In view of the considerations set forth in Chap. VI we can see, however, that the assumption that the external acid exerts an osmotic pressure is an unnecessary one and, indeed, inconsistent with the fact that the acid freely penetrates the gelatin and combines with it. In view of the known dependence of coagulation upon phenomena of dehydration (Cf. Chap. VI) and the fact that protein salts may be coagulated as such and without decomposition, we may infer that the shrinkage of gelatin jellies upon addition of concentrated salts to the external medium is not due to the osmotic pressure of the salts, since gelatin is known to be permeable to inorganic ions, but to the competition between the inorganic salt and the gelatin salt for water.

Procter has taken advantage of the fact that the acid-gelatin compound is coagulated without decomposition by concentrated salt solutions to determine the quantity of acid "bound" by gelatin in acid solutions of varying concentration. He finds that the proportion of acid which is bound per unit mass of gelatin does not agree with the requirements of the Ostwald dilution-law for a binary electrolyte, no values of the hydrolytic dissociation-constant and the molecular weight yielding calculated results which are consistent with the initial rapid and subsequent slow rise in combining capacity with increasing concentration of acid in the surrounding medium. Better agreement between theory and experiment is attained by assuming that gelatin behaves as a diacid base having a molecular weight of 839, which agrees with the molecular weight estimated by Paal from freezing- and boiling-point measurements (68).

The taking up of water by gelatin from acid solutions is ac-

counted for by Procter in much the same manner as that outlined above (76). He pictures the gelatin acid-compound as a coherent mass from which the gelatin molecules cannot diffuse or separate and which in most respects behaves like a single enormous complex molecule. It is reasonable, he considers, to visualize it as a felted mass of amino-acid chains held to each other by attractions which possibly attach only their ends, but freely admitting the passage of liquid between them. He assumes, in accordance with our former conceptions of the mode of formation and ionization of protein salts, that the compound yields acid anions, but these anions, although diffusible, are held within the mass by electrostatic forces, since they cannot pass beyond the sphere of attraction of their companion colloid ions which form the jelly and which are therefore immobilized. The only way, therefore, in which the osmotic pressure of the anions can take effect is, not by their movement but by the movement of water, resulting in the swelling of the entire jelly mass and its dilution by admixture with the outside solution.

Two very serious objections attach to this interpretation of the phenomena. In the first place, as Procter himself has pointed out, were this the actual mechanism of swelling, then the operative force compelling movement of the water would, in ultimate analysis, be the electrostatic tension which prevents the acid anions from moving outwards into the surrounding solvent. There should thus be a measurable potential difference between the gelatin jelly and the external medium. This potential difference has been sought for by Ehrenberg who was unable to detect any measurable potential between the interior of a jelly and the external medium (23). In the second place, as Procter also points out, another difficulty lies in the fact that the condition which would thus arise would offer no equilibrium, since the acid anions and the free acid itself could not simultaneously be equal in concentration within and without the jelly. Our more recent views regarding the mode of formation and ionization of protein salts reconcile both these difficulties, however, for, since we may assume that no inorganic ions, or at most a very small proportion, are yielded by the protein-acid compound, the swelling of the jelly must be due, just as it is in the case of gelatin immersed in neutral water, to the osmotic pressure of the colloid particles themselves, which, being unable to penetrate

the colloid network in which they are entangled necessarily compel the compensating migration of water. No electrostatic tension between the jelly and the external solution need be assumed and since no acid anions are yielded by the protein salts, simultaneous equality of concentration of the uncombined acid and the acid anions within and without the jelly will be assured by their normal and equal diffusion into the jelly. The increased swelling capacity of gelatin in solutions of acids or alkalies is merely the expression of the fact that the ionization of the protein salt leads to an increase in the number of colloid particles per unit volume of the jelly and possibly also in part of the fact that protein ions have a greater affinity for water than undissociated protein molecules.

This conception of the process of swelling would still yield no equilibrium or swelling-maximum were there no compensating force acting in an opposite sense to the osmotic pressure of the gelatin. Since gelatin plates when immersed in water do not swell indefinitely until swelling merges insensibly into solution but, on the contrary, display a more or less well-marked swelling-maximum, the osmotic pressure exerted by the colloid particles within the jelly must, upon attainment of this maximum, be balanced by an equal opposing force which Procter interprets as the tension of the elastic colloid network (77). Applying Hooke's law, Procter finds that this tension ($= E$) is defined by the equation:

$$E = C\left(V - \frac{1}{\text{sp. gr. of gelatin}}\right),$$

where E is the stress inducing swelling, V is the volume attained by one gram of gelatin, and C is the modulus of elasticity. He finds that $C = 0.00125$ at 7 degrees; 0.00055 at 15 degrees; and 0.00021 at 18 degrees. The value of E at first increases with acidity to a maximum and then decreases, slowly approaching zero.

It has been shown by Ostwald (64) and Chiari (16) that gelatin in very faintly acid media displays a well-marked minimum of swelling-capacity. This minimum occurs at a hydrogen ion concentration almost exactly coinciding with the hydrogen ion concentration of gelatin solutions in which the gelatin is "isoelectric," i.e., does not wander in an electrical field. In other

words minimal combining capacity and ionization of the jelly is accompanied by minimal swelling capacity.

Hofmeister has studied the effects of salts, acids and bases upon the rate and magnitude of the swelling which gelatin plates undergo when immersed in their solutions; we have had occasion to comment upon the significance and interpretation of his results in Chap. VI. Wo. Ostwald (65) has investigated the influence of the concentration of added salts upon the swelling of gelatin plates in water. He asserts that the degree of swelling attained after a given period does not vary continuously in a definite sense as the concentration of the salt is increased, the curve displaying the influence of the concentrations of the salt upon the degree of swelling exhibiting very marked maxima and minima. We shall have occasion to further dwell upon the significance of these results in the next chapter.

The phenomena which accompany and the processes which underlie the taking up or loss of water by *living tissues* have been investigated by Loeb (52) (53), Cooke (21), Bottazzi and Scalinci (6), Beutner (3), and Körösy (47). Bottazzi and Scalinci have drawn attention to the remarkable fact, previously pointed out by von Schroeder in the case of gelatin (93), that the crystalline lens swells very markedly in distilled water or physiological saline solution, but progressively *loses* water when suspended in saturated water vapor at the same temperature. This phenomenon undoubtedly involves a departure from the second law of thermodynamics, realizing the possibility which was depicted figuratively by "Maxwell's demon." It is not surprising, perhaps, that the exception to the rule should have been first encountered in the domain of colloid jellies in which definite structures of molecular dimensions play so pronounced a rôle in determining their behavior.

The significance of the phenomena of swelling in certain aspects of clinical medicine has been especially insisted upon by M. H. Fischer and discussed by him at length in his work upon œdema (26). Exception has, however, been taken by Moore to certain aspects of Fischer's interpretation of the phenomena of œdema (58).

3. The Gelatinization and Coagulation of Proteins. — If an insoluble gel, such as white of egg coagulated by fixatives, the gel of collodion produced by the action of chloroform upon an ether solution, common black india-rubber, or the hydrogel of

silica, be examined under high magnification they can all be demonstrated, Hardy states (33) (34), to possess a fine open spongy structure. When, for example, a 13 per cent solution of egg-white is fixed with sublimate, sections are found to show a sponge or net structure. Staining the section with iron-hæmatoxylin, with saturated solutions of acid and basic dyes or even by evaporation to dryness in solutions of such dyes, failed to produce the staining of any substance within the meshes of the net, while pressure applied to the gel resulted in the squeezing of fluid out of these interstices. The structure of the gel is therefore that of an open sponge-work of solid, containing fluid within its meshes. Direct experimentation with agar showed that in a gel containing 1 per cent agar the solid framework is a solution of water in agar, while the fluid contained in the interstices is a dilute solution of agar in water; upon the heating the system the two components become miscible in each other and we obtain a homogeneous solution. Upon the basis of these facts Hardy draws a far-reaching analogy between this system (and other jellies which are heat-reversible) and the system phenol-water, which, if it contains more than 71 per cent or less than 76 per cent of phenol separates, at temperatures below 80 degrees, into two phases, the one a solution of phenol in water, the other a solution of water in phenol. According to the view developed by Hardy, the two cases differ only in the fact that upon separation of the two phases in the agar-water system the system retains a structure, while in the phenol-water system no structure is retained. Essentially, he believes, the difference between the two systems consists in this, that when the phenol-water system separates into two phases the phases become separated by the minimum possible surface, namely a plane; while when the agar-water system separates into two phases they remain in contact over an area far larger than the minimum. In the latter case it would appear that the surface tension at the surface of separation of the two phases is very low, so that the force leading to a diminution of surface is indefinitely small.

Pauli and Rona (72) object to the use which Hardy has made of the term "phase" in this connection. They point out that the fluid which may be pressed out of an agar jelly may contain from 0 to 0.14 per cent of agar according to the magnitude and mode of application of the pressure, and they urge, in considera-

tion of this fact, that there can be no sharp separation into phases within an agar, and, presumably, within a gelatin jelly. They believe that a sharp distinction should be drawn between the *coagulation* of a protein by dehydrating agents and the gelatinization of its solution, since in the former case a sharp separation into two phases occurs while in the latter it does not. The experimental basis of this objection to Hardy's view is, however, not altogether a sufficient one, since if any two parts of a chemical system, which are of more than molecular thickness, are separated by a surface, they constitute, in the sense of the phase-rule, separate phases. The observation of Pauli and Rona shows, however, that, at least in agar jellies, if separate phases exist they *are not of definite or constant composition.*

The fact that in protein jellies which do consist of two phases the phases are not of constant composition is also very clearly revealed by Hardy's own results, of which a description follows:

The manner in which the structure of an insoluble gel is built up can, according to Hardy, be readily observed in the ternary mixture alcohol, gelatin, and water. If 13.5 grams of gelatin are mixed with 50 cc. of water and 50 cc. of absolute alcohol, a mixture is formed which is optically homogeneous at 17 to 20 degrees but which separates into two phases at temperatures below this. "As the temperature falls below the limit a clouding occurs which I find to be due to the appearance of fluid droplets which gradually increase in size until they measure 3 μ. On cooling further, these fluid droplets become solid and they begin to adhere to one another.* The framework is therefore an open structure which holds the fluid phase in its interstices." "When once formed the phases have considerable stability. If the droplets are composed of a solid solution one may, by the addition of water, cause them to increase to relatively vast dimensions without their being destroyed; as they increase in size their refractive index approximates more and more to that of the external phase until t ıey are finally lost sight of. The addition of alcohol, however, once more brings them into view and causes them to shrink. Owing to this stability, once a configuration has been established, one has to far overstep the conditions of its formation in order to destroy it. This would account for

* The formation of similar droplets has been observed by Pauli and Rona (72) in the coagulation of gelatin by salts.

the remarkable hysteresis observed in reversible gels. . . . When water is added to a ternary mixture so as to considerably swell the droplets the system is unstable and the two phases mix at once when it is mechanically agitated" (34).

In gels of this type which are dilute with respect to the colloid, therefore, the structure is that of an open sponge-work; the meshes being filled with water or a water-rich solution of the substance forming the gel, while the frame-work of the sponge consists of anastomosing threads composed of linearly arranged globules of the water-poor phase. In such gels, therefore, the surface of the water-poor phase is convex while that of the water-rich phase is concave; in other words, the water-poor phase is *internal* to the water-rich or *external* phase. If, however, to a ternary mixture of gelatin, alcohol and water which forms such a gel as that described above, more gelatin be added, the character of the gel changes entirely, the water-poor phase becomes concave and the water-rich phase instead of being, as formerly, concave becomes convex to it. On cooling such a mixture to a temperature below that at which it forms an optically homogeneous solution, droplets separate out which are poor in gelatin, while the interstitial portion of the system, which is rich in gelatin, solidifies. Thus the gel comes to possess a honeycomb structure, the droplets being poor in gelatin, rich in water. This is very clearly shown in the following determinations of Hardy.

TEMPERATURE OF THE MIXTURE 15 DEGREES

(Equal parts of water and alcohol)

Per cent gelatin in mixture	Per cent gelatin in droplets (internal) phase)	Per cent gelatin in interstices (external phase)
6.7	17.0	2.0
13.5	18.0	5.5
36.5	8.5	40.0

From these determinations it is also clear, as I have said, that the two phases are not of constant composition, but may, under different conditions of total concentration, etc., vary widely in their relative and absolute gelatin and water content. This system differs, therefore, from the system phenol-water not only

in the extent of the surface which separates the phases, but also in the variability of the composition of its phases, in this respect resembling rather the system hydrated silica-water, investigated by van Bemmelen (1).

An inversion of the external and internal phases of a diphasic system, similar to that observed by Hardy on increasing the concentration of gelatin in the system gelatin-alcohol-water, may also be observed in the system olive oil-alkaline water, on increasing the proportion of oil to water (84) (85), and is here manifestly due to the inability of a limited quantity of alkaline water to surround and envelop an unlimited quantity of oil.

The question has been raised whether the jelly which is formed by gelatin dissolved in water (instead of alcohol-water mixtures) really possesses a structure analogous to that observed by Hardy in ternary systems. It has been urged that this structure is an artefact arising out of partial coagulation of the protein, since it is not directly visible in binary systems. The action of coagulants upon jellies which already possess a structure of this type, however, is not to otherwise alter but merely to coarsen the structure. This is due to loss of water on the part of the colloid-rich droplets with a consequent diminution of the volume of the colloid-rich phase and an increase in the volume of the more fluid interstices. This can be shown, not only by direct observation, but also by the relative ease with which water can be expressed from the jelly before and after "fixation." From Poiseuilles' law for the outflow of liquids from capillary tubes, it follows that the pressure required to express the fluid from the interstices of a gel at a given rate must vary approximately as the inverse fourth power of the diameter of the meshes, although, of course, the variable viscosity of the expressed fluid will be a factor introducing departures from this simple law. Now a hydrogel containing 13 per cent of pure gelatin at a temperature of 15 degrees will endure a pressure of 400 pounds to the square inch without expression of water; after fixation with formalin or corrosive sublimate, however, the fluid can be expressed from the gel like water from a sponge, with simple hand-pressure (33).

Since more complete coagulation does not alter the *type* of structure possessed by jellies of partially coagulated protein, but merely coarsens it, it is a fair inference that jellies which

have undergone no measure of coagulation also possess the type of structure outlined by Hardy, but that owing to its fineness the details of this structure are not visible.

The existence of a structure in jellies formed by the solution of gelatin in water is also objectively demonstrated by the observation of Liesegang (51) that when silver nitrate diffuses into gelatin which is impregnated with potassium bichromate, the precipitation of insoluble silver bichromate does not occur indifferently in all parts of the area of diffusion but in concentric circles. It has also been shown by Rohonyi (91) that when thin films of gelatin are frozen the ice crystals are formed in concentric rings. It is difficult to clearly conceive any mechanism which would permit this in a perfectly homogeneous medium. The theory that crystallization is inhibited by the gelatin until a certain degree of super-saturation is attained might account for failure of precipitation or crystallization at certain points, but, provided the jelly is strictly homogeneous and structureless it fails to account for its appearance at other points. That the distinction between gelation and coagulation is merely a distinction of degree and not of kind has been shown by Buglia (12).

The experiments of Hardy show that on adding water to the system alcohol-water-gelatin, the gelatin-rich phase progressively imbibes water until it passes by a series of insensible transitions into a *solution* of gelatin. Having regard to this fact and to the probability, which will be indicated in the following chapter, that the structure of a jelly of uncoagulated protein is merely the continuation of a structure which pre-exists in the solutions which become gelatinized, it appears highly probable that *in uncoagulated protein jellies the structure is of molecular dimensions, so that the constituents of the jelly are not separated from one another by any definite interface.* This conception does not in the least militate against the view that the structure of a coagulated or partially coagulated jelly merely results from the *coarsening* of a pre-existing molecular structure. It is probable that the coagulation of a protein solution, without passing through the intermediate stage of gelatinization, is similarly accompanied and possibly (from a mechanical standpoint) accomplished by the coarsening of a pre-existing molecular structure.

4. The Coagulation of Proteins by Heat, Light and Hydrostatic Pressure. — Several aspects of the phenomenon of heat-

coagulation have been discussed at some length in Chap. VI, and it has been pointed out in that chapter, and incidentally, in Section **1** of this chapter that the application of heat to proteins in solution results, probably, not only in the abstraction of the elements of water from the protein, with the formation of anhydrides, but also in its polymerization.

Under definite conditions of concentration, reaction and salt-content of its solution, etc., the coagulation-temperature of a protein is tolerably constant, and Frédéricque (27), Halliburton (29) and others have utilized this property very extensively in the endeavor to separate and characterize different proteins.

The concentration of protein, and especially the presence of other substances in the solution, very markedly modifies the coagulation-temperature, however. The influence of inorganic salts, upon the "temperature of coagulation" has been incidentally discussed in Chap. VI. The influence of a variety of salts in acid and alkaline solutions upon the coagulation-temperature of proteins has been very extensively studied by Pauli and his pupils (32), and found to be in satisfactory accordance with the view that heat-coagulation, like salt- or alcohol-coagulation is accomplished through the dehydration of the protein molecules.

The very important observation has been made by Chick and Martin (17) that the heat-coagulation of hæmoglobin and egg albumin (in solutions of crystallized preparations of these substances) is not an instantaneous process, but that it proceeds with a definite velocity which decreases as the protein becomes coagulated and increases very markedly with rise in temperature. The relation between time of exposure to a temperature sufficient to bring about coagulation and the quantity of protein coagulated is, for hæmoglobin, that which is characteristic for the occurrence of a monomolecular chemical reaction, namely:

$$\log \frac{C_0}{C_t} = Kt,$$

where C_0 is the initial concentration of the substance (protein), C_t its concentration at time t and K is a constant (the velocity-constant of the reaction). The following are illustrative of their results:

COAGULATION OF HÆMOGLOBIN, 3 PER CENT SOLUTION

Experiment	Temp, degrees	Time in minutes	Concentration of hæmoglobin (first sample = 100 = C_0)	$\log_{10} C$	$K = \frac{\log_{10} C_0 - \log_{10} C_t}{t}$
1	60	0	100	2.000	.....
		30	54	1.732	0.0090
		90	13.5	1.130	0.0097
					Mean.....0.0094
2	62.6	0	100	2.000	.....
		20	42	1.623	0.019
		45	12	1.079	0.020
		70	4.8	0.681	0.019
					Mean.....0.019
3	65.6	0	100	2.000	.....
		10	35.5	1.550	0.045
		20	11.0	1.041	0.048
		30	5.0	0.699	0.043
					Mean.....0.045
4	67.6	0	100	2.000	.....
		3	61.4	1.788	0.071
		6	34.8	1.542	0.076
		9	24.9	1.396	0.067
					Mean.....0.071
5	70.4	0	100	2.000	.....
		2	52.5	1.720	0.14
		4	25.3	1.404	0.15
		6	14.1	1.150	0.15
		7.5	7.6	0.886	0.15
					Mean.....0.15

The temperature-coefficient of the process is seen to be very high, the velocity-constant being multiplied about 15 times by 10 degrees rise in temperature (from 60–70.4 degrees). The relationship between the temperature and the value of K is very satisfactorily represented by the Arrhenius equation:

$$\frac{K_1}{K_0} = e^{\frac{\mu}{2} \frac{T_1 - T_0}{T_1 T_0}},$$

a relation which, although not strictly deducible from the gas laws (60) is analogous in form to the van't Hoff equation connecting the value of the equilibrium-constant with the temperature and which is deducible from the gas laws. We are probably justified in concluding, therefore, as we did in the analogous case afforded by the variation in the dissociation-constant of

oxyhæmoglobin with temperature,* that immediately prior to its coagulation in the above experiments the hæmoglobin existed in the solution in a molecularly dispersed condition, obeying the law of Avogadro.

The high temperature-coefficient of the process explains, as Chick and Martin point out, the apparent, but unreal constancy of the "coagulation-temperature" which was so much insisted upon by the earlier investigators quoted above. With rising temperature a point is reached at which the reaction is so rapid as to appear almost instantaneous.

It is obvious that the monomolecular reaction-formula would apply either to the *dehydration* of protein according to the formula

$$H_2N.R.COH.N.R.COOH = \underbrace{HN.R.COH.N.R.CO} + H_2O$$

or to its *hydration* according to a formula of the type

$$\text{Protein} + H_2O = \text{coagulated protein},$$

since in the latter case, the mass of water being very much greater than that of the protein, the active mass of water would be appreciably constant throughout the reaction.

From the fact that *dry* protein, heated to high temperatures does not undergo typical heat-coagulation, i.e., does not lose its solubility in water (24) Chick and Martin conclude that the heat-coagulation of protein is a process of *hydration*. From the results of Pauli and myself, cited in Chap. VI, it is evident, however, that the process of heat-coagulation is not one of hydration but of *dehydration* of the protein,† according to some or all of the equations (i) to (iv) in Chap. VI, section **6**. From the fact that the base-combining capacity of casein diminishes with rising temperature, and also the solubility of some of the caseinates, we have concluded ‡ that with rising temperature this protein, at all events, undergoes some measure of polymerization on heating through the dehydration of the end $-NH_2$ and $-COOH$ groups of adjacent molecules.§ If this were a general phenomenon

* Cf. Chap. VI. † Cf. also Michailow (56) and Starke (98).

‡ Cf. Chap. VI also **1** of this chapter and T. Brailsford Robertson (83).

§ Mann (55) states that in his opinion heat coagul tion is brought about by one portion of the albumin molecule precipitating the remainder, a view which is essentially similar to that expressed above. Sutherland (99) has also expressed the view that coagulation of a protein is the result of polymerization through the neutralization of "Valencies which are usually latent."

then it would follow, from van't Hoff's "Principle of Mobile Equilibrium" that the *hydrolysis* of protein (hydration) is accompanied by an evolution of heat, which conclusion is in complete accord with experimental observation.* The apparent discrepancy between the conclusion reached by Chick and Martin and those which have been formulated by other investigators in this field may therefore be due to the fact that dry protein has been deprived of the elements of water in its end $-NH_2$ and $-COOH$ groups, and that these, consequently, cannot react to form polymers of the protein.† It is evident that in the process of dehydrating proteins by heat not only the reaction:

$$H_2N.R.COH.N.R.COOH = HN.R.COH.N.R.CO + H_2O$$

occurs, but also reactions of the type

$$2\,H_2N.R.COH.N.R.COOH = H_2N.R.COH.N.R.COH.N.R.COH.N.R.COOH + H_2O,$$

and it is the formation of these polymers which leads to the apparently irreversible character of the process; *apparently* and not actually irreversible because as Corin and Ansiaux (22) have shown, if a solution of protein be cooled and vigorously

* Cf. **1** and the results of Wiedemann and Lüdeking cited in this chapter. The heat of reaction of protein hydrolysis is extremely small; observers using the usual indirect methods of determination either failed to detect any change in the heat-content of the system or else have observed a very slight *disengagement* of heat (101), (49), (36). Henderson and Ryder, however, using the beautiful and excessively sensitive isothermal method of calorimetry of T. W. Richards, have demonstrated that the tryptic hydrolysis of casein is accomplished by a progressive evolution of heat (37). It should be clearly borne in mind in this connection that the effect of raising the temperature upon a chemical reaction is always twofold. It shifts the station of equilibrium in one sense or in the opposite and, always, it *accelerates* the reaction in *either* sense to a greater or less degree (i.e., it magnifies both velocity constants, but unequally). The action of heat upon proteins must always be to shift the station of *equilibrium* in the direction of polymerization (i.e., condensation), since the reaction of hydrolysis is exothermic. But the fact that fairly complete *hydrolysis* will occur at the temperature of steam shows that the shift in equilibrium between the *lower* protein complexes and the amino-acids which are the products of their hydrolysis is not so great as to extinguish the reaction of hydrolysis.

† It should also be recollected, in this connection, that reactions occurring in solid systems are notoriously extremely slow, owing to the high internal friction of the system and the consequent hampering of molecular motion.

shaken just as the first traces of heat coagulation appear the incipient coagula will again pass into solution. The apparent irreversibility of the later stages of heat-coagulation is probably attributable to the high internal friction of the floccula which are formed, leading to extremely slow molecular movement and the introduction of a time-element of very considerable magnitude.

This deduction receives decided support from the discovery by Chick and Martin (18) (19) (20) that the heat-coagulation of proteins consists of two processes which they severally term "denaturation" and "agglutination." In acid solutions the process of agglutination or aggregation takes place at a rate very greatly in excess of denaturation and therefore the rate of formation of flocculated protein is primarily determined by the rate of denaturation, i.e., by the slower process. In alkaline solutions, however, aggregation does not occur, but may be induced by subsequent acidification or by saturation with sodium chloride. In acid solutions the rate of denaturation is accelerated by H^+ ions and these decrease during the reaction through the binding of acid by the denatured protein. If the concentration of H^+ ions be kept constant the reaction follows the monomolecular reaction-formula, but if this precaution be omitted then the reaction velocity falls off more quickly than would be anticipated from the monomolecular formula (18) (100). Similarly in alkaline solutions of egg-albumin the OH' ion concentration decreases during denaturation, but if the OH' concentration be kept constant the reaction is of the first order.

The diminution in the acidity of acid solutions of protein during heat-coagulation has also been observed and quantitatively determined by Sörensen and Jurgensen (97) and Quagliariello (79).

We may conclude, therefore, that the results of Chick and Martin are in satisfactory harmony with the view of Hofmeister and Pauli that coagulation, including the heat-coagulation of protein, is essentially a phenomenon of dehydration of which the first stage, that of internal neutralization through the loss of the elements of water from end $-NH_2$ and $-COOH$ groups, probably corresponds to the phenomenon of "denaturation" while the subsequent or simultaneous polymerization of these anhydrides leads to the formation of particles so large as to assume the properties of matter in mass, i.e., flocculi.

These facts undoubtedly underlie and explain the apparent transformation of serum albumin into serum globulins observed by Moll on heating blood serum to which alkali had been added (57). As Gibson (28) and Schmidt (92) have pointed out, a transformation of serum albumin into globulin would involve a synthesis of glycocoll, which amino-acid is not contained in the albumin molecule. There can be little doubt that the protein which Moll regards as globulin produced from albumin is in reality "denatured" albumin which acidification will flocculate, just as it flocculates a globulin.

The reversible character of "denaturation" (presumably anhydride-formation without polymerization) is indicated by the observation of Berczeller (2) that while the surface tension of a protein solution which is so salt-free as not to coagulate on heating, nevertheless diminishes on heating, this diminution is reversible and spontaneously disappears on standing.

It has been shown by Bovie that protein solutions in quartz vessels are coagulated by exposure to ultraviolet light (7). This type of coagulation, like heat-coagulation, demonstrably consists of two separate processes. The first process, that of denaturation, has, like other photochemical processes, a very low temperature-coefficient and takes place almost as rapidly at 0° C. as at room temperature. The second process, that of agglutination or flocculation, has a high temperature-coefficient indicating that it is not primarily a photochemical process, but a spontaneous consequence of the photochemical denaturation which precedes it.

Fernan and Pauli (25) have shown that exposure to the radiations from radium leads to coagulation of protein (serum albumin) in acid or alkaline solutions. Unlike heat-coagulation the coagulation in acid solutions by radium radiations is accompanied by no diminution of their H^+ concentration.

It has been shown by Bridgman (9) that the application of very great hydrostatic pressures results in the coagulation of white of egg. The pressure is applied very slowly to avoid any rise in temperature due to the compression and that the effect is not due to heat is further demonstrated by the fact that it is more easily elicited at 0 degrees than at 20 degrees. The application of five thousand atmospheres produces stiffening of the white of egg; six thousand atmospheres compression, applied for

thirty minutes, produces an appearance of the white resembling that of curdled milk, while seven thousand atmospheres pressure brings about complete gelation.

5. The Crystallization of Proteins. — A number of proteins, particularly the vegetable proteins and hæmoglobin, are readily obtainable in the form of crystals. Other proteins, such as egg- and serum-albumin only yield crystals with considerable difficulty. For the crystallization of these latter proteins mere concentration of their solutions is insufficient; inorganic salts in very high concentration must also be present (39) (41) (42) (48) (94) (73). Thus egg-albumin may be prepared in a crystalline condition by adding to its solution an equal volume of saturated ammonium sulphate solution and acidifying with acetic acid. The coagulum which forms becomes crystalline on standing. It would appear that the crystalline product is not the uncombined protein but a salt of the protein, formed with an acid and also, probably, with the ammonium sulphate (Cf. Chap. VI) (59).

The protein crystals are optically true crystals. According to Wichmann all albumin-crystals are either crystallographically identical or else isomorphic. They very readily absorb impurities (106) (45) (95), and the circumstances of their preparation involve the occlusion of a considerable quantity of the mother liquor. Hence repeated recrystallization is required to remove from them even colloidal impurities (95). Contamination by crystalloidal substances, combined or physically associated, obviously cannot be avoided, and these must be subsequently got rid of by very prolonged dialysis of the dissolved crystals.

A monumental and most fundamentally important contribution to our knowledge of the crystallography of proteins has been furnished by the investigations of Reichert and Brown (82) on the relationship of the morphology of hæmoglobin crystals to the biological classification of the species from which it is derived. From an enormous number of measurements of crystal angles, etc., conducted upon hæmoglobins derived from a large variety of species, these investigators conclude in the first place that the crystals of the species of any genus belong to the same crystallographic system and generally to the same crystallographic group, and they have approximately the same axial ratios, or their ratios are in simple relation with each other. In

other words the hæmoglobin crystals of any genus are isomorphous. In some cases this isomorphism may be extended to include several genera, but this is usually not the case unless, as in the case of the dogs and foxes, for example, the genera are very closely related. On the other hand the oxyhæmoglobin obtained from the same species always crystallizes in the same form, although often with different habit when obtained by different methods of preparation. But upon comparing the hæmoglobins from different species of a genus it is found that they differ from one another to a greater or less degree in angles or axial ratio, in optical characters and particularly in those characters comprised under the general term "crystal habit," so that one species can usually be distinguished from another by the form of its hæmoglobin crystals. But these differences, within the limits of a given genus, are not such as to preclude the crystals from all species of that genus being placed in an isomorphous series.

A clear relationship is thus seen to subsist between the physico-chemical behavior of a constituent of organisms and their place in the phylogenetic scale of relationships as established by their gross morphology, and a long stride has thus been taken toward the establishment of a physico-chemical basis for morphological distinctions. The further and entirely independent question now arises, however, as to the *chemical* interpretation of the observed *physico-chemical* phenomena.

Our experience with the crystallography of inorganic and the simpler organic substances has led us to infer with a considerable degree of confidence that substances which show differences in crystallographic structure are different chemical substances. Crystal form is affected even by isomeric modifications which analysis, unaided by other methods of investigation, fails to reveal. Now the enormous number of atoms in a protein molecule encourages, at first sight, the supposition that an enormous and indeed, for all practical purposes, an infinite number of isomerides are possible between which the most refined methods of analysis would not enable us to distinguish, but which would very probably differ from one another in the morphology of their crystals. In point of fact, however, the available number of isomers would be very greatly restricted by the necessity of maintaining unaffected the amino-acid groupings of the protein moiety which could not differ materially in different species

without leading to decided differences in the chemical behavior of the hæmoglobins which have not been observed by any investigator. Further doubt is thrown upon this interpretation of the facts observed by Reichert and Brown by the observation of Hüfner, recently confirmed with the utmost precision by Butterfield (13), Heubner and Rosenberg (38), and Schumm (96) that the characteristic absorption bands and the ratio of the absorption of light in different parts of the spectrum of hæmoglobin is absolutely identical in species so far removed from one another as the horse and man (Schumm) or the rabbit, sheep and hog (Heubner and Rosenberg). Now these are properties which we should anticipate might be materially affected by internal differences of atomic arrangement.

Further reason for doubting the correctness of referring the differences of crystal structure displayed by the hæmoglobins of different animals to internal differences in the molecules of the hæmoglobins is supplied by the observation of Loeb and Brown (54) that *the crystal-form of the hæmoglobin of the mule is intermediate in character between that of the horse and that of the donkey.* For if we assume that each different crystal form represents a different internal atomic arrangement of the hæmoglobin molecule, then the number of such arrangements, even if very great, must nevertheless be limited. The number of possible forms of crystals must therefore also be limited and moreover the possible modifications of forms must be discontinuous, i.e., there must exist forms between which no intermediate forms are possible. This being the case it would be very remarkable indeed were the hybridization of two closely related species to lead to the synthesis of a new isomeric variety of hæmoglobin not yet appropriated by any existing species of animal and in addition lying *between* the hæmoglobins of the parent species. If analogous phenomena should be displayed by all hybrids and by all varieties and mutations that might have arisen or might conceivably arise in the future we would have to admit that the hæmoglobins already recognizable as differing from one another in crystal form are only a small proportion of those which are realizable.

A much more reasonable supposition is that embodied in the view that the differences in crystal form observed by Reichert and Brown are attributable, not to the internal variation of atomic grouping in the hæmoglobin molecules but to external varia-

tions in the milieu from which they are crystallized. The technique adopted by Reichert and Brown was to induce crystallization directly in the laked blood. Now we know from the observations of the immunologists that the blood plasma from any species of animal differs antigenically from that derived from any other species (62) and, since all known antigens are proteins, we infer that the proteins, or, more probably, the compound protein complexes (Cf. Chap. VII) in blood plasmas derived from different species are in certain definite respects different from each other. The crystals of each species studied by Reichert and Brown were therefore deposited from a different medium and it is not improbable that the observed differences in the crystals are attributable to these known differences in the media in which they were formed. It is well known that crystal-habit is modified by alterations of the medium from which the crystals are deposited. That modifications of this origin so great as to preclude inclusion of the crystals formed in different media in the same isomorphous series have not hitherto been observed in the domain of inorganic chemistry is not improbably attributable to the simpler character of the conditions accompanying crystallization in inorganic or non-colloidal media. We have seen that there are many reasons for supposing that proteins, even in solution, are disposed in a certain reticular structure and if, as the facts dwelt upon in Chap. VII would seem to indicate, characteristic protein complexes, formed by the union in differing proportions of a relatively small number of simpler protein components, exist in each type of blood plasma we may well suppose that the reticular structures of the solutions comprising these plasma would likewise differ from one another. Having regard to the markedly cohesive properties of proteins, crystallization within the meshes of such a reticulum might very conceivably, through external strains imposed by points of attachment to the reticulum, modify the effects of the internal strains which find their expression in crystal form.

This hypothesis finds decided support in the fact first observed by Halliburton (39) (31) and confirmed by Reichert (81) that the crystal form of oxyhæmoglobin derived from a given species may be profoundly modified by admixture with the blood of another species. The following are illustrative results obtained by Halliburton, the "normal" form of rat hæmoglobin crystals

being rhombic, those obtained from guinea-pigs being normally tetragonal and those from squirrel's blood hexagonal.

Blood of	Mixed with that of	Form of hæmoglobin crystals deposited from the mixture
Rat	Squirrel	Both rhombic prisms and hexagons present.
Rat	Guinea-pig	No rhombic prisms of the shape usually seen in rat's blood present. No tetrahedra. Crystals are all rhombic prisms with hexagonal habit.
Squirrel	Guinea-pig	Hexagonal plates and tetrahedra both present. Many tetrahedra imperfect. The tetrahedra all reduced to about half the size of those prepared from the unmixed blood of the same guinea-pigs.
Dog	Squirrel	Fine rhombic needles and hexagonal plates both present in abundance.
Dog	Guinea-pig	The greater number of the crystals formed are very small tetrahedra about a quarter the size of those prepared from the blood of the same guinea-pigs. The optical properties are, however, the same. Rhombic prisms, very slender, like those of dog's blood, are also seen.

According to Reichert the degree of modification of crystal form induced by admixture of two bloods depends very greatly upon the proportion in which they are mixed.

In view of these facts there can be little doubt that the nature of the milieu in which crystallization occurs does play an important part in determining the form of the crystals which are deposited, and having regard to the known individuality of the plasma from different biological species it would appear unnecessary to seek further for the origin of the differences in crystal form of the oxyhæmoglobins derived from blood of different species of animals.

We are now in a position, also, to interpret the changes in crystal-form which result from repeated re-crystallization of hæmoglobin (Halliburton (30) (31)), for as Wichmann (106) and, more recently, Katz (45) have shown, the crystalline proteins swell in or absorb the surrounding fluid menstruum in a manner analogous to the swelling of jellies. A number of re-crystallizations are therefore required to remove completely traces of the original menstruum in which crystallization occurred.

Bradley and Sansum (8) believe that the hæmoglobins from different animals are antigenically different because guinea-pigs sensitized to ox or dog hæmoglobin failed to display anaphylactic shock or reacted but slightly to hæmoglobins of other origins, while they reacted strongly to the hæmoglobin with which they were sensitized. As the hæmoglobin preparations employed by Bradley and Sansum were admittedly (with the exception, they believe, of the dog hæmoglobin) not free from contamination by serum the interpretation of these results is open to serious question. Doubt is especially thrown upon this evidence for the specificity of hæmoglobins from different species by the fact that the animals sensitized to the purest preparation of hæmoglobin employed, that of the dog, reacted strongly not only to to dog hæmoglobin *but also to dog serum.* Having regard to the investigations of Wichmann and Katz, cited above, revealing the marked ability of crystalline proteins to absorb the menstruum from which they are deposited, and to the observation of Schulz and Zsigmondy (95) that egg albumin must be recrystallized from 3 to 6 times in order to remove appreciable contamination by other proteins, we may infer that in all probability the specificities demonstrated by Bradley and Sansum are serum-specificities and not hæmoglobin-specificities.

According to Howell (43), fibrin must now be added to our list of crystallizable proteins. He finds that when fibrinogen is precipitated by thrombin, the fibrin, in media of normal H^+ concentrations, separates out in crystalline needles readily recognizable as such under the ultramicroscope. They vary in length from 10 to 30 microns and form a close mesh-work. The normal blood-clot is therefore a crystalline gel. The blood of invertebrates, however, yields a non-crystalline gel and a similar gel is yielded by mammalian fibrin in alkaline media. Such non-crystalline gels, however, fail to display the spontaneous contraction or "synæresis" which is so characteristic of normal blood-clots.

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CHAPTER XIII

CERTAIN PHYSICAL PROPERTIES OF PROTEIN SOLUTIONS, ETC.

1. The Viscosity of Protein Solutions. — Protein solutions are usually characterized by the possession of a high viscosity. The question has been raised whether the viscosity is similar in nature to that of a solution of a crystalloid substance, or whether it is not, rather, comparable with the viscosity of a suspension of solid particles (40). In support of this latter view it has been pointed out that Bottazzi's measurements of the influence of concentration upon the viscosity of protein solutions indicate that the viscosity of these solutions does not change continuously with change in concentration, as it does in solutions of crystalloids, but changes *per saltum* (12). The measurements of Bottazzi were made upon relatively few and widely separated concentrations, however, and subsequent observers have not confirmed his results (108) (59). Thus Sackur finds that the viscosity of solutions of the "basic" caseinates of sodium and ammonium (i.e., containing about 80×10^{-5} equivalents of base per gram and neutral to phenolphthalein) varies with the concentration according to the Arrhenius-Euler formula $\frac{\eta}{\eta_0} = A^n$, where η is the viscosity of the solution, η_0 that of the solvent, n is the concentration of the solution and A is a constant, the numerical value of which depends upon the nature of the dissolved substance and upon the temperature (2). The following are Sackur's results:

n (in equivalent) sodium)	$\frac{\eta}{\eta_0}$ (15 degrees)	log A
0.01830	1.870	14.8
0.01370	1.581	14.5
0.00915	1.363	14.3
0.00547	1.202	14.6
0.00458	1.165	14.5

A remarkable feature of these results is the extraordinarily high value of A, involving a very rapid increase in viscosity with

increasing concentration. For the majority of crystalloids the value of A is not greatly in excess of unity, while for sodium caseinate, as we see, it is of the order of 10^{14}. This fact alone would lead us to suspect that the mechanism which produces the viscosity of these solutions is different in nature from that which produces the viscosity of solutions of crystalloids. Sackur has endeavored to ascertain which constituent of the solutions of sodium caseinate plays the greater part in determining their viscosity. He arrived at a conclusion the correctness of which more recent investigations have fully established, by a process of reasoning, however, which recent investigations have shown to be in some respects fallacious. He argued that the viscosity of these solutions might be attributable, primarily, to undissociated sodium caseinate, or the product of its hydrolytic dissociation,* i.e., free casein, or to caseinate ions. Hydrolytic dissociation would, he believed, be diminished by the addition of alkali and increased by the addition of acid. In the former case, according to his view, the number of caseinate ions should be increased, in the latter decreased. He found that the addition of alkali *increased* and the addition of acid *diminished* the viscosity of a neutral (to phenolphthalein) solution of sodium caseinate; the following are among his results:

Per cent of casein	Normality of total sodium	$\frac{\eta}{\eta_0}$ (15 degrees)
0.716	0.0063	1.24
0.716	0.0126	1.36
0.716	0.0189	1.38
0.716	0.0252	1.34

hence, he argued, the viscosity of these solutions is primarily attributable to *caseinate ions*.

We have seen, however (Chaps. IV and VIII), that the caseinates do not undergo hydrolytic dissociation in solution. In fact the initial solution to which Sackur added alkali in order to *suppress* hydrolytic dissociation was neutral to phenolphthalein and therefore could not have contained more than 10^{-5} N NaOH derived from hydrolytic dissociation of the sodium caseinate. We have also seen (Chap. IX) that on adding alkali to a solution of a casein-

* Since free casein is insoluble this possibility may be dismissed.

ate which is neutral to phenolphthalein the added alkali does not remain unneutralized, as Sackur assumes, but, on the contrary, is partially bound by the casein until 180×10^{-5} equivalents are combined with every gram of casein. But we have also seen (Chap. IX) that each successive equivalent of combined alkali splits another $-NHOC-$ bond in the casein molecule and consequently gives rise to another pair of ions. With increasing content of combined base, therefore, the caseinate, apart from slight modifications of its degree of dissociation, yields a corresponding proportion of ions. The fact that the viscosity of caseinate solutions markedly increases with alkalinity is therefore in strong support of Sackur's thesis that the viscosity of these solutions is primarily attributable to protein ions, although not for the reasons which he advances. The fact that the increase in viscosity with increasing alkalinity attains a maximum at just about the same time that the combining capacity of the casein attains a maximum (Cf. above table and the tables and diagram in Chap. IX) lends very striking support to this hypothesis.

The view that the viscosity of protein solutions is in a remarkably high degree dependent upon the protein ions which they contain has also been advanced, with substantial experimental support, by W. B. Hardy (41) and by Bottazzi (14). The latter observer has shown that the viscosity of protein solutions is at a minimum when ionic protein is absent, when the protein is uncombined with base or acid, and that on adding either acids or bases to this solution the viscosity increases.

Now a very little consideration suffices to show that the viscosity of protein solutions is of a different type from the viscosity, for example, of solutions of sugar or glycerol in water. Apart from the extraordinary magnitude of A, alluded to above, the type of viscosity exhibited by solutions of proteins differs from the viscousness of a glycerol-water mixture in that it affords no hindrance, or very slight hindrance, to the motion of ions and of crystalloid molecules. The properties of agar are, in this respect, very similar to those of protein. Thus Graham (35) showed that the velocity with which crystalloids diffuse through gelatin jellies is remarkably near to that with which they diffuse through water, and Voigtlander (126) has confirmed this result for agar jellies. Similar results have been obtained by Hüfner (47). According to Bechhold and Ziegler (6) the rate of diffusion of (at any rate concentrated)

crystalloids is diminished by gelatin jellies, and the degree of hindrance is materially modified by the presence, within the jelly, of other dissolved substances, but the hindrance is extremely small in comparison with the enormous viscosity of the jellies.

Similarly Reformatzky (90) has shown that the velocity with which methyl acetate is decomposed by acids in agar jelly (i.e., the number of molecular collisions per second) is within 1 per cent of its value in pure water.

Lodge (65), Whetham (129) (130) and Masson (69) have shown that the specific mobilities of the majority of inorganic ions is the same in agar jellies as it is in water. Dumanski (28) has shown that if allowance be made for the diminution in the cross-section of the conducting field, due to the presence of gelatin $\left(= \left(\frac{g}{c}\right)^{\frac{2}{3}}\right.$, where g is the number of grams of gelatin per gram of solution and c the specific gravity of gelatin$\Big)$ the conductivities of inorganic salt solutions in gelatin jellies are very slightly less than those of equally concentrated solutions in pure water.

We have seen* that the dependence of the conductivity of protein solutions upon their dilution is of perfectly normal character, resembling the dependence of the conductivity of a crystalloidal electrolyte upon dilution, despite the fact that the protein solutions, as the above-cited results of Sackur reveal, vary enormously in their viscosity with dilution.

On the other hand, the intimate dependence of the conductivity of solutions of electrolytes upon the ordinary type of viscosity has been commented upon and quantitatively estimated by a host of observers, among whom Walden may be especially mentioned (127) (51) (37). Viscosities, very much less than those of the dilutest jellies, profoundly diminish the conductive power of electrolytes. Nor must it be imagined that the viscosity of a protein jelly is essentially different, in any respect save magnitude, from that of a protein solution, for, as von Schroeder (110) has shown, the viscosity of a solution of gelatin, cooled below the gelation-point, increases progressively and regularly with time, until the extremely viscous solution passes insensibly into a jelly.

Not only inorganic, but also protein ions are profoundly influenced in their mobilities by the type of viscousness which

* Cf. Chap. X, also W. B. Hardy (41).

alcohol- or glycerol-water mixtures exhibit. Thus we have seen, in Chapter XI, that the mobilities of caseinate ions in alcohol-water mixtures are almost exactly in inverse proportion to the viscosity of the solvent, and this despite the fact that the viscosity of the solvent, measured by the time which it takes to run through a capillary tube, is so profoundly influenced by the introduction of the caseinate itself as to be in many cases, doubled in magnitude. Solutions of KCl in alcohol-water mixtures are, comparatively speaking, unaltered in their viscousness by the KCl, yet the dependence of the conductivities of solutions of potassium caseinate upon the percentage (between 0 and 60 per cent) of alcohol which they contain obeys exactly the same law as that which applies to the conductivity of KCl solutions in alcohol-water mixtures, a law which implicitly involves the conclusion that the total effect is due solely to the alteration in the mobilities of the ions attributable to the viscousness of the *solvent*. To make the matter clear by reference to a numerical example: Referring to the tables in Chapter XI we see that the viscosity of $N/10$ KOH (dilute alkali) is doubled by the introduction of 3.125 per cent of casein; halving the concentration of the solution, according to Sackur's results cited above, must more than halve the effect of the caseinate upon its viscousness; yet the mobilities of the caseinate ions are unaltered by this. The viscosity of the solvent can be also doubled by the introduction of 40 per cent alcohol and this, on the contrary, halves the mobility of the caseinate ions. The alcohol considerably modifies the degree to which the caseinate alters the viscousness of the solvent, yet this fact does not at all disturb the regularity of the relation between the viscosity of the *solvent* and the mobility of the caseinate ions which are dissolved therein. *In estimating the influence of viscosity upon the mobilities of caseinate ions we can entirely disregard that portion of the viscosity of the solution which, although comparable in magnitude with the viscosity of the solvent, is attributable to the caseinate itself.*

Bearing in mind the possibility, which was indicated in the previous chapter, that protein solutions may contain a preformed molecular structure analogous to that of the jellies or coagula which they can form, we are strongly impelled towards the belief that the type of viscosity which solutions of proteins exhibit may in some manner owe its existence to this structure, and not to the type of internal friction which hinders molecular

and ionic motion. Thus a netlike structure, such as a tennis net, will offer no hindrance to the passage through it of a quickly moving body which is smaller than its meshes, other than that which is due to the fact that the material which composes the net occupies a small fraction of the area which the body must traverse, but to any force which involves deformation of the structure, for instance, a force which seeks to drag it through a small tube, it will offer a very considerable resistance. On the other hand the resistance which is offered to a small moving body by a viscous liquid (viscous, that is, in the ordinary sense) is accurately measured by the resistance which the liquid offers to passage through a tube. Now the *direct* methods which we employ to measure the viscosity of fluids are all such (rotation of a relatively large body within the fluid; passage of the liquid through a capillary tube, etc.) as would *involve the deformation of any molecular structure within the fluid.* Our *direct* methods of estimating viscosity do not enable us to distinguish between that type of viscosity which is attributable to a structure within the fluid and the other type of viscosity which we may term "true" internal friction. The indirect measurement which the conductivity estimations afford, however, only reveals the latter type of viscosity and, as we have seen, when we employ this method of estimation the presence of protein is found to leave the viscosity of the solvent unaltered.*

If we admit, however, that the viscosity of protein solutions is due to a molecular net-structure within them, then we are forced to a conclusion which, in the light of our present inadequate knowledge of the mechanics of ionization, appears very curious. We have seen (Cf. above) that the viscosity of protein solutions must be attributable, primarily, to the protein ions which they contain. We are led to conclude, therefore, that the net-structure

* Save to the degree involved by the fact that the material composing the net-structure occupies a certain proportion of the cross-area of the conducting field. As we have seen above, the results of Dumanski show that if allowance be made for this diminution in cross-area the conductivities of solutions of electrolytes in gelatin jellies are nearly identical with those of equally concentrated solutions in water. The slight deviations which Dumanski observed are probably attributable to the fact that the calculation of this diminution in area involved the specific gravity of the gelatin in its dissolved condition, and this is not necessarily the same as that of unhydrated gelatin, i.e., gelatin in its undissolved condition.

within protein solutions is built up out of protein ions.* Such a conclusion is totally out of harmony with the prevailing view that ions, in solutions of electrolytes, are mutually independent and physically discrete bodies, and would appear to invite a distinction between the mode of ionization of ordinary electrolytes and that of protein salts. It is very questionable, however, whether such a distinction would be valid. Many modern investigations point to the existence of some modification of the familiar "Grotthus-chain" in solutions of electrolytes, most impressive among which is possibly the fact that in watery solutions the most rapidly-moving ions are those which, by their combination, give rise to water itself, i.e., hydrogen and hydroxyl, while in other solvents hydrogen and hydroxyl are not the most rapidly moving, *but those which, by their combination, give rise to the solvent* (24) (125) (25) (60). The fact that the viscosities of solutions of crystalloid electrolytes have not so far revealed the presence of a net-structure within them is possibly attributable to the tenuity of the net and to the fineness of its framework; to revert to the analogy employed above, a net of the finest and most flexible silk will readily pass without appreciable resistance through a tube which would offer a considerable resistance to the passage of a net of coarse thread. The phenomena to which I have drawn attention may quite conceivably be of very general occurrence, and of greater physical magnitude in solutions of proteins simply because of the greater size of the protein molecules. In this connection the views regarding the nature of ionization which have been put forward by Wm. Sutherland (118) offer extremely tempting suggestions.

The hypothesis that gelatin solutions, even those which do not gelatinize, contain a structure has also been put forward by Wo. Ostwald (80). The influence of added inorganic salts upon the viscosity of gelatin solutions has been studied by von Schroeder (loc. cit.); this observer finds that the chlorides and nitrates of the alkalies diminish the viscosity of gelatin solutions while sulphates increase it. Moreover the alteration in viscosity induced by the added salts does not vary continuously in a definite sense

* Indeed, whether we assume that the viscosity of protein solutions is due to protein ions or not, it would be difficult to avoid this conclusion in the light of the fact that many highly viscous solutions of caseinates are over 90 per cent dissociated (Cf. Chap. X).

as more of the salt is added, but the curve displaying the influence of the concentration of the salt upon the viscosity of the solution exhibits very marked maxima and minima. Now Ostwald has found that the influence of added salts upon the swelling of gelatin plates in water (Cf. Chap. XII) is of a similar character to their influence upon the viscosity of gelatin solutions; that the degree of swelling runs closely parallel with the diminution in the viscosity of solutions, as determined by von Schroeder, since the concentrations of salts at which maxima of swelling occur are nearly identical with those at which minima in the viscosity of solutions are observed. From this Ostwald argues that the passage of gelatin into solution does not destroy the structure of the gel but that this structure persists in solution.

Sutherland (119) in developing the theories to which I have referred above, has also expressed the opinion that protein solutions possess a structure.*

Of great interest in this connection is the observation which a number of investigators have made (110) (59) (34) (109) (33) that the viscosities of gelatin solutions, insufficiently concentrated to gelatinize, change somewhat on standing. Garrett has also observed (33) that the logarithmic decrement of a disc oscillating in gelatin or albumin solutions is not a constant, as it is in water or other homogeneous fluids, but, on the contrary, increases as a linear function of the time. This was traced to adhesion between the disc and the protein; if the disc was taken out of the fluid and washed the initial value of the decrement was always the same. This is obviously to be explained, on the basis of the above hypothesis, by the adhesion of portions of the net-structure to the oscillating disc. Garrett has further observed that very slight mechanical disturbance of a gelatin solution produces considerable alteration in the magnitude of the oscillation-decrement.

Preliminary heating diminishes the viscosity of gelatin solutions and the magnitude of the effect is a function of the time of exposure to the higher temperature (110) (33). Garrett attributes this phenomenon to a partial destruction by heating of a structure within the fluid. Ostwald (79) has found that preliminary heating of gelatin increases the rapidity with which it swells in water. He also interprets this by supposing that the structure of

* Cf. also C. O. Weber (128).

the gelatin is partially destroyed by heating. Garrett has also found that in a solution of gelatin which had been boiled the logarithmic decrement of an oscillating disc does not increase with time.

According to Lichtwitz and Renner (61) the viscosity of serum albumin solutions falls with rising temperature from 15 to 60° C. in the same proportion as the viscosity of water. Chick and Lubrzynski (20) have also shown that the influence of temperature upon the viscosity of egg-albumin solutions containing up to 20 per cent of protein is slight and a linear function of temperature being similar to the influence of temperature upon the viscosity of a solution of a crystalloid. In solutions containing a higher percentage of protein, however, the relationship between viscosity and temperature is curvilinear, the decrease being most rapid at low temperatures. From these results it would appear reasonable to infer that a change of temperature at temperatures lying below those necessary to induce coagulation affects the viscosity of a protein solution chiefly through altering the viscosity of the solvent, a deduction which is in harmony with the view expressed above that proteins in solution are not in any large proportion present therein in the form of independently mobile particles.

It has been shown by Christiansen (22) that acid protein solutions attain a maximum of viscosity when a slight excess of free acid is present, presumably coinciding approximately with the attainment of maximum combining capacity for the acid. The acidity which produces maximum viscosity is also the optimum acidity for the digestive action of pepsin. The viscosity of the solution decreases during digestion, a fact which had previously been demonstrated by Spriggs (116).

2. The Cohesiveness of Protein Solutions. — Closely allied to the property of viscosity is the property which may be somewhat vaguely designated "cohesiveness." The influence of a variety of aqueous solutions of acids, bases and salts upon the cohesiveness of gluten has been investigated by Wood and Hardy (132). These observers prepared gluten by washing flour in a stream of water to remove the starch. The protein residue is a coherent stringy mass, insoluble in water and consisting essentially of a mixture of gliadin and glutenin (Cf. Chap. II). The action of solutions upon the cohesiveness of the gluten was estimated quantitatively by suspending a small mass of gluten on a bent glass rod in a beaker

containing 120 cc. of the solution. It was found that dilute acids very quickly led to a very marked diminution in cohesiveness, allowing the drop to fall off the rod and disperse, forming a cloudy solution. More concentrated acid, however, maintains the cohesion; in other words, with increasing concentration of acid the cohesiveness of the gluten is first lowered, until it reaches a minimum, and then raised until it regains its value in water. There is no simple relationship between the concentration of an acid in which cohesiveness is retained just sufficiently to hold the drop to the rod and the concentration of hydrogen ions in these solutions as indicated by their conductivities, although higher concentrations of weak than of strong acids are as a rule required. Salts lessen the power which acids and alkalies possess of weakening the cohesion of gluten, and the concentration of salt required to nullify the dispersive action of the various acids varies with the concentration of acid in a very characteristic manner which is illustrated by numerous curves in Wood and Hardy's paper. These authors interpret their results on the supposition that diminution in the cohesiveness of the gluten is brought about by the appearance of electric double layers upon the surfaces of the gluten particles.

3. The Elasticity of Protein Solutions. — The elasticity of protein solutions and jellies has been investigated by Rankine (89), Rohloff and Shinjo (98), Reiger (94), Leick (58) and Haycraft (42). They find that even dilute solutions of gelatin resist deformation to a slight extent, a fact which would appear to point to the existence within these solutions of some species of molecular (or ionic) structure such as that indicated in section **1.** This "form-elasticity" of gelatin solutions is diminished by heating.

The form-elasticity of a freshly prepared gelatin jelly increases with time until it attains a maximum. According to Haycraft, gelatin jellies obey Hooke's law, that is to say, for moderate (linear) strains the deformation which is produced is directly proportional to the applied force, and the recovery, when the strain is removed, is rapid and complete.

According to Henderson and Brink (43) the compressibility of gelatin solutions is somewhat less than the compressibility of water and lower the greater the concentration of gelatin in the solution. With varying compression the compressibility varies in much the same way as the compressibilities of other solutions.

4. The Diffusion of Proteins in Solution. — It was pointed out by Graham in his classic memoirs on diffusion, that the proteins, like other colloids, are very sparingly diffusible. Nevertheless they diffuse through water, albeit with the extreme slowness indicated by their relatively enormous molecular weight. From the law of Fick (10) (30) we have:

$$ds = Dq\frac{dc}{dx}dz,$$

where ds is the quantity of diffusing substance which passes in the time dz through a diffusion cylinder having the cross-section q, c is the concentration of the substance in the whole cross-section at the point x, $c + dc$ is the same quantity at the point $x + dx$ and D is a constant peculiar to the substance, expressive of its diffusibility, and known as the "diffusion coefficient."

The following are the diffusion coefficients of certain proteins, estimated by the observers named and cited after Wo. Ostwald (81) (45) (46). For the purpose of comparison the value of D for sodium chloride is placed at the head of the list.

Substance	Observer	D	Temperature, degrees
Sodium chloride	Voigtlander	1.040	20
Clupein sulphate	Herzog	0.074	18
Egg-albumin	Herzog	0.059	18
Ovomucoid	Herzog	0.044	18
Albumin	Graham-Stefan	0.063	13

Now it has been shown by Nernst (73) and Planck (82) that the driving force which produces diffusion is osmotic pressure. Hence we can conclude that the proteins, in solution, exert a definite, although small osmotic pressure.

From the diffusion coefficient of egg-albumin, Herzog and Kasarnovsky (46) have calculated that it exerts an osmotic pressure corresponding to a molecular weight of about 20,000. That proteins in solution exert a definite osmotic pressure is also shown by cryoscopic, ebullition, and direct measurements, of which a brief description will be found below.

It has been found by Dabrovsky (23) that the diffusion-coefficient of crystallized egg-albumin is decidedly greater when the diffusion takes place into a solution of ammonium sulphate than

when it takes place into distilled water. From the value of the coefficient of diffusion and the viscosity of the liquid into which diffusion occurs, it is possible, employing the formula of Einstein, to compute the relative volumes occupied by the particles of egg-albumin when diffusing into different solvents. Applying this method of computation, Dabrovsky finds that the volume occupied by the egg-albumin molecules when dissolved in distilled water is no less than six times as great as that which they occupy when dissolved in a 3.6 per cent solution of ammonium sulphate. The extraordinary diminution of molecular volume which ammonium sulphate brings about must undoubtedly be connected with the high coagulating power of this salt and is probably to be attributed to dehydration of the protein, i.e., to abstraction of a galaxy of associated water-molecules (Cf. Chap. VI, section **6,** also this Chapter, section **6**).

5. The Freezing- and Boiling-points of Protein Solutions. — As is well known, the depression of the freezing-point of a solvent which is produced by a dissolved substance is proportional to the osmotic pressure which the dissolved substance exerts in the solution. Similarly, the elevation of the boiling-point of the solvent is proportional to the osmotic pressure of the dissolved substance. Corresponding to the extremely high molecular weights of proteins the osmotic pressures of their solutions (containing practical percentages of protein) are low, and the depression of the freezing-point and elevation of the boiling-point of water which are brought about by the introduction of protein are small.

Consequently Sabanejev (112) found that the depression of the freezing-point which is brought about by egg-albumin could be almost entirely accounted for by the (estimated) pressure of the inorganic constituents of the protein, and Sebanejev and Alexandrov (114) estimated from their determinations that the molecular weight of albumin must be at least 14,000 in order to account for the extremely slight lowering of the freezing-point which could be attributed to the protein alone. The proteoses, Sebanejev estimated, also from cryoscopic determinations, to possess a molecular weight of from 2000 to 3000, while the molecular weight of the peptones was estimated to be 400 or less (113).

Tamman (120) measured the difference between the lowering of the freezing-point of the serum of the horse before and after coagulating the proteins by heat, and removing them, and found that

the difference amounted to only 0.006 degrees, the experimental error of the cryoscopic method being about 0.005 degrees. In connection with experiments such as these it should be recollected, however, that heating induces many modifications in protein-containing solutions which might conceivably affect the osmotic pressure (freezing-point) in a variety of diverse ways (92). Lüdeking found that even 40 per cent of gelatin in solution did not perceptibly alter the boiling-point (67) and Krafft and Wiglow (55) confirmed his results. Bugarszky and Liebermann (17) estimated the depression of the freezing-point due to dissolved egg-albumin, albumose and pepsin, and their ash, separately, and deducted the latter from the former. They estimated in this manner the molecular (or, in reality mean molecular and ionic) weight of egg-albumin to be 6400, that of albumose to be 2400 and that of pepsin to be 760. They also found that if egg-albumin, albumose or pepsin be added to solutions of acids or alkalies, the cryoscopic depression of the resultant solution is less than the sum of the depressions due to the acid or alkali and the proteins dissolved separately; while if the egg-albumin, albumose or pepsin were dissolved in salt solutions the cryoscopic depression of the mixture was found to be (within the limits of experimental error) identical with the sum of the cryoscopic depressions due to the protein and the salt dissolved separately. The significance of these latter results has already been commented upon in Chap. IV.

Bugarszky and Tangl (18) carried out an extended series of investigations aiming at the determination of the cryoscopic depression due to the non-electrolytes of the blood, among which they included the proteins. They determined the chlorine content of the blood and, from that, deduced the equivalent molecular concentration of the sodium chloride in the blood and its conductivity; they then measured the conductivity of the blood and subtracting from it that due to sodium chloride, considered the remainder as due to sodium carbonate and estimated therefrom the molecular concentration of the sodium carbonate. They then subtracted the cryoscopic depression due to the sodium chloride and sodium carbonate contents thus estimated, from the observed cryoscopic depression of the blood; the difference they ascribed to the non-electrolytes and proteins. In this way they estimated the concentration of non-electrolytes in horse's blood to be about 0.056 mol. per litre. The accuracy of this determina-

tion is, however, very questionable, since it depends upon the assumption that the proteins in blood are non-electrolytes. While it is probable as Hardy's results indicate (Cf. Chap. VII, section 6), that the proteins in normal blood are non-ionic, the possibility must not be lost sight of that they may participate, as isolated water soluble proteins frequently do, in the conduction of a current through their solutions (Cf. Chaps. VIII and X).

The majority of the above estimates were not carried out upon pure proteins. Robertson and Burnett (106), however, have investigated the cryoscopic depression due to dissolved caseinates of the alkalies and alkaline earths, employing pure casein. The following were the results obtained.

TABLE I

Caseinates containing 50×10^{-5} equivalents of base per gram
(Experimental error of determination $\pm$ 0.0025 degrees)

Base	Concentration of base neutralized by casein	Δ = depression of the freezing-point of water	Indicating a molecular plus ionic concentration of
NH_4OH	$\frac{m}{50}$	0.045	$\frac{m}{41}$
NH_4OH	$\frac{m}{50}$	0.035	$\frac{m}{53}$
NH_4OH	$\frac{m}{33.3}$	0.055	$\frac{m}{34}$
NH_4OH	$\frac{m}{33.3}$	0.055	$\frac{m}{34}$
NH_4OH	$\frac{m}{20}$	0.07	$\frac{m}{26}$
NH_4OH	$\frac{m}{15}$	0.095	$\frac{m}{19}$
KOH	$\frac{m}{50}$	0.0325	$\frac{m}{57}$
KOH	$\frac{m}{50}$	0.0375	$\frac{m}{49}$
KOH	$\frac{m}{33.3}$	0.0425	$\frac{m}{44}$
KOH	$\frac{m}{33.3}$	0.0475	$\frac{m}{39}$
KOH	$\frac{m}{20}$	0.05	$\frac{m}{37}$
KOH	$\frac{m}{20}$	0.075	$\frac{m}{25}$

TABLE I. — (*Continued*)

Base	Concentration of base neutralized by casein	Δ = depression of the freezing-point of water	Indicating a molecular plus ionic concentration of
KOH	$\frac{m}{15}$	0.10	$\frac{m}{18.5}$
LiOH	$\frac{m}{59.5}$	0.03	$\frac{m}{62}$
LiOH	$\frac{m}{40}$	0.045	$\frac{m}{41}$
LiOH	$\frac{m}{24}$	0.07	$\frac{m}{26}$
LiOH	$\frac{m}{18}$	0.08	$\frac{m}{20}$
$Ca(OH)_2$	$\frac{m}{91}$	0.015	$\frac{m}{120}$
$Ca(OH)_2$	$\frac{m}{91}$	0.015	$\frac{m}{120}$
$Ca(OH)_2$	$\frac{m}{91}$	0.0175	$\frac{m}{101}$
$Ca(OH)_2$	$\frac{m}{61}$	0.02	$\frac{m}{92.5}$
$Ca(OH)_2$	$\frac{m}{61}$	0.02	$\frac{m}{92.5}$
$Ca(OH)_2$	$\frac{m}{61}$	0.02	$\frac{m}{92.5}$
$Ca(OH)_2$	$\frac{m}{45.5}$	0.025	$\frac{m}{74}$
$Ca(OH)_2$	$\frac{m}{45.5}$	0.025	$\frac{m}{74}$
$Ca(OH)_2$	$\frac{m}{45.5}$	0.025	$\frac{m}{74}$

TABLE II

Caseinates containing 80×10^{-5} equivalents of base per gram
(Experimental error of determination ±0.0025 degrees)

Base	Concentration of base neutralized by casein	Δ = depression of the freezing-point of water	Indicating a molecular plus ionic concentration of
NH_4OH	$\frac{m}{36}$	0.04	$\frac{m}{46}$
NH_4OH	$\frac{m}{34}$	0.04	$\frac{m}{46}$
NH_4OH	$\frac{m}{27}$	0.0475	$\frac{m}{39}$
NH_4OH	$\frac{m}{25}$	0.05	$\frac{m}{37}$
NH_4OH	$\frac{m}{20}$	0.07	$\frac{m}{26.5}$
NH_4OH	$\frac{m}{18}$	0.055	$\frac{m}{34}$
NH_4OH	$\frac{m}{17}$	0.075	$\frac{m}{25}$
NH_4OH	$\frac{m}{15}$	0.10	$\frac{m}{18.5}$
KOH	$\frac{m}{50}$	0.035	$\frac{m}{53}$
KOH	$\frac{m}{33}$	0.04	$\frac{m}{46}$
KOH	$\frac{m}{33}$	0.055	$\frac{m}{34}$
KOH	$\frac{m}{25}$	0.06	$\frac{m}{31}$
KOH	$\frac{m}{20}$	0.07	$\frac{m}{26.5}$
KOH	$\frac{m}{17}$	0.0875	$\frac{m}{20}$
KOH	$\frac{m}{15}$	0.095	$\frac{m}{19}$
LiOH	$\frac{m}{40}$	0.04	$\frac{m}{46}$
LiOH	$\frac{m}{30}$	0.05	$\frac{m}{37}$
LiOH	$\frac{m}{20}$	0.07	$\frac{m}{26}$
$Ca(OH)_2$	$\frac{m}{91}$	0.015	$\frac{m}{120}$
$Ca(OH)_2$	$\frac{m}{68}$	0.02	$\frac{m}{92.5}$
$Ca(OH)_2$	$\frac{m}{45.5}$	0.0225	$\frac{m}{80}$

The significance of these results and of results which I have obtained with ovomucoid is commented upon in Chap. X. It may further be remarked here that the observed depressions could not have been due to impurities associated with the casein for the following reasons:

(1) Keeping the concentration of base constant it is evident that increasing the quantity of casein dissolved in it in the proportion of 8 to 5 does not alter the observed depression in any appreciable degree, whereas, if this were due to impurities associated with the casein, it should result in a proportionate increase in the observed depression.

(2) Keeping the concentration of casein constant and increasing the concentration of base bound by it in the proportion of 8 to 5 results in a proportionate increase in the observed depression.

The cryoscopic depression is, therefore, obviously conditioned primarily by the combined alkali. This might be interpreted to indicate that the depression is in reality due to the base and not to the protein. This, however, is not the case, since, as we have seen (Chaps. V and IX), these solutions were respectively neutral to litmus and to phenolphthalein and therefore contained no, or only a trace of, free base. Nor were the depressions due to inorganic ions, since, as we have seen (Chap. VIII), such solutions contain no inorganic ions. As I have explained in Chap. X, the observed depressions must be attributed primarily to protein ions, each equivalent of combined base yielding the same number of protein ions, derived through the splitting of successive $-N.HOC-$ bonds.

One fact should be especially commented upon here, and that is that the observed depressions in the two sets of solutions examined stood in direct proportion to the concentration of combined base. If this were so for other concentrations, then at zero concentration of combined base, if casein were soluble under such conditions, the freezing-point depression due to dissolved casein would be zero. In other words the possibility is indicated that base- and acid-free protein may exert an immeasurably small osmotic pressure. I have elsewhere attributed this to polymerization of the protein as the uncombined protein is set free (104) (106).

6. The Osmotic Pressure of Proteins in Solution. — The direct determination of the osmotic pressure of protein solutions is

a task fraught with immense difficulties, on account of the difficulty of preparing ideally pure proteins. The original investigations of Graham (35) appeared to indicate that colloids in general exert a high osmotic pressure. Subsequent investigators, however, attributed these results to an admixture of crystalloids. Starling endeavored to measure directly the osmotic pressure of the proteins in blood serum by using for his osmometer a membrane permeable to salts but impermeable to proteins (117) and this method has since then been employed in all accurate work upon the subject, since, as Reid has pointed out (92) it is the only method of procedure which is applicable to the problem. We have no assurance that any given protein preparation is totally free from (not necessarily conductive) impurities which may influence the direct measurement of osmotic pressure; it is, therefore, essential to employ a membrane which is permeable to such impurities and thus, if time be allowed for the system to come to equilibrium, differentiates between protein and non-protein constituents of the solution investigated. For this purpose Reid employs a membrane of vegetable parchment, which, as he has shown, is permeable even to nucleic acid although it is impermeable to the proteins employed by him in his investigations. By extremely prolonged purification Reid has succeeded in obtaining preparations of egg-albumin which exhibit no measurable osmotic pressure when examined by this method. In subsequent investigations (93), however, he obtained osmotic pressures, due to dissolved hæmoglobin, of perfectly constant value and such as to indicate a molecular weight of about 48,000. Barcroft and Hill (4) have, however, demonstrated by thermodynamical methods that in solutions containing hæmoglobin prepared by less prolonged dialysis the molecular weight of this substance is about 16,669. Roaf (96) employing the differential osmotic method just described, finds that the molecular weight of hæmoglobin, dissolved in distilled water, is about 32,000, while in sodium carbonate solutions it is 16,000. These results appear to confirm the view (Cf. also Barcroft and Hill loc. cit.) that non-ionic protein is polymerized and so exerts a considerably smaller osmotic pressure than ionic protein.

Benj. Moore and Roaf and collaborators (70) (71) (72) (95) (97) and R. S. Lillie (63) have made the extremely interesting discovery that the osmotic pressure which is exerted by proteins (deter-

mined differentially, as described) varies very pronouncedly with the nature of the inorganic acids, bases or salts which their solutions contain.*

The following are among Lillie's results, obtained when dilute acids and alkalies were employed as solvents:

TABLE III

1.5 Per Cent Gelatin in Dilute HCl Solutions

Solvent	Osmotic pressure of protein in mm. Hg.
Water	8.2
m/3100 HCl	6.8
m/2050 HCl	12.3
m/1550 HCl	17.9
m/1025 HCl	26.5
m/770 HCl	32.4
m/620 HCl	34.9
m/412 HCl	39.3

TABLE IV

1.5 Per Cent Gelatin in Dilute KOH Solutions

Solvent	Osmotic pressure of protein in mm. Hg.
Water	7.9
m/3100 KOH	14.1
m/620 KOH	23.7
m/412 KOH	25.1
m/310 KOH	29.0

In Lillie's words, "In the presence of either acid or alkali the osmotic pressure of gelatin thus shows a marked increase, which, within the above range of concentrations, exhibits a certain proportionality to the quantity of acid or alkali added. For equivalent concentrations, acid produces a somewhat greater increase than alkali. The change in osmotic properties is to be attributed to a finer subdivision of the colloid particles and a consequent increase in the surface of intersection between colloid particles and

* In this connection it is of interest to note that von Wittich (131), von Regeczy (91), Oker-Blom (76) and others have shown that proteins diffuse more rapidly into sodium chloride solutions than they do into distilled water. Cf. also Dabrovsky (23).

medium." These results are obviously in complete agreement with results obtained by Robertson and Burnett, employing the cryoscopic method, which are commented on above. The *decrease* in pressure observed in very dilute acid may be attributable to the neutralization by the acid of base bound by the protein preparation employed. Lillie draws an analogy between his results and those obtained by Ostwald (78) (79) in investigating the swelling of gelatin plates in dilute acid and bases. Egg-albumin, however, exhibits the opposite behavior, namely, the osmotic pressure which it exerts is slightly diminished by acids and bases.

The osmotic pressure of gelatin and egg-albumin is unaffected by the addition of non-electrolytes such as cane-sugar, dextrose, glycerol and urea, but is considerably affected by the addition of inorganic salts being (and this is true both for gelatin and egg-albumin) depressed thereby. According to Lillie the depression of the osmotic pressure exerted by this protein is a function of the nature of both the anion and cation of the added salt. It increases in the order alkali metals < alkaline earths < heavy metals (for cations); and CNS < I < Br < NO_3 < Cl < F < plurivalent anions, SO_4, tartrate, citrate, phosphate (for anions). This observation is of extraordinary significance when we recollect that this is the order in which, according to Hofmeister and Pauli, the various ions bring about the dehydration and coagulation of the salts which proteins form with bases (Cf. Chap. VI).*

Very remarkable phenomena are displayed by the solutions of soluble chitin which have been prepared by Alsberg and Hedblom (1) from the chitin of *Limulus polyphemus* by prolonged treatment with weak hydrochloric acid. The chitin when subjected to this treatment forms at first a gelatinous mass and later a colloidal solution. The analytical figures obtained for this "soluble chitin" and the gelatinized chitin obtained by adding KOH and then reneutralizing with HCl are best explained, according to the authors, by assuming that the chitin, on passing into colloidal solution, unites with water. Soluble chitin depresses the freezing point but slightly, so that its molecular weight is probably very high. It passes through collodion and parchment paper, but has the extraordinary property of carrying the water in which it is

* Moore and Roaf (72) have sought to explain the coagulation of proteins by salts by the formation of large aggregates, basing their argument upon data similar to these obtained by Lillie.

dissolved through the membrane so that the space within the latter may become nearly empty. It is probable that these phenomena depend rather upon the extreme affinity of this colloid for water than upon ordinary osmotic forces.

C. J. Martin (68) and W. M. Bayliss (5) find that the osmotic pressures of egg-albumin and of hæmoglobin solutions vary directly with the absolute temperature, thus obeying the law of Gay-Lussac.

7. The Nature of Protein Solutions. — The view has been held by many investigators that the proteins, and colloids generally, do not enter into true solution, forming molecularly dispersed systems which obey the law of Avogadro, but are to be regarded, on the contrary, as forming stable *suspensions* when they appear to pass into solution in water; a view which reaches its most extreme expression in the statement of Duclaux (26) that "Les Colloids doivent etre consideres comme etant, dans l'eau, d'une insolubilite absolue." Others, arguing* from the *a priori* assumption that "solutions" of colloids are necessarily, in reality, suspensions, have objected to the application of theoretical principles to them which involve the law of Avogadro (such as the mass-law, etc.). It is obvious that this is a reversion of the customary procedure of science; the applicability of a law is in the first place a question in itself, independent of any other generalizations, and, in the second place, the applicability of a law involving Avogadro's law may in itself be regarded as presumptive evidence that the law of Avogadro applies as well.

From what has been said above, it is evident that under certain conditions, particularly in the form of their salts, the proteins diffuse in water and exert a definite osmotic pressure. Hence Avogadro's rule must hold good, although the *time* required for the protein molecules to distribute themselves uniformly throughout any given volume of fluid may conceivably, in certain instances, be very great.

Apart altogether from direct observation, however, the possession of a definite osmotic pressure by proteins and certain other colloids in solutions is directly deducible from the fact that chemical reactions which involve them attain definite equilibria (101). The work which is performed in the transformation of a molecule of substance is the sum of two factors, the one the *chemical work*

* For example, W. Nernst (75).

which is involved in the transformation, the other the work performed in bringing the molecule to the pressure of the system (56); the latter factor is, of course, dependent upon the concentration of the substances within the system, while the former, equally obviously, is not. If the reacting components in a system exerted no osmotic pressure whatever, the expression for the work done in the transformation of a given mass of the components would, therefore, be a constant one (depending only upon temperature), and, consequently, at any stage of the reaction the work done in transforming unit mass would be the same, whatever the concentration of the reacting components. Under these conditions the reaction would always proceed to an end in one direction or the opposite; since the work performed at every stage of the reaction would be unaffected by the concentration of the reacting components. Now it has been shown that the reactions between toxins and anti-toxins, lysins, and anti-lysins, etc., attain definite equilibria (3) and hence these bodies must, in solution, exert definite osmotic pressure. The probable protein nature of these bodies has been commented upon in a previous chapter (Chap. VII, section **7**). Moreover, it has been shown that a definite equilibrium is attained between proteins and the antibodies which are produced in the serum when these proteins are injected into the circulation (3), and definite equilibria are attained between different proteins in solution (121) and between proteins and inorganic acids and bases (Cf. Chap. IX) and in protein digests (122) (123) (99) (102) (107). Many of these equilibria, it has been shown, can be approached from either direction, so that they are not "false" equilibria (27) attributable to the internal molecular friction or hysteresis of the systems. We may, therefore, safely conclude that in many instances, and especially in the form of their salts, proteins and bodies allied to proteins exert, when in solution, definite osmotic pressures; and are distributed in molecular dispersion throughout their solutions in accordance with the law of Avogadro.

It is, of course, not for a moment contended that this is the case in every apparent "solution" of protein. Proteins, like other bodies, and more easily than the majority of crystalloids, can also be obtained in a suspended condition. Under such circumstances they may form apparently stable suspensions, simulating true solutions in certain respects. An illustration of such suspension

is afforded by potassium caseinate in 75 per cent alcohol (Cf. Chap. XI).

Certain authors, following Graham, regard all colloidal solutions, including protein solutions, as being "supersaturated." The above considerations dispose of this view also. Linebarger (64) has also shown, by direct experimentation on the rate of coagulation, that solutions of proteins cannot be correctly regarded as supersaturated. Very numerous observations, detailed in previous chapters, will occur to the reader which also conflict with this view, for example, the constancy of the proportion between base and casein at "saturation" of the former with the latter.

8. The Opalescence of Protein Solutions; the Tyndall Effect. — The majority of protein solutions are decidedly opalescent, i.e., they contain particles of sufficient size to scatter and reflect a proportion of transmitted light. Even when the opalescence is very small, when the solution is viewed at right angles to the direction of a pencil of light traversing it, a certain degree of scattering (the Tyndall effect) is observed. Upon this fact many investigators have based the view that proteins in solution form heterogeneous and not molecularly dispersed systems.

It has, however, been pointed out by Konovalov (53) that the dust particles, which render it so difficult to obtain even the purest water in an "optically void" condition, may act as nuclei, under conditions in which the expenditure of energy necessary for an alteration in concentration is very small (i.e., when the osmotic pressure of the dissolved substance is very small), attracting layers of the dissolved substance and so producing opalescence. In this way it is possible that the observed opalescence in such solutions is due only to a minute proportion of the dissolved substance condensed by capillary action around accidental insoluble contaminations. Arrhenius (3) has advanced a similar view. "It may, however, be urged that the presence of some submicroscopic particles does not prove at all that the whole of the quantity of protein present, or even a considerable part of it, is in this state of pseudo-solution."

It is, however, not at all inconceivable that the opalescence of many colloidal solutions may be due to the grossness of the colloid molecules themselves,* but it is unlikely that this is the case in solutions of proteins. It has been shown by Lobry de Bruyn (16)

* C. O. Weber (128), p. 75.

that the smallest particles which are capable of scattering light are from fifty to a hundred times smaller than the mean wave length of light at 0.5 μ, the diameter of such particles could be not less than from 5 to 10 $\mu\mu$. Now we have seen in Chaps. VIII and IX, that the molecular weight of casein in solutions containing 11.4×10^{-5} equivalents of base per gram of protein, is probably about 17,600 (estimated from electrochemical data). From the analysis of Hammarsten (39) it appears that if casein be possessed of this molecular weight, then its empirical formula must be, approximately:

$$C_{777}H_{1241}N_{197}O_{250}S_4P_4.$$

Now it has been shown by Kopp (54) that the relative molecular volumes of organic substances can, with certain marked exceptions, be calculated as an additive function of their constituent atoms.* The mode in which the valencies of the atoms are distributed affects their contribution to the total volume, however, "carbonyl" oxygen contributing an amount differing from that contributed by hydroxyl oxygen. From the analysis of Abderhalden and others it would appear that if casein possesses the above molecular weight it must contain about four molecules of tyrosin, and one each of the other hydroxy-acids which it is known to contain, namely, serin and caseinic acid, or in all, neglecting the necessarily few terminal $-COOH$ groups (Cf. Chap. I) it would appear to contain only about 8 atoms of hydroxyl oxygen out of the 250 atoms of oxygen which are present. But it will be recollected (Chap. I) that there is strong reason to suspect that in many of the $-COHN-$ groups within the protein molecule the oxygen is in the hydroxy condition. We must, therefore, make two estimates of the molecular volume, one on the supposition that the molecule contains only carbonyl oxygen and the other on the supposition that it contains only hydroxyl oxygen. The true volume will, it is probable, lie between the two. Now the difference in molecular volume due to carbon is 11, that due to hydrogen is 5.5, to nitrogen (as in ammonia) 2.3, to carbonyl oxygen 12.2, to sulphur 22.6, to phosphorus 25.5, yielding, for the first estimate of the relative molecular volume of casein:

$$777 \times 11 + 1241 \times 5.5 + 197 \times 2.3 + 250 \times 12.2 + 4 \times 22.6 + 5 \times 25.5 = 19{,}060;$$

* The figures employed in the calculation which follows are cited after Ostwald (77).

a second estimate, regarding the oxygen as hydroxyl oxygen (difference due to one atom of hydroxyl oxygen = 7.8), yields the molecular volume 17,960. The volume, estimated in this way, therefore, lies between 18,000 and 19,000. The absolute volume of the molecule will therefore lie between $\frac{18{,}000}{5.5}$ and $\frac{19{,}000}{5.5}$ or about 3400 times that of a hydrogen molecule, and about 523 times that of a molecule of carbon dioxide. The diameter of the molecule will, therefore, be approximately $\sqrt[3]{523}$ = about 8 times that of a molecule of carbon dioxide. Now the diameter of a molecule of CO_2 is, according to Nernst (74), 0.3 $\mu\mu$, hence that of a molecule of casein must be about 2.4 $\mu\mu$, or about one-half the diameter of the smallest particle which will scatter transmitted light. Admitting the great uncertainty which, in our present state of incomplete knowledge, attaches to such estimations as these, we must yet admit that it is improbable that the opalescence of solutions of proteins such as casein is directly attributable to the protein molecules themselves; and it appears the more improbable when we recollect that in pronouncedly opalescent solutions casein salts, and many other protein salts are, as we have seen (Chaps. VIII–X), quite extensively ionized, that is, split up into particles which occupy, themselves, a fraction of the volume computed above. When we recollect that the non-filterable character of many proteins in solution (Cf. Chap. VII, section **6**) and the viscosity of protein solutions (Cf. the earlier part of this chapter) are alike attributable to *ionic* protein, the suggestion offers itself that the opalescence of protein *solutions* (as distinguished from that of *suspensions* of partially coagulated protein) may possibly be attributable to the peculiar characteristics of ionic protein. It is possible that the non-filterable character of ionic protein and its power of scattering transmitted light are alike due to a bulky water-complex, which is associated with each of the protein ions (Cf. Chap. VI, section **6** and Chap. VII, section **6**). In support of this view may be mentioned the remarkable fact that the addition of alcohol or of acetone, in quantities insufficient to initiate coagulation, to solutions of the caseinates leads to a very decided decrease in their opalescence. On the other hand, it is possible that the net-like structure which we have seen reason to believe that protein ions form in solution (section **1**, this chapter) is accountable, not only for the viscosity of solutions of ionic protein, but

also for the non-filterability of ionic protein and the opalescence of its solutions. Between these alternatives, if indeed they are alternatives, our data do not, as yet, suffice for us to decide.

The opalescence of protein suspensions has been made by Kober (52) the basis of a method of determining proteins quantitatively (Cf. Chap. III).

9. The Surface Tension of Protein Solutions. — The influence of dissolved protein upon the tension of air-water surfaces has been investigated by Quincke (85) (86), Zlobicki (134), Frei (31), Buglia (19), Iscovesco (59), Shorter (115), Bottazzi (13) (15), and Berczeller (9). Gelatin, egg-globulin and hæmoglobin diminish the air-water tension.* The diminution is greater the higher the temperature (between 0 and 24.4 degrees, Zlobicki). The surface-tension of gelatin solutions and blood serum is increased by the addition of small quantities of alkali, and diminished by the addition of small quantities of acid (Buglia, Frei).

According to Bottazzi (13) (15) the surface tension of serum albumin solutions is at a maximum when their ionization is at a minimum and at a minimum when their ionization is at a maximum. The maximum depression of the surface tension of water by serum albumin occurs at a reaction just on the acid side of neutrality. Undissolved protein does not affect the surface tension of water when shaken up with it (9).

According to Berczeller (9), the surface tension of a protein solution which is so electrolyte-free as not to coagulate on heating nevertheless diminishes on heating. This reduction of surface tension is reversible, for on standing for some time at ordinary temperature the solution regains its original surface tension.

The surface tension of protein solutions diminishes during digestion (9).

10. The Formation of Surface-films by Dissolved Proteins. — Proteins, when dissolved in water, would appear, as a rule, to diminish the surface tension at surfaces bounded by substances other than air, since they tend to become concentrated at such surfaces and form films of insoluble protein there, and, as Willard Gibbs pointed out in his classic memoir on equilibrium in heterogeneous systems, such a tendency is indicative of a diminution in the free energy of the surface at which such concentration occurs.

* According to Iscovesco, however, *pure* egg-albumin *raises* the air-water tension.

This phenomenon has received extended consideration at the hands of Hermann (44), Berthold (11), Ramsden (87) (88), and Shorter (115), who have shown that protein solutions can be wholly or partially coagulated by mere mechanical agitation, or by the purely surface-action of fine powders, such as burnt clay or charcoal. Ramsden also explains the formation of a film at the surface of heated milk and other protein solutions in the same way.*

The formation of these protein films by surface-action can be very conveniently illustrated by the following experiments (100) (103). If chloroform be shaken up with casein, gelatin or protamin solutions, it settles in fine particles or droplets, which, if numerous, form a milky layer at the bottom of the vessel; by transmitted light, however, they appear perfectly clear. These droplets are extraordinarily stable, and do not coalesce, however long they stand in contact; they may be repeatedly washed in water until all traces of protein have been removed from the supernatant fluid, and they still remain perfectly stable and distinct from one another; they may be shaken up in chloroform itself or treated with $N/10$ potassium hydroxide without impairing their form or stability. If, however, they be heated to nearly the

* The non-filterability of many proteins, that is, their inability to pass through the pores of a clay filter has, by some authors, also been attributed to surface-action. This factor alone, however, is insufficient to explain the total non-filterability of these proteins through certain filters. True, when solutions of substances which reduce the surface-tension at the surfaces of insoluble powders are filtered through such powders, the first portion of the filtrate which filters through contains less of the dissolved substance than the original solution (Cf. J. J. Thomson (124)); for example, when potassium permanganate solution is filtered through fine quartz sand the first portions of the filtrate may be colorless. But subsequent portions of the filtrate contain a greater proportion of the solute and, finally, the unaltered solution filters through, owing to the fact that the surfaces of the pores of the filter are now fully coated by a film of more than molecular thickness. If this were the only factor concerned in holding back dissolved proteins from passing through clay filters, the protein would sooner or later pass through the filter, which, in many cases it does not (Cf. F. W. Zahn (133), J. Lehmann (57)). True, it is possible that the first portions of the protein which are deposited upon the walls of the pores, partially clog up the pores and narrow them, thus assisting the filter to hold the protein back; but even if this be the case, the ultimate factor which determines the impermeability of such filters for proteins must either be the grossness of the protein particles themselves, or of their associated water-complexes, or else the existence within the protein solution of a structure connecting together the protein particles.

boiling point of chloroform, under a layer of water, the droplets burst and coalesce, forming a homogeneous layer of chloroform. If treated with alcohol they immediately dissolve, leaving a fine membraneous precipitate of protein floating in water. Thus, if we shake up chloroform with about twice its volume of a 1 per cent solution of protamin sulphate, and, after standing, separate the chloroform droplets by pouring off the supernatant liquid, and then, after repeatedly washing the droplets in water by decantation, add to the small amount of supernatant water about an equal volume of alcohol and gently agitate, the droplets which are thus stirred up into the alcohol-water layer can be seen to swell up rapidly and burst, and the fine membranes which surround them can be seen falling down through the alcohol-water. If we now add several volumes of alcohol and shake up the liquid, the chloroform droplets all disappear, and what we now have is a clear, homogeneous solution in which innumerable minute membranes can be clearly seen floating.*

That these surface films possess quite different properties from the protein in the body of the fluid is evident; this is shown by their great insolubility and by the fact that they are not readily acted upon by acids and alkalies. Now many investigators have pointed out that at the surface separating two phases of a system marked changes in chemical equilibrium frequently occur (74) (62) (48), owing to the alteration in chemical potential which occurs at such surfaces (124). It appears probable that at surfaces within protein solutions similar changes in equilibrium occur, leading to the formation of polymers or anhydrides of the protein.† These alterations in equilibrium are very slowly reversed upon removal of the protein from the surface which caused the change in equilibrium; this is shown by the insolubility of the coagula which are produced in this way. Evidently some phenomenon analogous to hysteresis prevents or greatly delays complete return

* The formation of these films also explains the power which proteins possess to render many emulsions stable, which are not stable in pure water (Cf., for example, Jamison and Hertz (50)). It also explains the tendency of protein solutions to form foams. For the analogous part which is played by soap films Cf. Quincke (84) and T. Brailsford Robertson (104).

† In this connection it should be noted that the increase in the concentration of proteins at such surfaces, due to the diminution in the surface energy which the protein causes, must be accompanied by a corresponding diminution of *the active mass of water* at these surfaces.

to the equilibrium which was disturbed. The occurrence of such phenomena of hysteresis in heterogeneous systems containing proteins, and their probable dependence upon the internal friction of the solid phase have been commented upon in connection with the heat-coagulation of proteins (Cf. previous chapter).

I have shown that the films which are formed by gelatin around chloroform droplets are permeable to alcohol and also, but less, permeable to chloroform. The membranes are permeable to various substances in the following order, those to which they are most permeable being placed first, alcohol, ether, ethyl acetate, scharlach R., chloroform, toluol, xylol, carbon bisulphide.

The interesting observation has been made by Shorter (115) that the surface-elasticity of freshly prepared protein solutions undergoes a progressive increase with time. These increases are not uniform but step-like, and represent, with irregular fluctuation, the deposition of successive layers of molecular thickness. Equilibrium is only attained with extreme slowness, a phenomenon which Shorter attributes to the fact that in solutions an increase in concentration of the protein at any point has a large effect upon surface-tension but only a small effect upon the osmotic pressure of the underlying layers of solution. In concentrated solutions of protein, after a very prolonged period, it was found in some cases that the progressive thickening of the surface-membrane was suceeded by thinning. It is not certain, however, where such long periods of time are concerned, that autohydrolysis of the protein may not have contributed toward modification of the results.

The possible biological significance of protein films of this character has been discussed at some length in my communications referred to above.

11. The Specific Gravities of Protein Solutions. — We have seen (Chap. XII) that the taking up of water by concentrated proteins (gelatin) is accompanied by a volume-contraction (67); hence in concentrated protein solutions the specific gravity cannot be calculated directly from the specific gravities of the solvent and solute respectively.

The specific gravities of *dilute* protein solutions have been investigated by Haebler (38), Lehnstein (66) and others. According to the latter author, the change in the specific gravity of a sodium hydroxide solution due to the introduction of protein is directly proportional to the percentage of protein introduced.

Chick and Martin (21) have compared the densities of proteins in the dissolved and in the dry conditions and find invariably an increase in density of the dissolved protein corresponding to the volume-contraction which accompanies the passage of proteins into solution. Thus dry casein suspended in benzol has a specific gravity of 1.318, while a 7.85 per cent solution of casein in dilute sodium hydroxide has a specific gravity of 1.0240, whence the density of the dissolved sodium caseinate = 1.42, while correcting for the effect due to sodium hydroxide, the dissolved casein itself has a density of 1.39.

The following table summarizes the results obtained with various proteins:

Protein	Density in solution	Density in the dry state
Casein	1.390	1.318
Crystallized egg-albumin	1.359	1.269
Crystallized serum-albumin	1.378	1.275
Serum globulin	1.374	1.293

The more dilute a solution of sodium caseinate, the greater its apparent density was found to be. This was not the case in solutions of serum globulin, or albumin.

12. The Magnetic Properties of the Proteins. — It was shown in 1845 by Faraday (29) and later by Plücker (83) that hæmoglobin contains iron; it is, nevertheless, like the other constituents of living tissues, decidedly *diamagnetic*. This observation has been confirmed in an extended series of investigations by Gamgee (32), who finds that not only oxyhæmoglobin but also CO-hæmoglobin and methæmoglobin are strongly diamagnetic bodies. The iron-containing radical, hæmatin, and its derivative hæmin are, on the contrary, as might be expected from their content of iron, strongly *magnetic* bodies.

Benedicenti (7) (8) finds that the presence of protein inhibits the lowering of the diamagnetic constant of water by the addition of dissolved ferric chloride or finely pulverized iron.

13. "The Gold-Number" of Proteins. — It was found by Zsigmondy (135) that colloidal gold is precipitated from its solution (or suspension) by the addition of sodium chloride, but that this precipitation can be prevented by the addition to the solution of many other colloids, among which may be reckoned the proteins.

This phenomenon was investigated more fully by Schulz and Zsigmondy (10), who define the number of milligrams of a colloid which is just insufficient to prevent 10 cc. of a colloidal gold solution from showing a change in color after the addition of 1 cc. of 10 per cent NaCl solutions as the "gold-number" of the colloid. It appears to be highly characteristic for certain proteins. The following are the values determined by Schulz and Zsigmondy for the various proteins contained in or derived from egg-white:

Globulin	0.02–0.05
Ovomucoid	0.04–0.08
Crystallized egg-albumin	2.00–8.00
Other (con-) albumin	0.03–0.05
Alkali-albuminate	0.006–0.04

The great difference between the crystalline albumin "gold-number" and that of the other proteins in egg-white made it possible to recognize the presence of very small quantities of colloidal contamination in the egg-albumin. Schulz and Zsigmondy were able to show by this method that egg-albumin must be recrystallized from three to six times to remove appreciable quantities of such contaminations.

The determination of the "gold-number" of cerebro-spinal fluid has been found to be of very great value in the differential diagnosis of the various forms of syphilitic sequelæ, the modifications observed being attributable to the marked increase in globulin-content which accompanies and characterizes certain of these conditions.

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CHAPTER XIV

OPTICAL PROPERTIES OF PROTEIN SOLUTIONS*

1. The Specific Rotatory Power of the Proteins. — It was observed by Hoppe-Seyler (16) (18) that the majority of animal proteins, in aqueous solutions, rotate the plane of polarized light to the left, and he measured the specific rotatory powers of several of the animal proteins. These investigations were continued by Fredericque (7) and Kühne (26) who proposed employing the specific rotatory power of a protein as a means of characterizing it and establishing its identity. The results which were obtained by these observers have been greatly extended and amplified by a large number of observers who have investigated this physical property of a variety of proteins. It appears that all of the naturally occurring proteins, excepting the nucleo-proteins and hæmoglobin, rotate the plane of polarized light to the left. The following are among the results which have been obtained by observers who have worked with proteins of animal origin:

TABLE I

Protein	Observer	Nature of solution	Degrees
Serum albumin	Hoppe-Seyler (20)	.....	−56.00
Serum albumin	Fredericque (7)	in water	−57.30
Serum albumin	Starke (62)	.....	−60.05
Serum albumin	Sebelien (59)	dialyzed solution in distilled water	−60.10 to −62.60
Serum albumin	Maximovitsch (31)	.....	−47.47
Egg albumin	F. G. Hopkins (15)	dilute ammonium sulphate	−30.70
Egg albumin	Osborne and Campbell (38)	water	−28.60 to −30.80
Lact-albumin	Sebelein (59)	dialyzed solution in distilled water	−36.40 to 36.98
α-crystallin from the crystalline lens	Mörner (33)	neutral solution in dilute ammonia	−46.90

* The opalescence of protein solutions has already been discussed (Chap XIII, 8).

TABLE I. — (*Continued*)

Protein	Observer	Nature of solution	Degrees
β-crystallin from the crystalline lens	Mörner (33)	dialyzed solution in dilute acetic acid	−43.30
Fibrinogen	Mittelbach (32)	dilute salt solution	−52.50
Casein	Hoppe-Seyler (20)	neutral solution	−80.00
Casein	Hoppe-Seyler (20)	faintly alkaline	−76.00
Casein	Hoppe-Seyler (20)	strongly alkaline	−91.00
Serum globulin	Fredericque (7)	dilute saline solution of mixed globulins	−47.80
Ovomucoid	Osborne and Campbell (38)	.....	−61.10 to −61.38
Alkali-albumose from egg-albumin	Maas (30)	.....	−49.40
Pepsin-peptone from fibrin	Borkel (3)	water	−36.36
Pepsin-peptone from fibrin	Borkel (3)	12.5 per cent ammonia	−39.42
Pepsin-glutin peptone	Scheermesser (55)	.....	−77.08 to −77.81
α-antipeptone, by tryptic digestion from fibrin	Muller (34)	water	−24.50
β-antipeptone by tryptic digestion from fibrin	Muller (34)	water	−32.40
α-antipeptone out of Witte's peptone	Siegfried (60)	water	−18.45 to −19.69
Antipeptone from gelatin	Kruger (25)	water	−100.80
Globin from hæmoglobin	Gamgee and Hill (9)	weakly acid watery alcoholic solution	−65.50
Globin from hæmoglobin	Gamgee and Hill (9)	solution in dilute acetic acid	−54.20 (red light)
Hæmoglobin	Gamgee and Hill (9)	water	+10.40 (red light)
Nucleo-histone from thymus	Gamgee and Jones (10)	dilute ammonia	+37.50 (red light)
α-nucleo-protein from pancreas	Gamgee and Jones (10)	dilute ammonia	+37.50 to +38.10 (red light)
Nuclein from pancreas	Gamgee and Jones (10)	water	+64.40 (red light)
Nucleo-protein of suprarenal glands	Gamgee and Jones (10)	water	+48.10 (red light)

The following results* are those which have been obtained by observers who have worked with vegetable proteins.

TABLE II

Protein	Observer	Nature of solution	Degrees
Edestin, hemp-seed	Chittenden and Mendel	10% NaCl	− 43.48
Edestin, hemp-seed	Alexander	10% NaCl	− 41.5
Edestin, hemp-seed	Osborne and Harris	10% NaCl	− 41.7
Excelsin, Brazil-nut	Alexander	10% NaCl	− 40.5
Excelsin, Brazil-nut	Osborne and Harris	10% NaCl	− 42.94
Globulin, flax-seed	Alexander	10% NaCl	− 38.5
Globulin, flax-seed	Osborne and Harris	10% NaCl	− 43.53
Globulin, squash-seed	Osborne and Harris	10% NaCl	− 38.32
Amandin, almond	Osborne and Harris	10% NaCl	− 56.44
Corylin, hazel-nut	Osborne and Harris	10% NaCl	− 43.09
Juglansin, English walnut	Osborne and Harris	10% NaCl	− 45.21
Juglansin, American black walnut	Osborne and Harris	10% NaCl	− 44.43
Juglansin, butternut	Osborne and Harris	10% NaCl	− 45.40
Phaseolin, kidney-bean	Osborne and Harris	10% NaCl	− 41.46
Legumin, horse-bean	Osborne and Harris	10% NaCl	− 44.09
Ricin, castor-bean	Osborne, Mendel and Harris	water	− 28.85
Gliadin, wheat	Osborne and Harris	80% alcohol	− 92.28
Gliadin, wheat	Kjeldahl	55% alcohol	− 92.0
Gliadin, wheat	Kjeldahl	glacial acetic acid	− 81.(
Gliadin, wheat	Kjeldahl	5.1% acetic acid	−111.0
Gliadin, wheat	Kjeldahl	phenol	−130.0
Gliadin, wheat	Mathewson	70% methylalcohol	− 95.65
Gliadin, wheat	Mathewson	70% ethyl alcohol	− 91.95
Gliadin, wheat	Mathewson	60% ethyl alcohol	− 96.66
Gliadin, wheat	Mathewson	50% ethyl alcohol	− 98.45
Gliadin, wheat	Mathewson	60% propyl alcohol	−101.10
Gliadin, wheat	Mathewson	70% phenol	−123.15
Gliadin, wheat	Mathewson	phenol, anhydrous	−131.77
Gliadin, wheat	Mathewson	paracresol	−121.00
Gliadin, wheat	Mathewson	benzyl alcohol	− 53.10
Gliadin, wheat	Mathewson	glacial acetic acid	− 78.60
Gliadin α, wheat	Lindet and Ammann	70% alcohol	− 81.6
Gliadin β, wheat	Lindet and Ammann	70% alcohol	− 95.0
Gliadin, rye	Kjeldahl	55% alcohol	−121.0
Gliadin, rye	Kjeldahl	"dilute acid"	−144.0
Gliadin, rye	Kjeldahl	glacial acetic acid	−105.0
Gliadin, rye	Kjeldahl	phenol	−157.0
Gliadin, rye	Lindet and Ammann	70% alcohol	− 87.8
Gliadin, barley	Lindet and Ammann	70% alcohol	− 87.8
Hordein, barley	Lindet and Ammann	70% alcohol	−137.5
Hordein, rye	Lindet and Ammann	70% alcohol	−137.5
Zein α, maize	Lindet and Ammann	70% alcohol	− 29.6
Zein β, maize	Lindet and Ammann	70% alcohol	− 40.0
Zein, maize	Osborne and Harris	90% alcohol	− 28.0
Zein, maize	Kjeldahl	75% alcohol	− 35.0
Zein, maize	Kjeldahl	glacial acetic acid	− 28.0

* Cited after T. B. Osborne (37), which consult for references to the literature.

From these results it is clear that the nature of the solvent plays a very considerable part in determining the rotatory power of proteins. Furthermore, it would appear that as a rule the salts which proteins form with acids and bases differ very markedly in rotatory power from the free protein (3) (20) (60) (1) (4) (6) (39). In an analogous manner the rotatory powers of the salts which the simple amino-acids form with acids and bases differ very markedly from those of the free acids (40). As a rule too little attention has been paid to this fact by investigators who have sought to characterize the proteins by their rotatory powers. It is evident that the specific rotatory power of a protein cannot be regarded as characteristic of it until the nature of the protein salt and of the solvent employed are rigidly defined.

According to Alexander (1) the rotatory power of a dissolved protein is considerably affected by exposure of the solution to a high temperature for a period preceding the measurement. It must not be forgotten, however, that partial hydrolysis of the protein may play a very considerable part in bringing about this change.

2. The Absorption of Light by Protein Solutions. — The peculiar absorption-spectra of solutions of hæmoglobin and its colored derivatives have been very extensively studied (17) (19) (63) (21) (22) (36) (5) (58) (14). From the results of these investigations it is evident that the absorption-bands (α and β) in the visible spectrum which are caused by these solutions are primarily attributable to the hæmatin- and not to the distinctively protein moiety of the hæmoglobin molecule. In addition to these bands, however, the absorption-spectrum of hæmoglobin and its derivatives reveals another absorption-band on the extreme edge of the violet end of the visible spectrum (61) (2) (8). According to Gamgee (8) this absorption-band is given not only by solutions of hæmoglobin but also by solutions of hæmatin; he therefore attributes it to the hæmatin, and not to the protein moiety of the hæmoglobin molecule. According to Soret, however, an absorption-band on the extreme edge of the ultra-violet can be demonstrated, by employing a fluorescent screen, in the absorption-spectra of solutions of a large variety of proteins, among which may be mentioned egg-albumin, mucin (from snails), casein, globulin and syntonin. This band is followed, on the ultra-violet side, by a region of especial transparency. Accord-

ing to Soret it is unaffected by acids, but on adding NaOH or ammonia to the protein solution the region of transparency in the ultraviolet disappears completely and the band of absorption is displaced towards the less refrangible end of the spectrum. On neutralizing the solution with acid the original absorption-spectrum is restored. A similar absorption-spectrum is yielded by solutions of tyrosin and it is affected in a similar manner by alkalies. Soret therefore attributes the absorption-band in the absorption spectra of protein solutions to the tyrosin radical which the proteins contain.

Kober (23) has recently carried out a spectrographic examination of solutions of a number of amino-acids and polyamino-acids. He finds that the aliphatic amino-acids display only general absorption in the extreme ultraviolet. The aromatic acids, tyrosin and phenylalanin do, however, show definite absorption-bands in the ultra-violet spectrum. The absorption-band in the ultraviolet which is displayed by proteins in solution is therefore attributable not only to tyrosin radicals, as Soret supposed, but also in some measure to phenylalanin radicals.

Since the occurrence of any photochemical reaction is dependent upon the absorption of the chemically active rays by some constituent or constituents of the reacting system it would appear possible that the markedly toxic action of ultraviolet light upon many unicellular organisms may be dependent upon the absorption of the ultraviolet light-rays by the proteins of the organisms affected. Strong confirmation of this view is afforded by the experiments of Harris and Hoyt (12) who have shown that if ultraviolet light be passed through a thin layer of gelatin or peptone solution its toxicity for *paramoecia* is diminished to a much greater extent than by passage through a similar layer of distilled water. Solutions of urea, sugar or alanin do not confer this protection, while solutions of the aromatic amino-acids protect the organisms very markedly. Leucin likewise confers a very marked protection, but this amino-acid is rapidly decomposed by ultraviolet light, yielding colored products, and the decomposition is accompanied by an increase in the protective power of the solution.

3. The Refractive Indices of Protein Solutions. — The influence of dissolved proteins upon the refractive indices of various solvents has been especially studied by Reiss (41) (43), Herlitzka (13), Schmidt (56) and myself (43–54).

Reiss measured the change in the refractive indices of dilute salt solutions which results from the introduction of varying amounts of the "pseudoglobulins" of blood serum. His pseudoglobulins were prepared by fractional coagulation with ammonium sulphate and purified by prolonged dialysis. Fraction I (pseudoglobulin I) was coagulated at 32 to 36 per cent saturation with ammonium sulphate, fraction II (pseudoglobulin II) was coagulated at 36 to 39 per cent saturation. The change in the refractive index of the solvent due to the introduction of these proteins was found to be directly proportional to the quantity of protein dissolved in it; the change due to the introduction of 1 per cent of the "pseudoglobulin I" being 0.00224 and that due to the introduction of 1 per cent of the "pseudoglobulin II" 0.00230. The difference between these determinations is not sufficient to constitute a basis for distinction between the two globulin-fractions, since it is not greater than that which might have arisen through experimental error. Reiss also measured the influence of other constituents of serum (especially crystallized and amorphous serum albumin) upon the refractive indices of dilute saline solutions.

I have amplified and confirmed these results of Reiss, employing for this purpose a variety of proteins (casein, paranuclein, ovomucoid, ovovitellin, serum globulin, gliadin, etc.) and not only aqueous solvents but alcohol-water mixtures, acetone-water mixtures, etc. A large number of determinations upon casein in aqueous solutions, between the concentrations which it is feasible to employ* show that the change in the refractive indices of aqueous solvents due to the introduction of this protein is very accurately proportional to its concentration (43) (56). Accordingly the refractive index of a solution of this protein is given by:

$$n - n_1 = a \times c$$

where n is the refractive index of the solution, n_1 that of the solvent, c the percentage of casein in the solution and a a constant which is characteristic of the protein (for example, casein) which is

* If the concentration of protein be too small (less than about 0.5 per cent) the experimental error of the determination becomes a significant proportion of the observed difference in the refractive index of the solvent, due to the introduction of the protein. If the concentration of protein be too large then the solutions are too opaque to enable an exact reading to be obtained.

employed and represents the change in the refractive index of the solvent which is brought about by dissolving one gram of the protein in 100 cc.

The following results are illustrative:*

TABLE III

Solvent 0.10 *N* NaOH

Concentration casein per cent	Refractive index	a for $n_1 = 1.33444$
0.5	1.3352	0.00152
1.0	1.3360	0.00156
1.5	1.3368	0.00157
2.0	1.3375	0.00153
2.5	1.3383	0.00154
3.0	1.3390	0.00152
4.0	1.3405	0.00152
5.0	1.3420	0.00151
6.0	1.3436	0.00153

In the first column of the table is given the amount of casein in grams which was dissolved in 100 cc. of the solution. In the second column is given the refractive index of the solution measured at 20° C. In the third is given the value of the constant a calculated from the above formula for the given value of the constant n_1 (i.e., the refractive index of the solvent, previously determined at the same temperature).

The equation $n - n_1 = a \times c$ also holds good for solutions of ovomucoid in water (44), of paranuclein in $N/10$ KOH (45), of potassium serum globulinate (46), of potassium caseinate in alcohol-water mixtures (47), of gliadin in various solvents (54) and of edestin in aqueous solvents (56).

The change in the refractive indices of aqueous solvents which is brought about by a given percentage of casein is independent of the nature of the acid or base with which the casein is combined. This is shown by the fact that this change is the same whether the casein be dissolved in dilute KOH, NaOH, LiOH, NH_4OH, $Sr(OH)_2$, $Ba(OH)_2$, $Ca(OH)_2$ or HCl solutions. Also, between 0.01 *N* and 0.1 *N* it is independent of the concentration of alkali

* In all of these determinations a Pulfrich refractometer was employed, reading the angle of total reflection accurately to within 1′, and a sodium flame was employed as the source of light.

which is employed as solvent. Similarly, the value of a, in the above equation, is the same for "insoluble" serum globulin whether this protein be dissolved in $N/10$ KOH or in $N/40$ HCl. The influence which a protein exerts upon the refractive index of its solution is therefore independent of the nature or proportion of acid or base which is combined with it, differing in this respect very strikingly from the optical rotatory power of dissolved protein, which, as we have seen, is very intimately dependent upon the nature and proportion of combined inorganic acid or base. The reason for this fact is readily perceived when we reflect that the power of dissolved protein to refract light is a function of the space which is occupied by the protein molecules. Now the molecular volume, and, indeed, the molecular refractivity is an additive function of the atomic volumes (or refractivities) of the atoms which together make up the molecule. In a molecule which contains over a thousand atoms, as a protein molecule does, the substitution of even several H atoms by K atoms or the addition of a few H or Cl atoms or OH groups might be expected not to exert an appreciable influence upon the volume or refractive power of the whole molecule. The refractive index of a protein solution also remains unaltered by hydrolysis, upon which fact I have based a method of determining the comparative activities of trypsin solutions (50).

From the investigations of Gladstone and Dale (11) and of Landolt (27) it appears that the expression $\frac{N-1}{d}$, where N is the refractive index of a substance and d its density, which is known as the specific refractivity, is very constant, being only slightly dependent upon the temperature. Moreover each particular substance in a mixture preserves its own specific refractivity nearly unchanged; hence the refractive index of a mixture or of a solution can generally be readily calculated from the refractive indices of its components. Now the density of a dilute protein solution is always very nearly that of water, that is 1, so that the specific refractivity of a dilute protein solution may, with a tolerable approach to accuracy, be taken as $n - 1$, where n is the refractive index of the solution. Suppose c per cent of protein were to be dissolved in a solvent of specific refractivity $N_1 - 1$ (for instance, a dilute acid or alkali, which is nearly equal in density to pure water), then, if Gladstone's law holds good, and the density

of the dissolved protein itself be d (Cf. Chap. XIII, section **11**) the refractive index of the mixture should be given by:

$$(n-1) = \frac{(N_1-1)(100-c)}{100} + \frac{(N-1)c}{100} \cdot \frac{1}{d},$$

whence $$100(n-1) = 100(N_1-1) - c\left(1 - \frac{1}{d} - N_1 + \frac{N}{d}\right)$$

and $$n = N_1 + \frac{c}{100}\left(1 - \frac{1}{d} - N_1 + \frac{N}{d}\right)$$

or $$n - N_1 = \left(1 - \frac{1}{d} - N_1 + \frac{N}{d}\right)\frac{c}{100}, \qquad \text{(i)}$$

which is identical with the relation $n - n_1 = a \times C$ which, as we have already seen, subsists between the refractive index of a protein solution and its percentage concentration.* Hence we may conclude that *for solutions of proteins in aqueous solvents Gladstone's law of mixtures holds good.* The constant a is obviously given by $\frac{1}{100}\left(1 - \frac{1}{d} - N_1 + \frac{N}{d}\right)$. For solutions of casein containing between 0.25 and 6.00 per cent the average value of a is 0.00125. Hence, by exterpolation, the refractive index of the pure protein should be 1.675, taking the refractive index of water as 1.333 and the density of dissolved casein as 1.39 (Cf. Chap. XIII, section **11**). For serum globulin this constant is 1.774. The refractive index of the pure protein does not readily admit of direct determination in these cases. The protamin salts, clupein sulphate and salmin sulphate can, however, readily be obtained in a fluid (oily) condition. Kossel (24) has determined the refractive indices of these fluids and finds them to be, respectively, 1.443 and 1.442, figures which are of the same order of magnitude as the above estimates.

If the density of the solvent be d' instead of 1 and c be small, so that the density of the solution is very nearly that of the solvent, then equation (i) becomes:

$$n - N_1 = \left(1 - \frac{d'}{d} - N_1 + \frac{d'}{d}N\right)\frac{c}{100}, \qquad \text{(ii)}$$

* From this it would appear that the value of a should vary with the refractive index even of a dilute solution of a base or acid which is not exactly the same as that of water, that is, with the nature and concentration of the (dilute) acid or base employed as a solvent. It will be observed, however, that in the experimental values of a only a one-hundredth part of the difference between the refractive index of the acid or alkali solution and that of water appears. This quantity, for the solutions employed, is far too small to be detectable.

which is the same *form* as (i). Obviously, however, in a solution of a protein in a solvent the density or refractivity of which departs at all widely from those of water, although the law $n - n_1 = a \times c$ might be expected to hold good, the constant a would not have the same value as in aqueous solvents. In other words, in non-aqueous solvents the law $n - n_1 = a \times c$ should hold good, but, for a given protein the value of a should vary with the nature of the solvent depending upon its refractivity and its density. In satisfactory correspondence with this deduction we find that the law $n - n_1 = a \times c$ holds good for solutions of potassium caseinate in alcohol-water mixtures containing from 0 to 75 per cent of alcohol, for solutions of serum-globulin in alcohol-water mixtures, and for solutions of gliadin in a variety of solvents. As we shall see, however, although the value of a for a given protein varies with the nature of the solvent in the *sense* demanded by the above theory, this variation cannot be quantitatively expressed by the formula $100\,a = N\frac{d'}{d} - N_1 + 1 - \frac{d'}{d}$.

In estimating the accuracy with which a can be determined in the equation $n - n_1 = a \times c$, it is essential to recollect that the experimental error in determining the angle of total reflection with the Pulfrich Refractometer is $\pm 1'$. This, for the instruments which I have employed, corresponds to an error of ± 0.00009 in the determination of the refractive index of a given solution. Now it is obvious that since the absolute error in the determination of $n - n_1$ is the same, the error in the determination of a must be less in proportion to the magnitude of c.

In order to assign to each determination its due weight in the estimation of the mean value of a for any solvent we must therefore add together all of the observed values of $n - n_1$ and divide this sum by the sum of the concentrations employed. This procedure has been adopted in my own estimates, cited below. The following are the values of a for various proteins and solvents which have so far been ascertained. The influence of temperature upon the magnitude of a appears to be very slight. In fact for casein I have found the value of a to be the same, within the experimental error, at 40 degrees as it is at 20 degrees. Hence the temperatures at which the observations were made are not specified in the accompanying table; they lay, in every case, however, between ordinary room-temperatures and 24 degrees.

TABLE IV*

Protein	Solvent	a	Observer
Pseudoglobulin II	Dilute saline	0.00230	Reiss (41) (42)
"Insoluble" serum globulin	Dilute acid or alkali	0.00229±0.00024	Robertson (46)
"Insoluble" serum globulin	25 per cent alcohol	0.00202±0.00016	Robertson (46)
"Insoluble" serum globulin	50 per cent alcohol	0.00119±0.00024	Robertson (46)
"Insoluble" serum globulin	25 per cent acetone	0.00227±0.00016	Robertson (46)
"Insoluble" serum globulin	50 per cent acetone	0.00146±0.00024	Robertson (46)
Pseudoglobulin I	Dilute saline	0.00224	Reiss (41) (42)
Crystallized serum albumin	Dilute saline	0.00201	Reiss (41) (42)
Amorphous serum albumin	Dilute saline	0.00183	Reiss (41) (42)
Amorphous serum albumin	$\frac{1}{4}$ saturated $(NH_4)_2SO_4$	0.00177±0.00008	Robertson (48)
Amorphous serum albumin	$\frac{1}{2}$ saturated $(NH_4)_2SO_4$	0.00150±0.00008	Robertson (48)
Edestin	Dilute alkali	0.00174±0.00006	Schmidt (56)
Globin	Dilute acid or alkali	0.00169±0.00005	Robertson (52)
Salmin	As chloride in distilled water	0.00167±0.00019	Robertson (49)
Salmin	As sulphate in distilled water	0.00173±0.00009	Robertson (49)
Gliadin	$N/10$ acetic acid	0.00161±0.00006	Robertson and Greaves (54)
Gliadin	$N/10$ KOH	0.00167±0.00006	Robertson and Greaves (54)
Gliadin	60 per cent ethyl alcohol	0.00143±0.00006	Robertson and Greaves (54)
Gliadin	70 per cent ethyl alcohol	0.00152±0.00006	Robertson and Greaves (54)
Gliadin	60 per cent propyl alcohol	0.00152±0.00006	Robertson and Greaves (54)
Gliadin	25 per cent acetone	0.00168±0.00006	Robertson and Greaves (54)
Gliadin	50 per cent acetone	0.00147±0.00006	Robertson and Greaves (54)
Gliadin	75 per cent phenol	−0.00038±0.00006	Robertson and Greaves (54)
Ovomucoid	Distilled water	0.00160±0.00004	Robertson (44)
Casein	Dilute acid or alkali	0.00152±0.00005	Robertson (43) (47)
Casein	25 per cent alcohol	0.00157±0.00005	Robertson (43) (47)
Casein	50 per cent alcohol	0.00149±0.00005	Robertson (43) (47)
Casein	75 per cent alcohol	0.00125±0.00006	Robertson (43)
Paranuclein	Dilute alkali	0.00140±0.00008	Robertson (45)
Paranuclein A	Dilute alkali	0.00140±0.00012	Robertson (45)
Ovovitellin	Dilute alkali	0.00130±0.00010	Robertson (44)

* All of the percentages are percentages by volume of solution.

Calculating the theoretical values of a, on the assumption that Gladstone's law of mixtures holds good for solutions of protein in *any* solvent we at once find that although a varies with the nature of the solvent in the sense demanded by Gladstone's law, yet the law does not hold good, for different solvents, with any approach to quantitative exactitude. Thus the calculated value of a for solutions of casein in 50 per cent alcohol is 0.00095, assuming the density of casein dissolved in 50 per cent alcohol to be the same as that of casein dissolved in water, while if we assume the density of casein dissolved in 50 per cent alcohol to be that of undissolved casein (Cf. Chap. XIII, section **11**) the calculated value of a becomes 0.00121; the experimental value, however, is 0.00149; the calculated value of a for insoluble serum-globulin in 50 per cent alcohol is 0.00169, the experimental value is 0.00119. Hence Gladstone's law of mixtures holds good for solutions of protein in a specified solvent, but no longer holds good if we vary the nature of the solvent. Now it is a characteristic of the quantity $\frac{N-1}{d}$ that although it is independent of temperature and of concentration, it varies *when the state of aggregation varies* (35). We may conclude, therefore, as we have had occasion to indicate before, that the physical condition (number of associated water molecules, degree of dispersion, etc.) of proteins in alcohol-water mixtures and other non-aqueous solvents is not the same as it is in water.*

Comparing the above cited determinations by Robertson and Greaves, of the refractive indices of gliadin in various solvents, with Mathewson's determination of the rotatory power of gliadin in various solvents, it is evident that, as might have been expected, there is no correspondence between the effects of different solvents upon these two physical properties of dissolved protein.

It will be observed that the value of a for solutions of gliadin in 75 per cent phenol is *negative*. This is due to the fact that the refractive index of 75 per cent phenol is nearly equal to that of gliadin itself, and its density is greater so that a mixture of the two substances has a refractive index which is less than that of the 75 per cent phenol employed as solvent.

The influence of temperature upon the refractive indices of protein solutions has been determined by myself and by Herlitzka (13). As stated above, I find the effect of temperature upon the

* Cf. Chaps. I and XIII.

value of a for solutions of caseinates in water to be very small. In fact, if allowance be made for the alteration in the refractive index of the *water* with varying temperature, I have been unable to observe any alteration in a, distinctly greater than the possible experimental error, between 20 and 40 degrees. According to Herlitzka, however, if egg-albumin be employed, a distinct influence of temperature upon the refractivity of the protein itself can be observed. He interprets his results in the light of the Lorentz-Lorentz modification of Gladstone's law of mixtures (28) (29).

The utilization of the refractometric method for determining the concentrations of various proteins in solutions and in body-fluids has been discussed in Chap. III.

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PART IV

CHEMICAL DYNAMICS IN PROTEIN SYSTEMS*

* Any survey of this subject must, of course, involve an incidental exposition of the properties and behavior of the enzymes, to which reference must necessarily be made. Nevertheless any attempt to enter fully into a discussion of the properties of enzymes would, of course, lead us into regions quite foreign to the scope of this work. For exhaustive treatments of the general subject of enzyme-action the reader is referred to the works of Oppenheimer (15), Bredig (8), Taylor (19), Vernon (20), Euler (9) and Bayliss (7).

CHAPTER XV

THE HYDROLYSIS OF THE POLYPEPTIDS

1. The Hydrolysis of Polypeptids by Proteolytic Enzymes. — By the aid of the various hydrolysing agencies the synthetic polypeptids are capable of being split, with successive additions of the elements of water, into their constituent amino-acids. Among these hydrolysing agencies must be reckoned a number of the protein-splitting enzymes which are found in a variety of tissues, tissue-extracts and secretions.

The action of the enzymes of the pancreas (trypsin and, presumably, others) upon the dipeptids was first investigated by Fischer and Bergell (12) who acted upon solutions of various dipeptids with pancreas-extract. They found that:

glycyl-glycin β-naphthalenesulphoglycyl-d-alanin β-naphthalenesulpho-d-alanyl-glycin Di-β-naphthalenesulphotyrosyl-dl-leucin	were not hydrolysed.

while

β-naphthalenesulphoglycyl-l-tyrosin β-naphthaleneglycyl-dl-leucin carboxethyl-glycyl-dl-leucin glycyl-l-tyrosin leucyl-alanin alanyl-leucin leucyl-leucin	were hydrolysed.

In the course of these investigations the exceedingly interesting fact was encountered that the racemic compounds are hydrolysed asymmetrically by the pancreas extract; thus only l-leucin was obtained by the action of the extract upon carboxethyl-glycyl-d-l-leucin, the remainder (containing d-leucin) not being acted upon. *Now l-leucin is the naturally occurring variety, the form, that is, which is found in the proteins.*

These investigations were greatly extended by Fischer and Abderhalden (11) who employed a pancreatic juice prepared by Pavlov from a pancreatic fistula and activated by entero-

kinase obtained from the succus entericus of the duodenum. The result of these investigations was to show that the synthetic polypeptids can be divided into two distinct classes, members of the one class being hydrolysed or (if racemic) partially hydrolysed by the trypsin; members of the other class not being hydrolysed by trypsin. The various polypeptids employed by Fischer and Abderhalden were distributed between these two classes as follows:

Hydrolysed

* Alanyl-glycin
* Alanyl-alanin
* Alanyl-leucin A
* Leucyl-isoserin A
Glycyl-l-tyrosin
Leucyl-l-tyrosin
* Alanyl-glycyl-glycin
* Leucyl-glycyl-glycin
* Glycyl-leucyl-alanin
* Alanyl-leucyl-glycin
Dialanyl-cystin
Dileucyl-cystin
Tetraglycyl-glycin
Triglycyl-glycin ester
d-alanyl-d-alanin
d-alanyl-l-leucin
l-leucyl-l-leucin
l-leucyl-d-glutamic acid

Not Hydrolysed

Glycyl-alanin
Glycyl-glycin
Alanyl-leucin B
Leucyl-alanin
Leucyl-glycin
Leucyl-leucin
Aminobutyryl-glycin
Aminobutyryl-aminobutyric acid A
Aminobutyryl-aminobutyric acid B
Valyl-glycin
Glycyl-phenylalanin
Leucyl-prolin
Diglycyl-glycin
Triglycyl-glycin
Dileucyl-glycyl-glycin
d-alanyl-l-alanin
l-alanyl-d-alanin
l-leucyl-d-leucin
d-leucyl-l-leucin

The compounds which are marked by asterisks were racemic. *Of all these the hydrolysis proved to be asymmetric and only the naturally-occurring stereo-isomer was attacked.*

There is thus a remarkable correlation between the ability of these enzymes to attack certain amino-acid unions and the occurrence of these unions among the natural proteins which these enzymes attack in the ordinary course of events. As we shall see in the following chapter there are many excellent reasons for believing that the action of the proteolytic enzymes, like that of many of the inorganic catalysors, is dependent upon the formation of intermediate compounds of the enzyme with the substrate. It appears highly probable, therefore, that the enzyme

is itself possessed of an optical structure which fits it to unite with certain stereo-isomers and only with them. To pursue Emil Fischer's classical illustration, the enzyme and these particular stereo-isomeric unions fit one another as a key does a lock. The position is therefore this, that the proteolytic enzymes are only adapted to "fit" certain modes of union between amino-acids and *that these are precisely the modes of union which occur in the proteins which it is their function to digest.* To a certain type of mind, a teleological "explanation" of this fact would doubtless prove very inviting. But the biochemist demands something more satisfactory than teleology, or even than the conveniently comprehensive neo-Darwinian generalization of "adaptation through survival of the fittest." Our physico-chemical view-point inclines us to suspect the existence of some physico-chemical *mechanism* which, in the building up of these bodies has necessarily brought about their mutual adaptation to and dependence upon one another. The nature of this mechanism is, I believe, not far to seek. In Chap. XVII we shall see that not only the *hydrolysis* but also the *synthesis* of the proteins is brought about through the agency of enzymes, and that the enzymes which bring about the synthesis of the proteins are, in all probability, but slight modifications (probably anhydrides) of the enzymes which bring about their hydrolysis. Synthesis, just as hydrolysis, must involve the formation of intermediate compounds between the enzyme and the substrate (in this instance the constituent amino-acids out of which the protein is to be built up). Since the enzyme is only adapted to unite with certain stereo-isomers of the amino-acids only these stereo-isomers can be transported into the structure which the enzyme is engaged in building up. In other words, the stereo-isomeric structure of the naturally occurring proteolytic enzymes necessarily determines the stereo-isomeric structure of all of the naturally occurring proteins, and for the same reason that these enzymes can only *split* certain amino-acid unions, they can only cause these unions to arise. Hence the occurrence of only those stereo-isomeric groups within the protein molecule which are open to *attack* by the proteolytic enzymes may be regarded as a *consequence* of their power to react with the digestive enzymes (17).

From the above-cited results of Fischer and Abderhalden it is evident that not only the stereo-isomeric structure of the

amino-acid radicals in a polypeptid is of great importance in determining the susceptibility of the polypeptids to hydrolyis by trypsin, but also the *order* in which the amino-acid groups are combined and the *nature* of these groups themselves, apart altogether from their optical configuration. Thus alanyl-glycin, $CH_3.CH_2NH_2.CO.NH.CH_2.COOH$, is readily hydrolysed; while the isomeric glycyl-alanin, $NH_2.CH_2.CO.NH.CH.CH_3.COOH$, is not. When alanin is the acyl radical, as in alanyl-glycin, alanyl-alanin and alanyl-leucin, hydrolysis occurs, but it does not when leucin, valin or aminobutyric acid are the acyl radicals (for instance, leucyl-alanin, leucyl-glycin and leucyl-prolin).

The number of amino-acid groups within the molecule is also of great importance. Thus tetraglycyl-glycin is hydrolysed in the presence of trypsin, while glycyl-glycin, diglycyl-glycin and triglycyl-glycin are not; other things being equal it is evident that mere length of the peptid chain *per se* confers upon it greater susceptibility to attack by this enzyme.

It will be observed, on surveying the above results, that a number of polypeptids are not hydrolysable by pure trypsin which nevertheless are built up out of naturally occurring radicals. Particularly resistant to hydrolysis are those peptids which contain a preponderance of glycin, phenylalanin or pyrrolidin-carboxylic acid.* In the light of the theory presented above, therefore, the question forces itself upon us, how can such peptid groups come to form, as they do, constituent parts of the molecules of many proteins? Obviously, if they cannot be *digested* by trypsin and our theory is valid then they cannot have been *introduced* into the protein molecule through the agency of trypsin; they must have been introduced through the agency of some other enzyme or enzymes.

In this connection the very significant fact will be observed that whereas Fischer and Bergell succeeded in hydrolysing leucyl-alanin through the agency of pancreas-extract, Fischer and Abderhalden were unable to secure the hydrolysis of this substance through the agency of pure trypsin. This pointed to the existence in the extract of enzymes other than trypsin which, unlike

* Emil Fischer and E. Abderhalden (10). The connection between these results and the theory of Kühne that the proteins are built up of hemi- and anti-groups, respectively, hydrolysable and non-hydrolysable by pepsin and trypsin is sufficiently evident to require no further comment here.

trypsin, are able to bring about the hydrolysis of leucyl-alanin. The possibility was thus clearly indicated that a variety of enzymes might be found to exist in the various tissues and tissue fluids, distinguishable from one another in their power to hydrolyse various polypeptids, but only with difficulty distinguishable from one another in their action upon proteins, since every protein, it might be anticipated, contains unions susceptible to attack by any one of these enzymes.

The possibility thus indicated has been rendered a certainty, thanks to the extensive researches of Abderhalden and his collaborators (2). These investigators have employed the extracts and press-juices of various organs, prepared by Buchner's method of grinding up with sand and kieselguhr and pressing out at a pressure of 100 to 300 atmospheres, which contain a number of different enzymes capable of bringing about the hydrolysis of various polypeptids. These enzymes are not so selective in their action as pure trypsin, they can hydrolyse polypeptids which trypsin cannot, and the press-juices of different organs exhibit characteristic differences in regard to the polypeptids which they can and those which they cannot attack. Most striking differences were observed between the enzymatic activities of the red blood-corpuscles and those of the plasma which bathes them. The red corpuscles of the horse (but not those of the ox) hydrolyse glycyl-l-tyrosin, which is not hydrolysed by white corpuscles nor by plasma or serum; they also hydrolyse diglycyl-glycin which, it will be recollected, is not hydrolysed by trypsin. Plasma and serum both hydrolyse d-l-alanyl-glycin, diglycyl glycin and triglycyl glycin; hence the enzymatic activity of the serum is not attributable to trypsin or erepsin absorbed from the intestinal wall.

Pure pepsin, prepared by Pavlov, does not act upon any of the polypeptids, whereas it splits the natural proteins into some half-dozen peptones and proteoses, although it does not split these substances (which are in reality polypeptids) any further, and hence yields no amino-acids. Of this fact two alternative explanations are offered (16). Either the proteins contain certain types of union (such as ether-like combinations and so forth) which are not present in the synthetic polypeptids and which are the only points of union which pepsin can attack; or else the great *length* of the amino-acid chain in the proteins confers

upon it greater susceptibility to pepsin than the shorter polypeptid chains possess. In support of the former view it is pointed out that although erepsin, obtained from the succus entericus, is able to hydrolyse peptones and polypeptids, yet it cannot hydrolyse proteins, a fact which would appear to point towards the existence of a few linkages in proteins which are not present in polypeptids. In support of the latter view it is pointed out that polypeptid-chains of greater length are more susceptible to attack by other enzymes (i.e., trypsin) than the shorter chains, so that the possibility cannot be overlooked that the susceptibility of proteins to attack by pepsin may merely be attributable to their great complexity, i.e., to the extreme length of the polypeptid-chain.

2. The Kinetics of the Hydrolysis of Polypeptids by Proteolytic Enzymes. — The progressive hydrolysis of diglycyl-tyrosin in the presence of trypsin was followed by Taylor (19). He states that the results which he obtained were irregular and unsatisfactory, but he regarded these irregularities as being attributable to analytical errors.

The optically active dipeptids, d-alanyl-d-alanin, d-alanyl-l-leucin and glycyl-l-tyrosin, have been employed by Abderhalden and Koelker, Abderhalden and Michaelis and Abderhalden and Gigon (3) (4) (6) (14) in investigating the time relations of their hydrolysis by trypsin. The degree of hydrolysis at any moment can readily be followed by observing the optical rotation of the solution, the rotation due to d-alanyl-d-alanin, for example, being negative ($\alpha_D^{20°} = -21.2°$), while that of the products of complete hydrolysis is positive. The change in the optical rotation is, of course, directly proportional to the degree of hydrolysis.*

The relation between the time and the extent of hydrolysis is, as Abderhalden and Michaelis have shown, susceptible of fairly simple formulation. The form of relation which is characteristic for a monomolecular reaction (that is, a transformation which involves only one species of molecule) is expressed by the differential equation:

$$\frac{dx}{dt} = k\,(a - x),$$

* Subject to a slight correction due to the fact that the specific rotatory powers of the dipeptids are not absolutely independent of their concentrations. Cf. Koelker (14).

in which x is the amount transformed after time t, a is the initial quantity of substrate and k is the velocity-constant of the reaction. When integrated this yields the equation:

$$\log \frac{a}{a-x} = kt. \qquad \text{(i)}$$

In the derivation of this equation it is to be noted that the velocity of the reverse reaction is regarded as being negligible, so that no station of equilibrium is reached until the transformation is practically complete. This condition is tolerably well fulfilled in the reaction under consideration, for the hydrolysis of the polypeptid does not cease until the quantity of undecomposed polypeptid is practically unappreciable. In the following table are given the values of the constant k for the hydrolysis of d-alanyl-d-alanin by liver extract at 37 degrees calculated by Abderhalden and Michaelis from the experimental values of $a - x$ and t obtained by Abderhalden and Koelker. In every case the initial concentration of the dipeptid was such that 1.45 grams of the substance were dissolved in 6 cc. of the digest.

Time in minutes	1.45 units of dipeptid + 6 cc. of ferment solution k =	Time in minutes	1.45 units of dipeptid + 4 cc. of ferment solution k =	Time in minutes	1.45 units of dipeptid + 3 cc. of ferment solution k =	Time in minutes	1.45 units of dipeptid + 2 cc. of ferment solution k =
3	0.0453	3	0.0192	5	0.0125	5	0.00710
7	0.0390	7	0.0183	6.5	0.0132	11	0.00847
11	0.0342	10	0.0202	7.5	0.0134	15	0.00834
13	0.0336	11	0.0197	16	0.0142	23	0.00901
18	0.0380	16	0.0214	22	0.0161	31	0.01020
20	0.0373	17	0.0203	23	0.0163	41	0.01216
24	0.0399	25	0.0268	28	0.0192	53	0.01474
27	0.0451	30	0.0294	29	0.0196	65	0.01920
34	0.0430	34	0.0342	30	0.0209	80	0.01810
....	.....	35	0.0359	38	0.0232	....	.....
....	.....	....	.....	45	0.0265	....	.....

It is evident that, within the experimental error, k calculated from formula (i) is tolerably constant for the higher concentrations of ferment solution (6 cc. of ferment solution); for lower ferment-concentrations, however, the value of k does not even approximate to constancy, but rises markedly as hydrolysis proceeds; in other words the velocity of the transformation does not fall

off so rapidly as it should were it a simple monomolecular reaction of which the velocity constant is simply enhanced in magnitude by the presence of the ferment.

In the derivation of equation (i) it is assumed that the velocity of transformation at any moment is proportional to the mass of substrate which is at that moment undergoing change. The theoretical velocity therefore falls off in direct proportion as the substrate is used up. The next simplest assumption, taking cognizance of the fact that the velocity of transformation does not decrease with the rapidity demanded by the monomolecular formula, is that the velocity of transformation is independent of the mass of the substrate, and is therefore expressed by the equation:

$$\frac{dx}{dt} = k_1$$

which, when integrated, yields

$$x = k_1 t. \qquad \text{(ii)}$$

Calculating the values of k_1 from this formula, for the above four series of experimental data Abderhalden and Michaelis obtained the following results:

1.45 units of dipeptid + 6 cc. of ferment solution k_1 =	1.45 units of dipeptid + 4 cc. of ferment solution k_1 =	1.45 units of dipeptid + 3 cc. of ferment solution k_1 =	1.45 units of dipeptid + 2 cc. of ferment solution k_1 =
0.1300	0.0600	0.0380	0.0240
0.0843	0.0529	0.0400	0.0255
0.0713	0.0540	0.0400	0.0240
0.0708	0.0518	0.0369	0.0239
0.0639	0.0494	0.0368	0.0242
0.0595	0.0494	0.0365	0.0241
0.0538	0.0456	0.0368	0.0228
0.0504	0.0420	0.0366	0.0212
0.0411	0.0397	0.0314	0.0180
.....	.....	0.0332	.....
.....	.....	0.0302	.....

It is evident that k_1, calculated from formula (ii), tends to approximate constancy for the *lower* ferment concentrations (2 cc. and 3 cc.), while it altogether fails to do so for the higher ferment-concentrations. To the latter solutions, as we have seen, equation (i) applies tolerably well. Neither equation, therefore, taken by itself, represents the entire process; each applies under

certain limiting conditions. The possibility was thus indicated that a *combination* of the two formulæ might be found to adequately represent all of the phenomena. Obviously the process represented by equation (i) plays a predominant part when the ferment-concentration is high; that represented by equation (ii) plays a predominant part when the ferment-concentration is low. We must therefore introduce into the combined equation some factor ϵ which will denote the proportionality between the two processes, and which will be dependent upon the mass of ferment. We thus obtain the equation:

$$\log_{\text{nat.}} \frac{a}{a-x} + \epsilon x = k_2 t; \qquad \text{(iii)}$$

introducing the modulus of the natural logarithms (0.4343) we obtain

$$\log_{10} \frac{a}{a-x} + 0.4343\, \epsilon x = 0.4343\, k_2 t,$$

which may be written

$$\log_{10} \frac{a}{a-x} + 0.4343\, \epsilon x = k_3 t. \qquad \text{(iv)}$$

The following are the values of k_3 calculated by Abderhalden and Michaelis from equation (iv) for the four series of determinations cited above:

1.45 units of dipeptid + 6 cc. of ferment solution $\epsilon = 0.7$ $100\, k_3 =$	1.45 units of dipeptid + 4 cc. of ferment solution $\epsilon = 1.5$ $100\, k_3 =$	1.45 units of dipeptid + 3 cc. of ferment solution $\epsilon = 3.0$ $100\, k_3 =$	1.45 units of dipeptid + 2 cc. of ferment solution $\epsilon = 10.0$ $100\, k_3 =$
(8.49)	5.74	6.20	11.1
6.63	6.52	6.54	11.9
5.49	5.90	6.56	11.2
5.52	5.51	6.22	11.3
5.68	5.25	6.41	11.5
5.54	5.91	6.40	11.7
5.63	5.91	6.72	11.1
6.04	6.15	6.53	11.1
5.55	6.17	6.17	(9.6)
.....	.....	6.64	.....
.....	.....	6.58	.....

With the exception of the two values enclosed in brackets, which are obviously influenced by experimental errors, the value of k_3 for each set of determinations is evidently, within the ex-

perimental error, constant. The value of ϵ, it is evident, rises with decreasing ferment-concentration, but not in direct proportion.

It is of great interest to observe that equation (iii) and (iv) is of the same form as that which Henri (13) (6) has found to hold good for the inversion of cane sugar by invertase. The theoretical foundation of Henri's equation, therefore, calls for consideration in this connection.*

Henri started from the point of view that the ferment, in the presence of a substrate which is undergoing digestion, may conceivably exist in three modifications, namely, in combination with the substrate (concentration = F_s), in combination with the products (concentration = F_p), and in the free condition (concentration = F_f). Calling the total concentration of ferment F, we obviously have:

$$F = F_s + F_p + F_f. \tag{v}$$

Assuming that in the formation of the ferment-substrate compound one molecule of ferment unites with one molecule of substrate we have, from the mass-law:

$$F_f\,(a - x) = \frac{1}{m} F_s, \tag{vi}$$

in which $\frac{1}{m}$ is the equilibrium-constant of the reaction.

Similarly assuming that in the formation of the ferment-products compound one molecule of ferment unites with one molecule of the hydrolysis-products we have:

$$F_f x = \frac{1}{n} F_p, \tag{vii}$$

in which $\frac{1}{n}$ is the equilibrium-constant of the reaction.

Combining equations (v), (vi) and (vii) we obtain:

$$F_f = \frac{F}{1 + m\,(a - x) + nx}, \tag{viii}$$

and

$$F_s = \frac{mF\,(a - x)}{1 + m\,(a - x) + nx}, \tag{ix}$$

* The derivation of Henri's equation which follows is, essentially, quoted from Taylor (19).

According to Henri, the velocity of hydrolysis might conceivably be directly proportional to the mass of the ferment-substrate compound ($= F_s$) or to the mass of the free ferment and that of the substrate. In the former case we obtain:

$$\frac{dx}{dt} = \frac{kmF\,(a - x)}{1 + m\,(a - x) + nx}, \tag{x}$$

in which k is the velocity-constant of the reaction. In the latter case we have:

$$\frac{dx}{dt} = \frac{kF\,(a - x)}{1 + m\,(a - x) + nx}. \tag{xi}$$

Obviously both equations are identical in form; the former when integrated, leads to the equation:

$$(1 + na) \log \frac{a}{a - x} + (m - n)\,x = k_3 t, \tag{xii}$$

in which k_3 is a constant which is directly proportional to the total concentration of ferment, and n and m are independent of the mass of ferment or substrate, but are obviously dependent upon the temperature and the conditions (reaction, etc.) of the experiment. Putting

$$\frac{m - n}{1 + na} = \epsilon,$$

we obviously regain the equation of Abderhalden and Michaelis. The condition that m and n should be independent of the mass of ferment, however, is obviously not fulfilled by Abderhalden and Michaelis' results, since ϵ varies notably with the concentration of the ferment; while k_3 does not approximate to direct proportionality to the mass of ferment.

In the derivation of Henri's equation it will be evident that many simplifying assumptions are made which are not justified by anything save the fact that they afford the simplest conception of the relations. Thus it is assumed that the *active* mass of the ferment in so far as hydrolysis is concerned is directly proportional to its actual mass, that *one* molecule of ferment reacts with *one* molecule of substrate, that *one* molecule of ferment reacts with *one* molecule of the products of hydrolysis, that the concentration of the ferment-substrate compound is

evanescent in comparison with that of the substrate and so forth. If we try to generalize Henri's equation by removing these simplifying assumptions we obtain equations which are, mathematically speaking, unmanageable. The surprising thing is, therefore, not that Henri's equation fails to adequately represent Abderhalden and Koelker's results, but that, if the conditions in the system are as complex as those which Henri depicts, *any* simple relation can be found which will express the progress of the reaction with any approach to fidelity.

Computing the average values of $100\,k_3$ calculated from the equation (iv) for the various ferment-concentrations employed by Abderhalden and Koelker we obtain:

cc. of ferment solution in 6 cc. of digest	$100\,k_3$	cc. of ferment solution in 6 cc. of digest	$100\,k_3$
2	11.6	4	6.01
3	6.45	6	5.76

from which it is evident that the velocity-constant of hydrolysis, when computed from Henri's equation, far from being directly proportional to the ferment-mass is actually greater the more dilute the ferment. It appears distinctly within the bounds of possibility that the intensity of the hydrolysing activity of the ferment is relatively greater the more dilute its solution. This, in turn, suggests the possibility that the ferment may exist both in an active and inactive form (active and inactive, that is, with respect to the acceleration of *hydrolysis*). The proportion of inactive ferment would, of course, depend, not only upon the total mass of ferment in the system, but upon the mass of uncombined active ferment. If this were the case then k and ϵ in Abderhalden and Michaelis' equation, accepting Henri's derivation of the equation, might be expected to be functions, not only of the total ferment mass and of the relation between the mass of free active ferment and that of the substrate and products respectively, but also of the relation between the proportion of ferment in the inactive condition and the mass of the uncombined active ferment (Cf. Chap. XVII).

On the other hand, it is possible that the coincidence between Abderhalden and Michaelis' equation and that of Henri is merely formal, that, as Abderhalden and Michaelis suggest, the true

equation expressing the course of hydrolysis is in reality equation (i), but that as hydrolysis proceeds the reaction is subject to an acceleration, possibly attributable, either directly or indirectly, to the products of the reaction.

Euler (9) has studied the hydrolysis of glycin by erepsin, obtained from the intestinal wall. The method employed was that of following the hydrolysis by measuring the progressive alteration of the electrical conductivity of the solution, direct proportionality between the alteration in conductivity and that of the dipeptid-concentration having previously been established. He finds that the velocity of hydrolysis is very intimately dependent upon the alkalinity of the solution, thus:

1/10 *N* GLYCYL–GLYCIN. 5 G. EREPSIN POWDER IN 100 CC.

Alkali-concentration *N*	0	0.04	0.05	0.075	0.10
Reaction-velocity constant × 1000	0.05	7.0	6.2	2.6	0.2

For a given initial NaOH, dipeptid and erepsin concentration the monomolecular formula represents the progress of this reaction with tolerable fidelity, as the following results show:

0.10 *N* GLYCYL GLYCIN. 5 GRAMS EREPSIN IN 100 CC.

0.04 *N* Na			0.05 *N* Na		
Minutes	1000 $(a - x)$	1000 k	Minutes	1000 $(a - x)$	1000 k
0	920	. . . . .	0	935	. . . . .
7	819	7.20	10	806	6.46
15	721	7.08	17	739	6.00
22	649	6.88	25	654	6.18
30	579	6.70	30	622	6.90

Within certain limits the reaction-velocity is independent of the initial concentration of this dipeptid, but this holds good only within certain limits of the proportion enzyme : substrate. If the enzyme-concentration is small, for a given Na-concentration the reaction-velocity rises with increasing concentration of glycyl-glycin. Euler attributes this fact to neutralization of injurious excess of NaOH by the additional glycyl-glycin. The progressive change in the reaction-constant as hydrolysis proceeds may doubtless be attributed to the fact that the substrate and products are possessed of different combining capacities for bases, so that the proportion of free base alters as the reaction proceeds.

Euler has arrived at the conclusion that in solutions of glycyl-glycin which contain a base, only the *salt* of the dipeptid undergoes hydrolysis. In terms of recent theories of catalysis (18) this may be held to indicate that the substance which actually undergoes hydrolysis is the dipeptid ion.

For high ferment-concentrations the velocity of the hydrolysis of glycyl-glycin is, according to Euler, directly proportional to the concentration of erepsin. If, however, the ferment concentration is low, then the value of k, calculated from the monomolecular formula, increases much more rapidly than the concentration of erepsin.

The influence of various added substances (salts, amino-acids, and so forth) upon the rate of hydrolysis of dipeptids by liver-extract has been studied by Abderhalden and Gigon (3). They find that dilute solutions of KCN accelerate while strong solutions greatly retard the hydrolysis of d-l-leucyl-glycin; sodium fluoride strongly retards the hydrolysis of d-l-leucyl-glycin and of glycyl-l-tyrosin; "physiological salt solution" has no effect upon the rate of hydrolysis. In high concentrations both magnesium chloride and sulphate depress the rate of hydrolysis; calcium chloride accelerates the hydrolysis and strontium chloride is indifferent, the substrates being d-l-leucyl-leucin and glycyl-l-tyrosin.

The effect of amino-acids upon the rate of hydrolysis is of surpassing interest since these are the products of the reaction, and if they exert an influence upon the rate of hydrolysis they will induce more or less marked deviations in the relation between the duration and the extent of hydrolysis from that which would be indicated by the monomolecular law or by Henri's modification of the monomolecular law, described above. Employing glycyl-l-tyrosin they find that:

The hydrolysis is accelerated by	The hydrolysis is unaffected by	The hydrolysis is slightly retarded by	The hydrolysis is strongly retarded by
l-alanin	glycocoll	d-l-alanin	d-alanin
.....	d-leucin	d-valin	d-l-valin
.....	.....	d-l-leucin	l-leucin
.....	.....	d-l-serin	l-serin
.....	.....	.....	l-tyrosin
.....	.....	d-isoserin	d-l-isoserin
.....	.....	.....	l-isoserin

In addition it was observed that d-glutamic acid, d-tryptophane, l-diamino-trioxydodecanic acid, d-l-aminobutyric acid and phenyl-alanin depress the rate of hydrolysis.

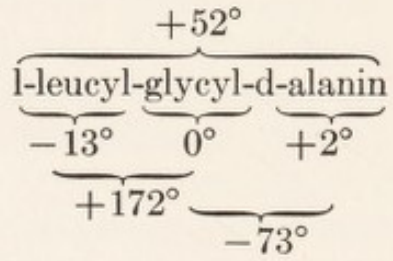
In every case it will be observed that the effects of optical antipodes are considerably different, in quantity or even, in the case of alanin, in sense, from one another and that the racemic body affects the hydrolysis in an intermediate manner. Of great significance is the fact that l-tyrosin, which is itself a product of the hydrolysis of glycyl-l-tyrosin, strongly retards the hydrolysis, although glycocoll, which is the other product, does not. Abderhalden and Gigon attribute this to a binding of the ferment by the tyrosin. The affinity between glycocoll and the ferment is slight and so this product does not retard hydrolysis so markedly. It is possible that the low degree of affinity between glycocoll and the majority of the proteolytic ferments is responsible for the difficultly digestible character of many of the peptids which are rich in glycocoll.

The influence of temperature upon the rate of hydrolysis of glycyl-l-tyrosin and d-l-leucyl-glycin by liver and pancreas-extracts has also been studied by Abderhalden, Caemmerer and Pincussohn (2). In each case, within certain limits, the rate of hydrolysis is very greatly accelerated by a rise in temperature. Very high temperatures, naturally, delay or prevent hydrolysis by destruction of the enzyme. For liver extract, working upon these substrates, the temperature-optimum proved to be about 55 degrees; for pancreas-extract between 45 and 50 degrees.

3. The Order in which Amino-acids are Split off from Polypeptids by Proteolytic Ferments. — The optical properties of certain polypeptids and of their possible decomposition-products have been utilized by Abderhalden and Koelker (4), Abderhalden and Brahm (1) and Abderhalden, Koelker and Medigreceanu (5) in the attempt to ascertain which point in a tri- or tetra-peptid is first attacked by a proteolytic ferment. Thus l-leucyl-glycyl-d-alanin has a molecular rotation * of +52 degrees. It might conceivably yield in the first place either l-leucyl-glycin (+172 degrees) and d-alanin (+2 degrees), or else glycyl-d-alanin (−73 degrees) and l-leucyl (−13 degrees), or else, through simultaneous splitting of both −NHOC− bonds, all three of the products

* The rotations given are 1/100 of the molecular rotations. Cf. Koelker (14).

l-leucyl (−13 degrees), glycin (0 degrees) and d-alanin (+2 degrees). If the first link to be broken, therefore, is that between the glycyl and alanyl groups the positive rotation of the original solution should increase until it reaches a maximum. If this link were the only one attacked then this maximum rotation should be that of a mixture of l-leucyl-glycin and d-alanin, i.e., over three times that of the original solution. If the first link to be broken were that between the leucyl- and the glycyl-groups the original positive rotation should decrease and finally become negative. If the two links were split simultaneously the positive rotations should decrease until it became very slightly negative. These possibilities are made clear by the following diagram:



The experimental result, employing trypsin, is that the rotation at first increases to an extent of about 40 per cent. Hence l-leucyl-glycin must be liberated and the point of first attack (or, at least, most rapid attack) must be the bond between the glycyl and the alanyl groups. After attaining a maximum positive rotation only 40 per cent in excess of that of the original solution, however, the rotation declines again, owing to the hydrolysis of the l-leucyl-glycin. Hence the enzyme does not complete the decomposition of the one bond before attacking the other. In other words the two reactions proceed side by side but at different velocities, that of the splitting of the glycyl-alanin bond being the most rapid. The leucyl-glycin bond, however, does not appear to be attacked at all while it is bound up in the tripeptid molecule, there being no evidence of the formation of glycyl-d-alanin. The progress of the reaction is therefore, it would appear, the following, first the glycyl-alanin link is broken and l-leucyl-glycin and alanin are split off. The velocity of this reaction is so much greater than that of the other possible reactions (splitting of the leucyl-glycyl link) that the latter reaction does not occur to any appreciable extent. The l-leucyl-glycin which is thus formed is immediately attacked by

the ferment. The substrate of this second reaction being, however, a product of the first, its velocity is at first low, since the substrate concentration is low; but as velocity of the first reaction declines, owing to the consumption of the substrate, that of the second reaction increases, owing to the increase in the mass of its substrate.

When however, instead of trypsin, yeast endotryptase is employed as the ferment, not the glycyl-alanin bond but the leucyl-glycyl bond is first attacked. There is thus no question but that the mode of action of yeast endotryptase is quite different from that of trypsin and a means of sharply distinguishing between different enzymes is clearly indicated. It will also be evident that in digests containing a mixture of proteolytic enyzmes, such as occurs in tissue-extracts, etc., the conditions of hydrolysis must be much more complex than they are in digests containing only a single enzyme; since under such circumstances two or more parallel reactions may be occurring, possessed of different specific velocities, at each step in the progressive hydrolysis of of the peptid or protein.

Glycyl-d-alanyl-glycin is first attacked by trypsin at the glycyl-d-alanyl junction, by yeast endotryptase at the alanyl-glycin junction; d-alanyl-glycyl-glycyl-glycin is first attacked by trypsin at the glycyl-glycin junction.

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CHAPTER XVI

THE HYDROLYSIS OF THE PROTEINS

1. The Proteolytic Enzymes as Catalysors. — The fact that proteins, in the presence of water, can be hydrolysed to amino-acids by prolonged heating, e.g., by superheated steam (126) (80) (81) (76) (69) (70) (87) (115) without the addition to the system of any acid, alkali or ferment, indicates that the process of hydrolysis is occurring, although slowly, at all temperatures and in the absence of catalysors other than, possibly, the hydrogen or hydroxyl ions of water itself. The fact that, at ordinary temperatures, catalysors can bring about the hydrolysis of proteins shows that even at these temperatures the proteins are not in equilibrium with their products. The influence of rising temperature upon a chemical reaction is always twofold; it shifts the station of equilibrium in one sense or in the opposite and, always, it accelerates the reaction to a greater or less degree (i.e., magnifies the velocity-constant). The action of heat upon proteins must be in all cases to shift the station of equilibrium in the direction of polymerization (i.e., condensation) since the reaction of hydrolysis is faintly exothermic, but the fact that fairly complete hydrolysis occurs at temperatures above 100 degrees shows that the shift in the equilibrium between *the lower protein complexes and the amino-acids which are the products of their hydrolysis* is not so great as to extinguish the reaction of hydrolysis. The effect of high temperatures in accelerating the auto-hydrolysis of proteins is to be looked upon, therefore, as that of rendering readily detectable, through acceleration, a reaction which occurs, although slowly, at all temperatures (133). It is possible to demonstrate directly, however, and without appeal to inference, that the hydrolysis of proteins in neutral watery solutions does occur at normal temperatures; the velocity of hydrolysis is, however, usually very low under these conditions. Taylor has shown that an appreciable proportion of pure sterile globulin kept in distilled water at ordinary temperatures for 18 months is hydrolysed to proteoses; he has also found that

leucin may be recovered from a sterile suspension of casein in pure water and that arginin may be recovered from a solution of protamin sulphate in pure water, both after the lapse of a year or more (133); other examples of the slow autohydrolysis of proteins in pure water might be adduced (133) (131). In a few cases, however, the autohydrolysis occurs at a readily measureable velocity. I have found (110) that the velocity-constant of the hydrolysis of casein in milk at 36 degrees (in the presence of excess of toluol) is 0.000546, common logarithms being employed and the time expressed in hours (using the monomolecular formula, Cf. previous chapter equation (i)). For a 2.8 per cent solution of casein in NaOH, carefully neutralized to litmus and therefore containing H^+ and OH' ions in the concentrations in which they exist in pure water, the velocity-constant at 36 degrees, similarly estimated, proved to be 0.000518. In the first experiment the extent of hydrolysis was estimated by determining the residue of undigested casein after 32 days, in the second experiment the undigested residue was determined after 20 days. Now in the second experiment, at all events, no ferments were present and in both experiments the solutions were almost exactly neutral. We must therefore regard the observed hydrolysis as being not due to catalysors but to the action of the solvent water itself or of its ions.* Expressed in numerical terms, the above-cited results mean that in absolutely neutral solution, in the absence of any proteolytic enzymes, one-half of a caseinate of sodium or calcium is hydrolysed in about 24 days.

The hydrolysis of neutral caseinates by trypsin or pepsin affords, therefore, an unusually favorable example, among protein reactions, of the action of an enzyme in accelerating an already progressing reaction.

* It might be inferred from these results that normal milk contains no proteolytic enzymes. This inference would not be altogether a safe one, however, since the milk which was employed was obtained in the open market and there is reason to suspect that it had been manipulated in a manner which, it is possible, may have destroyed pre-existing enzymes. No difficulty was encountered in keeping this milk sterile, throughout the course of the experiments, by the simple addition of toluol. When milk obtained from the University Experimental Farm was employed, however, which had not been pasteurized, it was found impossible to keep the milk sterile, for the length of time required, without employing, as sterilizing agents, substances which might conceivably destroy or injure proteolytic enzymes.

The autohydrolysis of the caseinates in neutral and faintly alkaline solutions has been more extensively studied by E. H. Walters (147) who has shown that the neutral caseinates of lithium, sodium and potassium in sterile solution undergo comparatively rapid autohydrolysis, approximately five per cent of the protein being hydrolysed in ninety-six hours at 37.5° C. The "basic" caseinates (neutral to phenolphthalein) of the same bases undergo autohydrolysis at a slightly higher velocity. The velocity of the autohydrolysis of the "basic" caseinates of calcium and barium is about three times as great as that of the autohydrolysis of the caseinates of lithium, sodium or potassium, indicating very clearly that some factor other than the H^+ or OH' ions plays a part in determining the velocity of the autohydrolysis.

The proteolytic enzymes have long been regarded, by the majority of investigators * as true catalysors; but the evidence which has been brought forward in support of or in opposition to this view has varied very much in character from time to time. With the rapid gain in exact knowledge in the field of physical chemistry which characterized the scientific advance in the latter part of the last century, the characteristic features of many catalytic processes became very thoroughly known and certain catalytic reactions came to be regarded as "typical" for no better reason than that they were the best studied and therefore the best known. Now these so-called "typical" instances of catalysis had in the first place attracted the attention of investigators simply because of the marked peculiarities which they exhibited and which appeared to differentiate them sharply from other chemical reactions, and thus, by a not unnatural process of circular reasoning these peculiarities came to be regarded as "typical" and diagnostic of "true" catalysis. Our conceptions and definitions of "catalysis" have therefore altered and enlarged as the number of "typical" instances has grown, and, correspondingly, features in the mode of action of ferments have been brought forward, at one time in support of the thesis that the ferments are not true catalysors, and at another time in support of the exact converse of this view. Unquestionably attempts have been made by many authors to shape the phenomena of fermentation into accord with prematurely rigid

* Starting with Berzelius and Liebig. Cf. Jacob Berzelius (8).

conceptions of catalysis based upon insufficiently extended investigation.

A phenomenon which at a very early date greatly impressed the investigators in this field was the extraordinarily small quantity of a catalytic agent which, in "typical" instances of catalysis, was found sufficient to bring about the chemical alteration of enormous quantities of material. Thus $\frac{4}{10}$ of a milligram of colloidal platinum will bring about the combination of the hydrogen and oxygen in no less than 10 litres of gas, *without the least reduction in its efficiency as a catalysor* (25); 0.000001 grams of potassium permanganate in 10 cc. of solution notably accelerates the reduction of mercuric chloride by oxalic acid (64); the rate of oxidation of an aqueous solution of sodium sulphite is perceptibly accelerated by the presence of 0.0000000000001 N $CuSO_4$, or even by merely dipping a strip of clean metallic copper in the water for less than a minute (136). Were any appreciable proportion of the catalyst used up during the process of catalysis it is obvious that quantities so minute as these would be incapable of bringing about the conversion of such enormous quantities of material, and, in fact, we find in many cases, even when relatively large quantities of catalysor are employed, the catalysor is not appreciably used up during the progress of the reaction; thus a solution of cane sugar contains the same amount of acid after hydrolysis as it did before (19) (67). But if the catalysor is unaltered at the end of the reaction which it accelerates *then it must have accomplished this acceleration without adding any energy to or subtracting any energy from the reacting system*, in other words it cannot in any way have affected the final equilibrium of the system, but only hastened the attainment of that equilibrium, since any shift in equilibrium must in general be accompanied either by an expenditure or an absorption of energy. This theoretical deduction has been confirmed in a great variety of instances in the field of non-fermentative catalysis*; in the field of fermentations data bearing upon this deduction are either lacking or fail to establish its validity (27) (14).

If, however, the station of equilibrium in any reaction is unaffected by the presence of a given catalysor, then that catalysor

* Cf. especially Turbuba (137) (138), who has shown that the equilibrium between aldehyde and paraldehyde is the same whether sulphur dioxide, zinc sulphate, hydrochloric acid, oxalic acid or phosphoric acid is used as catalysor.

must accelerate, and accelerate equally, the attainment of that equilibrium from either side; it must in other words accelerate the forward and the reverse reactions equally. This is readily seen when we reflect that equilibrium in the reacting system is attained when the velocity of the forward is equal to that of the reverse reaction. If the velocity of the forward reaction is increased by any agency, therefore, and the station of equilibrium is unaffected, the velocity of the reverse reaction must also have been increased and in the same proportion. The correctness of this theoretical deduction has also been established in a number of "typical" instances of non-fermentative catalyses, but although it has been shown that yeast (58) (59), kephirlactase (28), diastase (22), emulsin (24), lipase (7) (65) (55) (9) (130) (132) (98) (99), trypsin (135) and pepsin (108) (109) (111) (112) (35) will not only accelerate the hydrolysis of maltose, lactose, glycogen, amygdalin, fats, protamin and paranuclein, respectively, but also the synthesis of these substances from the products of their hydrolysis, yet it has in no instance been shown that the velocity of the reverse reaction is accelerated in the same proportion as that of the forward reaction; indeed such evidence as exists tends to show that this is not the case (14) (111).

From the study of a limited number of instances of non-fermentative catalysors, however, it appeared, as I have said, that the catalysors are substances which remain unchanged at the end of the reaction which they accelerate and which do not shift the station of equilibrium and therefore cannot initiate, but only accelerate reactions (89) (90) (91) (92) (93).

Recent investigations, particularly those of Stieglitz (127) (128) and Euler (27), have shown that the above definition of catalysis is too rigid and that the cases which it adequately covers are merely extreme instances of a much more general phenomenon.

It has long been known that in many instances of unquestioned catalysis the acceleration of the reaction in question is accomplished, or at least accompanied by, the formation of intermediate compounds between the substrate and the catalysor. The classic illustration of a catalysed reaction of this type is that afforded by the "continuous etherification process" for the production of ether from alcohol. In this process sulphuric acid is employed as catalysor and, as is well known, the sulphuric

acid first combines with the alcohol, forming a substance which can be isolated, namely ethyl-sulphuric acid — and this compound reacts with another molecule of alcohol, forming ether and regenerating the sulphuric acid, in accordance with the equations

$$C_2H_5OH + \begin{matrix} HO \\ HO \end{matrix}\!\!\diagdown\!\!\diagup SO_2 = H.OH + \begin{matrix} C_2H_5O \\ HO \end{matrix}\!\!\diagdown\!\!\diagup SO_2$$

$$\begin{matrix} C_2H_5O \\ HO \end{matrix}\!\!\diagdown\!\!\diagup SO_2 + C_2H_5OH = \begin{matrix} HO \\ HO \end{matrix}\!\!\diagdown\!\!\diagup SO_2 + C_2H_5{-}O{-}C_2H_5$$

and, at the end of the reaction, the sulphuric acid molecule which is set free is unaltered and can carry another molecule of alcohol through the same series of transformations. Hence, as in the "typical" cases of catalysis cited above, the only things which prevent the reaction from proceeding indefinitely, with the conversion of an indefinite quantity of alcohol through the agency of a limited quantity of sulphuric acid, are the failure of the supply of alcohol, or the continued accumulation of the products; thus, if the water which is formed in the above reaction be allowed to remain in the system the sulphuric acid becomes, finally, so highly diluted that the velocity of the transformation sinks to a negligible magnitude, i.e., for practical purposes, the reaction ceases.

Another example of the same kind is the use of nitric oxide as a "carrier" of oxygen in the manufacture of sulphuric acid. The first reaction is the combination of NO with O to form nitrogen peroxide:

$$2\,NO + O_2 = 2\,NO_2.$$

This is brought into contact with SO_2 and steam, sulphuric acid is formed and nitric oxide regenerated:

$$SO_2 + H_2O + NO_2 = H_2SO_4 + NO$$

and the NO is now free again to "carry" oxygen into the sulphurous, converting it into sulphuric acid.

In a large number of instances in which the intermediate reactions have not been defined and their occurrence established, it has nevertheless been shown that the catalysor forms compounds with the substrate or with modifications of the substrate,

of such a character as to strongly suggest that they play an important part in determining the course of the reaction. Thus in the presence of aluminium chloride, sulphuryl chloride and sulphur chloride react thus:

$$SO_2Cl_2 + S_2Cl_2 = SO_2 + 2\,SCl_2$$

and it appears that the aluminium chloride aids the reaction through the formation of double compounds with the various molecular species which participate in the reaction, for many such compounds are known, for example $Al_2Cl_2 \cdot 2\,SO_2$, $AlCl_3SCl_4$, and so forth (20).

A very decisive series of instances of catalysis through the formation of intermediate compounds has been established by the painstaking and extensive researches of Stieglitz (1) (127) (128). In the presence of acids the iminoesters (in aqueous solution) are decomposed into ammonia and the corresponding, sparingly ionized organic acid. It has been shown by Stieglitz, with the utmost quantitative precision, that the catalytic action of the mineral acid, in this reaction, is accomplished through the formation of salts of the catalysing acid with the ester, since the molecular species which actually undergoes hydrolysis in this system is the ester-ion *and the salts of the ester are much more completely electrolytically dissociated than the ester itself, so that the active mass of the substrate is increased by the presence of the catalysor*. Similarly, in the catalytic decomposition of methyl acetate by acids an oxonium salt of the ester with the acid is formed and it is the positive ion of this salt which undergoes hydrolysis. Now this latter is a "typical" instance of catalysis. The catalytic agent is not used up during the reaction, it can be recovered from the system unaltered when the reaction is complete, and the equilibrium of the reaction is not measurably disturbed by the presence of the catalysor. Nevertheless, in a comprehensive mathematical analysis of the conditions which must actually obtain in such a system, Stieglitz has shown that the point of equilibrium must be shifted by the catalysor, albeit to an immeasurably slight degree, and that *in the specific instance under consideration* the equilibrium is only inappreciably shifted simply because the quantity of the compound which is formed at any moment, between the catalysor and the substrate, is evanescently small, since these salts are subject to very extensive

hydrolytic dissociation. When the salt formation is at any instant extensive then, as Stieglitz points out, although in every respect the mechanism of the acceleration of the hydrolysis by the catalysing acid is the same, yet a definite shift in equilibrium results from the presence of the catalysor and, concurrently, the catalysor is in some measure used up during the progress of the reaction and some energy has to be expended in order to recover it, unaltered, from the system in its final condition of equilibrium. I quote from Stieglitz' article:*

"In accordance with the results, our views concerning catalytic action must be modified in regard to all three of the commonly assumed fundamental characteristics of catalytic action, (1) that the acceleration must be proportionate to the concentration of the catalytic agent present; (2) that the agent must not appear to combine with any of the substances undergoing change; and (3) that the ultimate condition of equilibrium must not be measurably modified by the presence of a catalysor. These characteristics are practically true only for limiting cases where the amount of salt-formation is so small as to be beyond the scope of our measurements. None of them is absolutely true under any conditions. When the amount of salt-formation becomes measurable, as for iminoesters, they need not hold even approximately, and *still the fundamental mechanism and mode of the catalysis is the same in these cases* as in the others. The one vital fact, then, of an acceleration due to an increase in the active mass or concentration of a reacting component in a catalytic action is the only fundamental fact common to all catalytic actions."

With this enlarged conception of catalysis in mind we need no longer have scruples in regarding the fermentative reactions as instances of true catalysis. They exhibit the essential phenomenon of *acceleration* through the presence of a chemical agency which is added to the system, in other words the ferment. Our task is therefore, not to compare the fermentative reactions with a special and arbitrarily chosen class of catalytic reactions but, as in all instances of catalysis, to attempt to unravel the *chemical mechanism* of the observed acceleration.

In a system so complex as that afforded by a protein mixed with an enzyme in aqueous solution several possibilities present

* Julius Stieglitz (127). Cf. also Hans Euler (26).

themselves, which are too frequently overlooked, and which doubtless play a part in determining the complexity of the phenomena which are experimentally observed.

In the first place, we have seen that certain of the proteins, or protein salts, undergo hydrolysis at a measureable rate *in the absence of proteolytic enzymes.* Now, from the principle of the mutual independence of different reactions (21) (53) (82) it follows that when the proteins are acted upon by proteolytic enzymes both the catalysed and the uncatalysed reactions must be proceeding side by side, albeit the latter, possibly, at a reduced velocity.* Hence if the catalysed reaction is not overwhelmingly more rapid than the uncatalysed reaction the progress of the latter must disturb the time- and mass-relations of the former. The velocity-constant which is actually measured will be the sum of the constants for the catalysed and the uncatalysed reactions; only the former constant will bear a specific relation to the mass of the catalysor; the *sum* of the constants, which would be the quantity actually measured, would not, therefore bear this relation to the mass of the catalysor, but exhibit departures from it of greater or less magnitude according to the magnitude of the constant for the uncatalysed reaction.

In the second place, we have seen, in discussing the hydrolysis of the polypeptids, that a tripeptid may undergo hydrolysis by splitting at *either* linkage, and that the hydrolysis due to the splitting of the one linkage may be accelerated by one proteolytic enzyme, and that due to the splitting of the other linkage by another enzyme. In so complex a peptid as a protein many linkages vulnerable to the enzyme under consideration must exist and it is entirely within the bounds of probability that any given enzyme shares its attack between two or more vulnerable linkages and that, consequently, what we directly observe is not the progress of one definite reaction, or even a catenary series of reactions, but a number of parallel chains of successive hydrolyses. On the other hand, we know that the vulnerability of the various linkages in the protein molecule to various proteoclastic enzymes, differs so that one group of linkages will be preferentially attacked by one enzyme, and another group by yet another enzyme, while no enzyme or group of enzymes will open all of the

* Owing to reduction of the active mass of the free protein through the formation of a protein-ferment compound.

—COHN— linkages which are susceptible to hydrolysis by acids (29) (114) (56) (2). Hence if the enzyme-preparation which we employ is impure, i.e., contains an admixture of proteolytic enzymes, then the number of possible parallel reactions and number of simultaneous points of attack in the protein molecule must be greatly enhanced. It is probably to this fact that we must in part attribute the much greater activity of organ-extracts and of organs and tissues *in situ* than of isolated ferments in bringing about the hydrolysis of proteins (133).

In the third place if the proteolytic enzymes are proteins or peptids, as in many cases seems highly probable, they must be subject to hydration and dehydration, just as the proteins are, and in any case we know that they are thermolabile and subject in aqueous solutions to loss of activity, which is usually attributed to hydrolysis, and to modifications leading to precipitation following dehydration by concentrated inorganic salts. The possibility is thus indicated that the proteolytic ferments may exist in two or more modifications, nor can we assume that only one of these modifications is capable of reacting with the substrate, or its products, and influencing the velocity with which they undergo modification. The activity of the ferment in accelerating either hydrolysis or synthesis of protein may therefore vary with the conditions under which it acts and *among these conditions must be reckoned both the relative and the absolute concentrations of the substrate and products of the reaction*, since they must modify, through combination with them, the equilibrium between any different forms of the enzyme which may exist in the system. In quite general terms, however, and without assuming the existence of more than one form of the ferment, it may be stated that if the ferment enters, at any stage of the reaction of protein-hydrolysis, into combination with the substrate to any appreciable extent (and we shall see that in many instances it does), since the final equilibrium of the reaction must therefore be affected by the ferment, the ferment must to some extent be used up in the hydrolysis *and the activity of the ferment must vary as the reaction proceeds.*

2. The Evidence for the Existence of Intermediate Compounds between the Proteolytic Ferments and their Substrates. — The existence of compounds between proteins and proteolytic enzymes has been established in a number of cases by a variety

of observers. Thus Vernon (145) has shown that serum albumin, paraglobulin and particularly egg-albumin, when added to a tryptic digest, markedly delay the hydrolysis of another protein by the trypsin. Obviously, if the enzyme did not enter into combination with its substrates, the rate at which it attacks one substrate should not be influenced by the presence of another. Hedin (46) has shown that the power of egg-albumin to delay the hydrolysis of another protein by trypsin is much greater if the egg-albumin and the trypsin are mixed together *before* the trypsin is added to the digest than *after*. Evidently the egg-albumin-trypsin compound is but slowly reversible; when none of the trypsin is bound by another substrate the active mass of free trypsin is greater and the proportion bound by albumin is therefore greater. Once the trypsin is bound by albumin it is only very slowly abstracted from the combination by another substrate.*

Dauwe (23) has shown that trypsin can be extracted from its solution by coagulated egg-white or by fibrin, and the ferment can be regained from the compound by prolonged washing with water. From what has been said in Chap. V, section **3,** it will be clearly realized that this latter fact does not in the least militate against the view that the combination between the ferment and the substrate is *chemical* in character.

A very striking instance of the way in which the formation of compounds of this type may interfere with the time- and mass-relations in the main hydrolysis under observation has been discovered by Hedin (49). When trypsin acts upon casein, the time required for the attainment of a given degree of hydrolysis ($= t$) is inversely proportional to the concentration of ferment ($= p$). In other words pt = constant. But, if egg-albumin be present, a proportion of the trypsin is bound by the albumin *and this proportion is greater the more dilute the ferment,* so that the quantity of ferment which is free to act upon the trypsin is no longer proportionate to its concentration and the law no longer holds good. From this it is clear that if we wish to obtain readily interpretable time- and mass-relations in a protein digest we must employ only *pure proteins.* We have seen, also, that we

* Hedin has also shown that a greater proportion of trypsin is bound at higher than at lower temperatures. But if excess of trypsin is attached to the albumin by temporarily raising the temperature, on lowering the temperature the trypsin is not given up again.

must employ only a *single enzyme.* Anyone who possesses an extensive knowledge of the literature on protein hydrolysis by enzymes will readily admit that these conditions have very rarely been realized.

3. The Kinetics of Protein Hydrolysis by Enzymes. — In several instances it has been found that very small quantities indeed of proteolytic enzyme will suffice to convert large quantities of protein; thus one part of rennet will curdle from 400,000 to 800,000 parts of milk (42) and a pepsin powder has been prepared which in seven hours dissolved 500,000 times its weight of fibrin. From these facts it has been urged by many observers that the fermentative splitting of proteins is an instance of "typical" catalysis, in the limited sense of the term described in section **1**. The justification for this view is totally inadequate, for it has not been shown that at the end of extensive hydrolysis the enzyme can be recovered from the digest, wholly unaltered in efficiency, without the expenditure or absorption of any energy. In fact, in many cases it is known that the activity of the enzyme is impaired during the hydrolysis. Since this impairment of activity is even more pronounced when proteins are not present, it is usually conceded that autohydrolysis of the enzyme itself suffices to account for the whole of this effect; this is clearly not necessarily the case; while autohydrolysis of the enzyme renders it very difficult to ascertain whether the enzyme is actually exhausted by the hydrolysis or not, yet it does not exclude the possibility that such exhaustion occurs. Now it must be constantly borne in mind that the proteins are very nearly thermoneutral substances; their hydrolysis (Cf. section **1**) is accompanied by the disengagement of heat, it is true, but this disengagement of heat is very minute. Hence the energy change which is involved in a shift of the equilibrium between protein and the products of its hydrolysis must be very minute and a shift in the equilibrium of a correspondingly minute quantity of a less thermoneutral substance might very well suffice to provide or absorb the requisite energy to bring about a very decided shift in the equilibrium between protein and its products. We cannot assume, therefore, from the smallness of the amount of enzyme which will transform large quantities of protein, that the enzyme does not exert any influence upon the equilibrium which the protein finally attains with its products.

If we assume, however, (i) that the ferment does not enter appreciably into combination with the substrate or products and hence does not shift the equilibrium between the protein and its products, (ii) that the products of the reaction do not appreciably depress the velocity of hydrolysis, and (iii) that the *reaction* of the digest either does not affect the velocity of hydrolysis or is unaltered by its progress, then *for a certain period of the hydrolysis*, we may regard the transformation as concerning, on the whole, only one molecular species, and the equation

$$\log \frac{a}{a-x} = kt \qquad \text{(i)}$$

should hold good.

It will be well, before proceeding further, to clearly understand what is implied in the statement that this equation will hold good *for a certain period of the hydrolysis*. When the first −COH.N− bond in the protein molecule splits (considering, for the present, only one of the possible parallel reactions, since, in a system of parallel reactions of the same order, the reaction-constants of the different reactions simply add themselves together to produce the gross resultant) a product p, is the result. This product, however, now becomes the substrate for a second reaction involving the splitting of another −COH.N− bond and the production of a second substance p_2. Two possibilities now exist. The transformation $p_1 \rightarrow p_2$ may be specifically more rapid than the transformation $p \rightarrow p_1$ or it may be specifically less rapid. In either case it will initially be in an absolute sense less rapid than the primary reaction since its substrate-concentration is initially zero and, consequently, the absolute velocity of the second reaction will rise until it attains a maximum value, and the combined reactions will then proceed with the velocity and time-relations of the specifically slowest reaction. If this should chance to be the primary reaction then equation (i) will hold throughout; if it should chance to be the secondary reaction then equation (i) will hold *after the secondary reaction has attained its maximum velocity* but not before.

The meaning and derivation of equation (i) will be clear when it is recollected that the reaction of protein hydrolysis consists in the addition of a molecule of water to a −COH.N− bond in the protein molecule. In dilute solutions the active mass of

water will not be appreciably affected by the inclusion of a minute proportion of the water in the products of hydrolysis. Only one molecular species is appreciably changing in concentration, therefore, namely the protein. The number of molecular collisions per second between the water and the protein molecules will therefore be proportionate, at any instant, to the concentration of unaltered protein at that instant. If the initial concentration is a and the amount hydrolysed after time t is x, the velocity of hydrolysis $\left(\frac{dx}{dt}\right)$ is given by $\frac{dx}{dt} = k\,(a - x)$, in which k is the velocity-constant (*specific* velocity, or velocity per unit concentration) of the reaction. Integrating this, and recollecting that when $t = 0$, $x = 0$ we obtain equation (i).

The simplifying assumptions which we have been compelled to make in deriving this formula are very numerous and very many of them devoid of either theoretical or experimental justification. Hence it is not surprising that it has not often been found to hold good by those who have followed the time-relations of protein hydrolysis.

Victor Henri and Larguier des Bancels (54) studied the digestion of gelatin and casein by trypsin, following the hydrolysis by observing the increase in the conductivity of the digest, under the assumption that each molecule of hydrolysed protein contributes equally to the observed increase in conductivity. They found that the values of

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

were tolerably constant for brief periods of digestion and for varying values of a (= initial substrate-concentration), in none of their experiments, however, did they approach the stage of complete digestion, and Arrhenius (3) has shown that their results are equally well expressed by the formula:

$$x = k_1 \sqrt{t} \qquad \text{(ii)}$$

which is the well-known "rule of Schütz" to the effect that the quantity of protein which is digested by a given quantity of proteolytic ferment is proportional to the square root of the period occupied in digestion.

Bayliss, also employing the conductivity method (5), studied the hydrolysis of casein by trypsin. He found that the constant,

calculated from the monomolecular formula, falls off rather rapidly as digestion proceeds. The decrease in the value of the constant is not attributable wholly to autodestruction of the ferment. Arrhenius has, moreover, shown that Bayliss' results are also in satisfactory accord with the rule of Schütz.

Taylor (133), studying the hydrolysis of protamin sulphate by trypsin, found a tolerable accord with the monomolecular law:

Substrate 0.150. Vol. 100. Ferment 0.001. Temp. 34 degrees.

time	= 15	30	45	60	75	90	105	150	
$k \times 10^4$	68	69	69	76	66	64	62	60	Average = 67

Substrate 0.100. Vol. 100. Ferment 0.001. Temp. 34 degrees.

time	= 15	30	45	60	75	90	105	150	
$k \times 10^4$	79	84	80	76	73	70	64	68	Average = 74

Substrate 0.0075. Vol. 100. Ferment 0.001. Temp. 34 degrees.

time	= 15	30	45	60	75	90	105	150	
$k \times 10^4$	90	97	92	88	83	88	79	74	Average = 86

but it will be observed that the constant rises in value as the substrate-concentration decreases. Therefore, as Taylor points out, in a physical sense the direct proportionality between the velocity of hydrolysis and the concentration of unhydrolysed protein "is only spurious, since it holds but for each particular system."

On the other hand, Samojlov (116), Walter (148), Sjöquist (125), Schütz and Huppert (124), Gross (41) and Meyer (79), working with pepsin and employing a variety of substrates, have concurred in finding that for a considerable period of digestion the Schütz rule holds good; Borissov (11), employing trypsin, has also confirmed the Schütz rule and, as we have seen, Arrhenius has shown that the results of Bayliss and of Henri and des Bancels are in accord with this rule. Weis (150) working with the enzyme contained in malt-extract, with wheat-protein as substrate, has also confirmed the Schütz rule, although in very dilute solutions of ferment the exponent of the time tended to rise and approach unity, i.e., the relation tends to become

$$x = k_3 t. \qquad \text{(iii)}$$

Arrhenius (loc. cit.) has shown, from the results of Weis, that the quantity of protein hydrolysed by a given quantity of

ferment in a given time is directly proportional to the initial concentration of the substrate, i.e., the true form of equation (ii) is

$$\frac{x}{a} = k_2 \sqrt{t},$$

a result which had previously been established by Schütz and Huppert (loc. cit.)

Arrhenius (loc. cit., p. 64) has pointed out that the Schütz rule may be derived from the monomolecular formula provided we assume that the catalysor in the system enters into combination with and so is inactivated by the products of the reaction. This derivation is, however, only valid for the early stages of hydrolysis, during which x is small in comparison with a.

Regarding the dependence of the velocity of hydrolysis upon the ferment-concentration, Taylor (loc. cit.) finds that the time required to hydrolyse a given quantity of protamin sulphate to a given degree is inversely proportional to the quantity of ferment present. I have found (107) that the velocity-constant of the hydrolysis of calcium and barium caseinate in dilute solutions, also calculated from the monomolecular formula, is directly proportional to the concentration of trypsin, but at higher substrate concentrations ($N/400$ $Ca(OH)_2$ neutralized by casein) the ratio k/F increases with increasing ferment-concentration. Hedin (47) (48) (49) has studied the hydrolysis of casein by trypsin very carefully from this standpoint and finds that the relation

$$Ft = \text{constant}, \qquad \text{(iv)}$$

where F is the mass of ferment and t the time required to attain a certain degree of hydrolysis, holds good over a wide range of ferment-concentrations.

Employing the method of observing the rate of solution of coagulated protein enclosed in capillary tubes Schütz found that the rate of digestion is proportional to the square root of the ferment(pepsin)-concentration. Taylor (loc. cit.) and Arrhenius (loc. cit.) have, however, pointed out that if this method of measurement be employed, processes of diffusion and solution are liable to be confused with the process of hydrolysis. From the results of Hedin, however, which are cited above, since Ft = constant and the rule of Schütz $(x = k\sqrt{t})$ holds good in a large number of instances of protein hydrolysis, it

would appear that in these instances, at least, the extent of hydrolysis after a given time must be proportional to the square root of the ferment-concentration (41).

The influence of the formation of compounds between the substrate and the enzyme upon the kinetics of hydrolysis is very clearly revealed by the experiments of Bogdándy (10) who has shown that when a large excess of substrate is present the velocity of protein hydrolysis by pepsin depends only upon the mass of the ferment, while, when a large excess of ferment is present, it depends only upon the mass of the substrate. This is obviously what we should expect to be the case were the actual substance undergoing hydrolysis a compound of the substrate and the enzyme.

The hydrolysis of caseinates by trypsin has been exhaustively investigated by Walters (146) who has shown that the relation between the time of hydrolysis and the amount of sodium caseinate hydrolysed is, for all stages of the reaction, almost exactly what would be expected from the monomolecular formula. Moreover the velocity of hydrolysis is directly proportional to the concentration of trypsin and there is a less exact proportionality between the initial concentration of the substrate and the velocity of hydrolysis, the velocity-constant decreasing slightly as the concentration of the substrate increases. The nature of the base combined with the casein has little or no influence upon the process of hydrolysis by trypsin (although it has a decided influence upon the velocity of *autohydrolysis* (147)) from which we may infer, since caseinates of the alkalies and of the alkaline earths are hydrolysed with equal velocity, that the degree of electrolytic dissociation of the caseinate has little or no effect upon the velocity of its hydrolysis by trypsin.

We thus see that under different conditions of enzyme- and substrate-concentration and with different substrates very different laws are found to express the relationship between time and the extent of hydrolysis of the proteins and polypeptids. None of these relations holds good outside certain definite limits of substrate- and enzyme-concentration and each of them only holds good for a limited portion of the hydrolysis. We are inclined to suspect that each of these relations is but part of some more general relation which, it is possible, holds good for all of the cases cited above and for all stages of the hydrolysis.

The task of seeking for such a relation, when we recall all of the factors which may very possibly enter into it, might well seem, for purposes of practical utility, well-nigh hopeless, were it not for the fact that the limiting cases of this general law have already been ascertained to hold good over quite extensive and practically attainable ranges of time and concentration. Evidently the number of the factors which play an *appreciable* part in determining the phenomena observed is not so great as we might be inclined to anticipate.

We have seen that Henri's law does not suffice; it is not sufficiently general to cover all of the time- and mass-relations in the hydrolysis of the polypeptids (Cf. the previous chapter, section **2**); we are led to suspect that some important factor has been omitted in the derivation of Henri's equation.

In the following chapter we shall see that pepsin is capable of exerting synthetic activity under conditions such that its hydrolytic activity is absent. We have already had occasion to indicate that the proteolytic ferments may exist in more than one modification. In the light of these facts we shall make the following assumptions regarding the mode of action of the proteolytic ferments upon the proteins.

Let us suppose that the proteolytic ferments act as "carriers" of water into the protein molecule, just as nitric oxide, in the manufacture of sulphuric acid, acts as a "carrier" of oxygen into sulphurous acid. We must therefore assume that these ferments can exist both in a hydrated and in a dehydrated form, just as nitric acid can exist in the reduced form of nitrous oxide. Moreover we must conclude that at the splitting of each $-COH.N-$ bond in the protein molecule some such reactions as the following occur:

(A)
$$-COH.N- + \underset{\text{(hydrated ferment)}}{HFFOH} = -COH.\overset{\overset{HF}{|}}{N}-\underset{\text{(ferment-substrate compound)}}{\underset{|}{FOH}}$$

(B)
$$\underset{\text{(ferment-substrate compound)}}{-COH.\overset{\overset{HF}{|}}{N}-\underset{|}{FOH}} = \underset{\text{(products of hydrolysis)}}{-COOH + H_2N-} + \underset{\text{(dehydrated ferment)}}{FF}$$

So far we have, in essentials, made only two assumptions, the one the original assumption that the ferment acts as a "carrier" of water, the other that *one* molecule of ferment reacts with *one* $-COH.N-$ bond.

Combining the above two equations we obtain:

(C) $$-COH.N- + HFFOH = -COOH + H_2N- + FF$$

from which it is evident that the point of equilibrium in the reaction:

(D) $$FF + H_2O \rightleftarrows HFFOH$$

must be shifted in some measure towards the left by the presence of the substrate and the extent of this shift must bear a constant proportion (α) to $(a - x)$. But this equilibrium must also be shifted towards the right by the presence of the products of the hydrolysis of the protein, and this shift must bear a constant proportion (β) to x^2.

Let us now analyse the physical meaning of Henri's equation. This equation, as we have seen in Chap. XV, may be written:

$$\log \frac{a}{a-x} + \alpha x = kFt, \qquad \text{(v)}$$

in which α and k are constants and F is the total mass of ferment present in the system.

Differentiating this equation we obtain:

$$\frac{dx}{dt} = \frac{F}{1 + \alpha(a-x)} k(a-x), \qquad \text{(vi)}$$

which means that the actual "active mass" of the proteolytic ferment, that proportion, namely, which accelerates the hydrolysis by multiplying the velocity-constant, is not F but

$$\frac{F}{1 + \alpha(a-x)}.$$

In other words, the process of combination between the ferment and the substrate and its products which Henri depicts, results in the inactivation of a certain constant proportion of the ferment by each molecule of the substrate. The mechanism of this will be clear from equation (C); by the same equation it will also be clear that a proportion of the ferment is at the same time *activated* (rendered available for the acceleration of the hydrolysis). The quantity of ferment thus activated must evidently bear a

constant proportion β to x^2. Introducing this factor into equation (vi) we obtain:

$$\frac{dx}{dt} = \frac{F}{1 + \alpha(a - x) - \beta x^2} \cdot k(a - x). \qquad \text{(vii)}$$

Integrating, we obtain:

$$(1 - \beta a^2) \log \frac{a}{a - x} + (\alpha + \beta a)\, x + \frac{\beta}{2} x^2 = kFt. \qquad \text{(viii)}$$

In the derivation of Henri's equation it is assumed either that the ferment is unaltered during the processes which accomplish the introduction of the elements of water into the protein molecule or else that the equilibrium in equation (D) lies so far to the right that for all practical purposes the ferment is present wholly in the hydrated, proteolytically active form. Equation (viii) is Henri's equation generalized to the extent of including *any* species of equilibrium in equation (D).

Equation (viii) may be written:

$$\log \frac{a}{a - x} + \frac{\alpha + \beta a}{1 - \beta a^2} x + \frac{\beta}{2(1 - \beta a^2)} x^2 = \frac{kFt}{(1 - \beta a^2)} \qquad \text{(ix)}$$

from which it is evident that it contains three mutually independent constants. This fact deprives it of any great utility for the purpose of comparing numerical data with the theoretical deductions, since an equation containing three mutually independent constants may be made to fit with tolerable exactitude any continuous curve by an appropriate choice of constants. In the absence of very numerous and very exact data, a numerical comparison between theory and experiment involving this equation, therefore, would be devoid of utility. It is of interest to observe, however, that all the relationships between x, F and t found by the various observers, whose investigations we have cited above and in the previous chapter, are especial cases of the general relation which is expressed in equation (ix). Thus if β be small, that is, if the shift in the equilibrium between the hydrated and unhydrated forms of the enzyme due to unit mass of the products of the hydrolysis is very small, or if the equilibrium in equation (D) lies far to the right, then we regain Henri's equation. If not only β but α is small, i.e., if the part played by the protein and its products in determining the equilibrium between the different forms of ferment is small, then we

regain the monomolecular formula. If βa^2 is large in comparison with 1 and α is small then we obtain the relation

$$\log \frac{a}{a-x} + \frac{x}{a} + \frac{x^2}{2\,a^2} = \frac{kF}{\beta a^2} t, \qquad \text{(x)}$$

which, when x is small, yields the relation:

$$\frac{x}{a} = kFt \text{ (equation (iii)).}$$

It is obvious from equation (vii) that if the velocity constant k were to be calculated from the monomolecular formula throughout the reaction of hydrolysis, k would tend to fall off as hydrolysis proceeded, i.e., as x increased, and also, it will be evident, even for small values of x the constant would decrease with increasing initial substrate-concentration. This obviously accords with the facts observed.

The way in which the relationship expressed in equation (ix) may simulate the monomolecular formula, the Schütz rule, etc., under certain conditions, is very well shown by the following table. The values of t corresponding to various values of x are calculated from formula (ix) on the assumption that $a = 10$ and that

$$\frac{\alpha + \beta a}{1 - \beta a^2} = \frac{\beta}{2\,(1 - \beta a^2)} = \frac{kF}{(1 - \beta a^2)} = 1.$$

From these values of t and the given values of x are calculated the constants corresponding to the law of direct proportionality between the quantity digested and the time, to the Schütz rule ($t = kx^2$) and to the monomolecular formula:

x	t	$k = \frac{t}{x}$	$k = \frac{t}{x^2}$	$k = \frac{t}{\log_{10} \frac{a}{a-x}}$
0.1	0.1144	1.144	11.44	26.0
0.2	0.2488	1.244	6.22	28.3
0.5	0.7723	1.545	3.09	34.6
1.0	2.046	2.046	2.05	44.5
2.0	6.097	3.049	1.52	62.9
3.0	12.155	4.051	1.35	78.4
4.0	20.222	5.055	1.26	91.1
5.0	30.301	6.060	1.21	100.7
6.0	42.398	7.066	1.18	106.5
7.0	56.523	8.075	1.15	108.1
8.0	72.699	9.087	1.14	104.0
9.0	89.000	9.889	1.21	89.0

It will be seen that from $x = 0.1$ to $x = 0.5$ the law of direct proportionality very nearly holds good; from $x = 3.0$ to $x = 9.0$ the Schütz rule very nearly holds good, while from $x = 4.0$ to $x = 8.0$ the monomolecular formula adequately expresses the relationship between x and t.

4. The Influence of Acid and Alkali upon the Rate of Protein Hydrolysis by Enzymes. — Of great importance in determining the rate of hydrolysis of proteins by proteolytic enzymes is the reaction (H^+ or OH' concentration) of the solution in which digestion is occurring. The activity of pepsin is greatly enhanced by a low degree of acidity, greater acidity hindering its activity through destruction of the enzyme itself by the excess of acid. The activity of trypsin, on the other hand, is very markedly favored by a very slight alkalinity, excess of alkali being even more destructive to trypsin than excess of acid is to pepsin. An excess of acid very rapidly destroys the activity of trypsin, while an alkaline medium leads to the somewhat less rapid destruction of pepsin. According to Taylor $\frac{N}{1100}$ to $\frac{N}{1400}$ OH' is the optimum alkalinity for the action of trypsin upon protamin sulphate (129).

The nature of the part played by the alkali and acid in protein hydrolysis by enzymes is by no means clear. Since acids and alkalies (H^+ and OH' ions) are well-known hydrolysing agents, a very natural assumption regarding their influence upon protein hydrolysis by enzymes is to suppose that they play the part of accessory catalysors, the catalytic action of the acid or alkali being added to that of the enzyme. As regards the influence of acids upon the hydrolysis of proteins by pepsin, however, there are many well-known facts which speak against this view.

The influence of acids is by no means proportional to their degree of dissociation as would be expected were their influence due to a catalytic action of the hydrogen ion (6) (68). On the contrary, hydrochloric acid has an almost specific action upon the hydrolysis of proteins by pepsin, so that many observers have inclined to the belief that the real ferment is in this instance a compound of pepsin with hydrochloric acid (121). Loeb (74) has suggested that the part played by the acid, in accelerating the hydrolysis of proteins by pepsin, is analogous to the part

played by acids in accelerating the hydrolysis of the imino-esters by increasing the active mass of the substrate through salt formation (Cf. Stieglitz' experiments, cited in section **1**). Loeb believes that the portion of the pepsin which is active in bringing about the hydrolysis of proteins is that portion of it which is ionized, and that acids increase the active mass of the ferment by forming ionized salts with it. Referring to the theory of protein ionization which is developed in earlier chapters of this work, and the intimate relation which, as we have seen, subsists between the degree of ionization and the degree of hydration of a protein (and presumably of these enzymes) this view would appear very probable, especially if we accept the account of the mechanism of the hydrolysis of proteins by enzymes which is given in the latter part (equations (A) to (D)) of the preceding section.

As regards the influence of alkalies upon the activity of trypsin, many of the facts appear, at first sight, to be more in harmony with the view that the alkali acts as an accessory catalysor, since it is stated that the hydroxides of the alkalies and alkaline earths act in proportion to their degree of dissociation (63) although divergencies from the proportionality of the action to the degree of dissociation have been found (6).

It has frequently been pointed out that the progress of hydrolysis of proteins is accompanied by marked changes in the acidity or alkalinity of the solutions in which the hydrolysis occurs (39) (40) (129) (107) (113). This is to be attributed to the splitting of $-COH.N-$ bonds, in hydrolysis, which do not, while bound up in the protein molecule, assist in the neutralization of bases and acids,* but which, when converted into $COOH-$ and H_2N- groups may be presumed to play the part which such groups ordinarily play; hence any excess of acid or base tends to disappear during hydrolysis. One might expect that this change in hydrogen or hydroxyl concentration would

* We have seen that the $-COH.N-$ groups provided by dicarboxyl- and diamino-acid groups are chiefly involved in the neutralization of bases and acids by proteins. In hydrolysis not only these bonds are split, but also the $-COH.N-$ bonds linking monocarboxyl and monoamino-acid groups. It is for this reason that the combining capacity for bases of the products of protein hydrolysis is greater, although only slightly greater, than that of the protein from which they are derived.

lead to deviation from regularity in the relationship between the degree and the time of hydrolysis which is experimentally observed. Nevertheless, if the degree of alkalinity be sufficient, this is not the case. Thus in Taylor's experiments upon the hydrolysis of protamin sulphate the alkalinity of the system changed markedly with the progress of hydrolysis, yet he obtained results in satisfactory accord with the monomolecular law.

The reason for this fact is very clearly revealed by the experiments of Robertson and Schmidt (113). We measured the rate of change in the alkalinity of tryptic digests of sodium caseinate and of protamin sulphate (dissolved in dilute alkali) by means of the gas chain. The progressive change in hydroxyl-concentrations of these digests, it was found, can be expressed by a monomolecular formula when the total OH′ concentration is greater than 10^{-6}, but thereafter, or when the OH′ concentration initially is less than this, the progressive change in alkalinity is no longer to be expressed by a monomolecular formula, but is represented by the formula

$$kt = \frac{x}{B\,(B - x)^2}$$

(where t is the time since the gas-electrodes first came into equilibrium with the solution and B is the OH′ concentration when $t = 0$), which is characteristic of a *bimolecular* reaction. For a short range of intermediate alkalinities the order of the reaction is indeterminate. These facts are illustrated by the tables on the following page.

The point at which the order of the reaction changed is indicated by an asterisk. It will be seen that it corresponds tolerably closely with $C_{OH'} = 10^{-6}$; and *it will also be observed that it is the same for substrates so very different as casein and protamin sulphate.* This indicates that the change in the order of the reaction at a certain OH′ concentration depends upon an alteration in some relationship between the alkali and the ferment and not upon the alteration of such a relationship between the substrate and the ferment.

The most probable interpretation to be placed upon these results is that the active agent in bringing about the hydrolysis is not uncombined trypsin but a compound of trypsin with the base which the solution contains. The efficiency of bases in

TABLE I. CASEIN

To $N/100$ NaOH containing about 2 per cent of casein was added an equal volume of $N/300$ NaOH. To 50 cc. of this solution were added 10 milligrams of trypsin. Temperature 15 to 18 degrees.

Time in minutes after mixing	$t - t_1$	$C_{OH'}$	x	k (monomolecular)	k (bimolecular)
51	....	28.4×10^{-7}	.....	.....	.....
59	0	27.3×10^{-7}	.....	.....	.....
69	10	26.2×10^{-7}	1.1×10^{-7}	180×10^{-5}	154×10^{-6}
73	14	24.2×10^{-7}	3.1×10^{-7}	375×10^{-5}	336×10^{-6}
99	40	20.6×10^{-7}	6.7×10^{-7}	305×10^{-5}	296×10^{-6}
126	67	16.2×10^{-7}	11.1×10^{-7}	338×10^{-5}	376×10^{-6}
153	94	12.8×10^{-7}	14.5×10^{-7}	352×10^{-5}	445×10^{-6}
197	138	9.3×10^{-7}	18.0×10^{-7}	340×10^{-5}	516×10^{-6}
225	166	7.6×10^{-7}	19.7×10^{-7}	336×10^{-5}	574×10^{-6}
246	187	6.7×10^{-7}*	20.6×10^{-7}	326×10^{-5}	600×10^{-6}
276	217	5.95×10^{-7}	21.35×10^{-7}	305×10^{-5}	604×10^{-6}
303	244	5.45×10^{-7}	21.85×10^{-7}	293×10^{-5}	627×10^{-6}
338	279	4.7×10^{-7}	22.6×10^{-7}	275×10^{-5}	635×10^{-6}
368	309	4.0×10^{-7}	23.3×10^{-7}	270×10^{-5}	690×10^{-6}
408	349	3.7×10^{-7}	23.6×10^{-7}	250×10^{-5}	670×10^{-6}
443	384	3.5×10^{-7}	23.8×10^{-7}	232×10^{-5}	642×10^{-6}
470	411	3.3×10^{-7}	24.0×10^{-7}	224×10^{-5}	654×10^{-6}
547	488	2.8×10^{-7}	24.5×10^{-7}	203×10^{-5}	660×10^{-6}
601	542	2.6×10^{-7}	24.7×10^{-7}	190×10^{-5}	656×10^{-6}
647	588	2.5×10^{-7}	24.8×10^{-7}	178×10^{-5}	627×10^{-6}
722	663	2.5×10^{-7}	24.8×10^{-7}	157×10^{-5}	556×10^{-6}

TABLE II. PROTAMIN SULPHATE

Ten grams of protamin sulphate in one litre of solution. Ten milligrams of trypsin and 2.5 of $N/10$ NaOH to 25 cc.

Time in minutes after mixing	$t - t_1$	$C_{OH'}$	x	k (monomolecular)	k (bimolecular)
60	....	28.5×10^{-7}	.....	.....	.....
85	0	24.3×10^{-7}	0	.....	.....
108	23	20.7×10^{-7}	3.6×10^{-7}	305×10^{-5}	311×10^{-6}
136	51	15.0×10^{-7}	9.3×10^{-7}	411×10^{-5}	500×10^{-6}
181	96	10.5×10^{-7}	13.8×10^{-7}	380×10^{-5}	564×10^{-6}
241	156	7.0×10^{-7}*	17.3×10^{-7}	346×10^{-5}	647×10^{-6}
307	222	5.3×10^{-7}	19.0×10^{-7}	298×10^{-5}	664×10^{-6}
356	271	4.5×10^{-7}	19.8×10^{-7}	270×10^{-5}	668×10^{-6}
400	315	4.0×10^{-7}	20.3×10^{-7}	249×10^{-5}	664×10^{-6}
453	368	3.5×10^{-7}	20.8×10^{-7}	227×10^{-5}	663×10^{-6}
502	417	3.1×10^{-7}	21.2×10^{-7}	213×10^{-5}	676×10^{-6}

activating trypsin is thus readily understood. When the concentration of unneutralized base is in excess of 10×10^{-6} all of the trypsin is combined with the base and in the form of the proteolytically active salt;* but when the alkalinity falls below this limit a proportion of the trypsin is no longer combined with alkali and this proportion is inactive. Below this limit of OH′ concentration, therefore, the velocity-constant of hydrolysis (calculated from the monomolecular formula) must fall off in proportion as $C_{OH'}$ decreases, in other words, the relation between $C_{OH'}$ and time will be expressed by the bimolecular formula.

This explanation of our results is especially supported by the fact that when the velocity of hydrolysis has fallen very low, owing to decreasing alkalinity, the velocity can be raised again, and the order of the reaction again made monomolecular, by simply increasing the alkalinity of the solution. This is shown in table III in which the break indicates that fresh alkali was added.

We may therefore conclude that in all probability pepsin and trypsin are only able to exert their proteolytic activity (i.e., act as carriers of water) when they are present in their solutions in the form of *salts* with acids and bases respectively.

The above experiments establish a lower limit of hydroxyl-concentration, below which the alkalinity is not sufficient to secure the greatest velocity of transformation. An upper limit, above which the alkalinity is too great to secure the greatest velocity of transformation, is indicated by the fact that trypsin undergoes autohydrolysis in solution and this autohydrolysis is greatly accelerated by alkalies (144) (145); directly, therefore, the rate of destruction of the trypsin by the excess of alkali measurably affects the progress of the reaction an upper limit of alkalinity is reached. The experiments of Taylor, cited above, establish this upper limit at about $\frac{N}{1500}$ OH′; between $\frac{N}{1{,}000{,}000}$ and $\frac{N}{1500}$ OH′, therefore, all alkalinities are equally favorable.

* Subject, of course, to dehydration as in equations (C) and (D) in the preceding section. In equations (viii) and (ix) it is the value of F which is to be considered as being affected by the alkali, not the proportion of the hydrated to the dehydrated form of the enzyme, although this may also very possibly be affected by the OH′ concentration.

TABLE III

Ten grams of protamin sulphate in 1 litre of solution. Ten milligrams of trypsin and 2.1 cc. of $N/10$ NaOH to 25 cc.

Time in minutes after mixing	$t - t_1$	$C_{OH'}$	z	k (monomolecular)	k (bimolecular)
29	.....	23.3×10^{-7}	.....	.....	.....
38	0	22.4×10^{-7}	0	.....	.....
63	25	16.25×10^{-7}	6.15×10^{-7}	559×10^{-5}	676×10^{-6}
78	40	13.3×10^{-7}	9.1×10^{-7}	567×10^{-5}	765×10^{-6}
93	55	10.5×10^{-7}	11.9×10^{-7}	601×10^{-5}	920×10^{-6}
109	71	8.9×10^{-7}*	13.5×10^{-7}	565×10^{-5}	954×10^{-6}
124	86	7.6×10^{-7}	14.8×10^{-7}	545×10^{-5}	943×10^{-6}
132	94	7.0×10^{-7}	15.4×10^{-7}	537×10^{-5}	1046×10^{-6}
13	.....	27.3×10^{-7}	.....	.....	.....
23	0	26.3×10^{-7}	0	.....	.....
33	10	24.3×10^{-7}	2.0×10^{-7}	334×10^{-5}	313×10^{-6}
51	28	20.7×10^{-7}	5.6×10^{-7}	371×10^{-5}	367×10^{-6}
72	49	18.3×10^{-7}	8.0×10^{-7}	323×10^{-5}	339×10^{-6}
90	67	16.9×10^{-7}	9.4×10^{-7}	286×10^{-5}	316×10^{-6}
111	88	13.8×10^{-7}	12.5×10^{-7}	317×10^{-5}	391×10^{-6}
123	100	12.8×10^{-7}	13.5×10^{-7}	314×10^{-5}	401×10^{-6}
152	129	11.3×10^{-7}*	15.0×10^{-7}	285×10^{-5}	391×10^{-6}
170	147	10.5×10^{-7}	15.8×10^{-7}	272×10^{-5}	389×10^{-6}
199	176	9.7×10^{-7}	16.6×10^{-7}	246×10^{-5}	369×10^{-6}
232	209	7.9×10^{-7}	18.4×10^{-7}	250×10^{-5}	423×10^{-6}
251	228	7.6×10^{-7}	18.7×10^{-7}	236×10^{-5}	411×10^{-6}

5. The Influence of Added Substances upon the Rate of Protein Hydrolysis by Enzymes. — The influence of added substances upon the rapidity of the hydrolysis of proteins by enzymes has been extensively studied, owing to its supposed importance in connection with the digestion of protein in the alimentary canal (97) (119) (51) (149) (73) (16) (96) (45) (18) (151) (30) (66) (15) (100) (152) (120). Despite this extended investigation, however, the results obtained by different observers have been conflicting in the extreme. Investigations which I have carried out on the influence of salts upon the hydrolysis of caseinates by trypsin indicate the probable source of the discrepancies between the results of different observers (107). I find that the order of efficiency of salts in accelerating the hydrolysis of casein by trypsin is the order of their efficiency in accelerating the *solution* of casein by alkalies. If this rule holds good for other proteins, then one might anticipate that the influence of salts upon their hydrolysis by enzymes would vary

somewhat with the nature of the protein or protein salt employed as substrate.

The effect of salts in accelerating the hydrolysis of casein by trypsin is, in some instances, very marked. Thus in a solution of "neutral" calcium caseinate containing 143 milligrams of casein in 200 cc. plus 1 cc. of a 0.025 per cent solution of Gruebler's trypsin at 36.5 degrees the velocity-constant of hydrolysis was 11 (calculated, in arbitrary units, from the monomolecular formula) while, upon the addition of 0.05 *N* NaCl the value of the constant rises to 44 (in the same units).

The influence of the concentration of the added NaCl upon the acceleration of the tryptic hydrolysis of caseinates which it brings about is of great interest, since, so far as my observations have extended, it is of the same nature as its influence upon the degree of swelling which gelatin plates undergo when immersed in water. It will be recollected (Cf. Chap. XII) that when gelatin plates are immersed in solutions of NaCl of varying concentration, the degree of swelling which they attain after a given period does not increase continuously with the concentration of the solution but exhibits marked maxima and minima at definite concentrations (94). Exactly similar phenomena occur when NaCl in varying concentration is added to a tryptic digest of casein. The acceleration in hydrolysis which is produced by the added NaCl does not continuously increase with increasing concentration of the NaCl, but exhibits marked maxima and minima.

6. The Action of the Coagulating Ferments, Rennet and Thrombin. — As is well known, rennet occurs, together with pepsin, in the gastric juice and in the juices of many plants and brings about the clotting of milk through the transformation of casein into paracasein. Thrombin occurs in some part of the circulating blood, possibly the leucocytes, and brings about the coagulation of blood through the transformation of fibrinogen into fibrin. The modes of action of these ferments have been the subject of very many investigations (102) (103) (31) (84) (75) (61), which cannot be dwelt upon in detail here. In brief, however, it may be stated that rennet converts casein into paracasein, whether calcium salts are present or not, and the paracasein having been formed (which is soluble in the absence of salts of the alkaline earths), it is coagulated (or *precipitated;*

Cf. Chap. VI) by calcium salts or other salts of the alkaline earths. The part played by calcium in forwarding the coagulation of blood is very different; here the dependency is one of the ferment (or a pro-ferment), and not of the coagulation of the substrate, upon the presence of calcium salts; it would appear that calcium salts *activate* a pro-thrombin, converting it into the ferment thrombin, which will then induce coagulation whether calcium salts be present or not. The function of calcium in these two processes is therefore very different.

Fuld (32), Gerber (37) and Madsen (77) have shown that for the coagulation of milk by rennet $F \times t = \text{constant}$; i.e., the velocity of coagulation is proportional to the concentration of ferment. Regarding the influence of calcium salts, very interesting results have been obtained by Reichel and Spiro (104). These observers added to 8 cc. of milk different quantities (R) of rennet solution and different amounts (p) of calcium chloride, and diluted with water to a total volume of 100 cc. They then measured the time required for coagulation at a constant temperature. The following were their results:

$CaCl_2$ in per cent p	Time of coagulation			Constant = $(p + 0.6)\,t$		
	R = 1 cc.	R = 0.5 cc.	R = 0.25 cc.	R = 1 cc.	R = 0.5 cc.	R = 0.25 cc.
0.00	95.0	48.0	24.0	57.0	28.8	14.4
0.05	88.6	45.6	23.0	57.6	29.6	15.0
0.1	79.0	41.6	22.0	55.3	29.1	15.4
0.2	66.4	36.0	19.0	53.1	28.8	15.2
0.5	48.0	26.4	14.0	52.8	29.0	15.4
1.0	30.0	18.2	10.6	48.0	29.1	17.0
2.0	17.0	11.0	6.8	44.2	28.6	17.7
5.0	10.0	7.4	5.4	56.0	41.4	30.2
10.0	13.0	9.2	6.2	137.8	97.5	65.7
20.0	22.0	15.0	8.6	453.2	309.0	177.2

It is evident that the product $(p + 0.6)\,t$ is constant for each value of R, provided p does not exceed 2 per cent. The velocity is also proportional to R, so that the complete relationship is $R\,(p + 0.6)\,t = \text{constant}$. The quantity 0.6 which must be added to p in the above equation is to be regarded as the (percentage) concentration of calcium ions present already in milk, before any calcium chloride is added. According to Reichel and Spiro this indicates that only about 15 per cent of the calcium in milk

is ionic; this obviously corresponds very well with the statement in Chap. VIII and elsewhere in this work that the calcium bound in calcium caseinate is not electrolytically dissociated as such.

In the opinion of Nencki and Sieber (86), Schoumov-Simanovski (123), Pavlov and Parastschuk (95), Savjallov (118), Gevin (38), Migay and Savitsch (83), Van Dam (139) (140) (141), Savitsch (117), Herzog (57), Funk and Niemann (34), Laqueur (71) (72) and Bosworth (12) (13), rennet and pepsin are identical substances, the transformation of casein into paracasein being the first step in hydrolysis or else a side-hydrolysis (135). On the other hand, Hammarsten (43) (44), Hedin (50), Rakoczy (101) and others are of the opinion that rennet and pepsin are distinguishable from one another. The evidence for the identity of the two enzymes lies in the fact that in no way, either quantitatively or qualitatively, can the activity of rennet be separated from that of pepsin, statements to the contrary effect (4) (52) (135) (62) (122) (43) having been disproved or else shown to rest upon experimental data obtained under conditions necessarily involving inhibition, or apparent inhibition, of the one form of action or of the other (Cf. especially Van Dam, (139) (140) (141)). This is very well illustrated by the following experiment of Morgenroth (85). If mixtures of calcium caseinate and rennet are kept at low temperatures no coagulation occurs, but the digestion process does occur; after these mixtures have been held for some time at the low temperature, if they are heated to 20 degrees they coagulate instantly. Thus the process which underlies the clotting takes place at the lower temperature but cannot be evidenced by actual clotting until the temperature has risen. It has repeatedly been pointed out (105) (106) (72) that the difference between the behavior of paracasein towards calcium salts and that of casein is essentially this, that calcium paracaseinate is coagulated by calcium salts *at lower temperatures* than calcium caseinate.

Now it has been shown by Van Slyke and Hart (143), Geake (36) and Van Slyke and Bosworth (142) that the paracasein freed from its combination with calcium is analytically indistinguishable from casein, and Van Slyke and Bosworth have shown that the minimal combining-capacity of paracasein for bases is exactly double that of casein. These facts admit of only one interpretation, namely, that casein is hydrolytically

split by rennet into two equal parts. The correctness of this interpretation has been completely confirmed by Bosworth (12) who has shown that in the first place no substance other than paracasein results from the action of rennet upon casein, while, in the second place, no matter what proteolytic enzyme (rennet or trypsin) be employed to split the casein, the first step in hydrolysis is the production of paracasein (13).

As regards the kinetics of the action of fibrin-ferment (thrombin) Fuld (33) finds that the Schütz rule (velocity proportional to the square root of the ferment-mass) very nearly holds good, the actual relation between the velocity (v) of coagulation and the mass of fibrin-ferment (E) being:

$$\frac{v_1}{v_2} = \left(\frac{E_1}{E_2}\right)^{0.585}.$$

C. J. Martin finds that for the coagulation of blood by thrombin $F \times t$ = constant (78).

Fibrin-ferment undergoes marked exhaustion during the process of coagulation (84) (60).

7. The Influence of Temperature upon the Velocity of Protein Hydrolysis. — The influence of temperature upon the hydrolysis of gelatin by trypsin has been carefully studied by Madsen and Walbaum (cited after Arrhenius (3)). The results are in good agreement with the formula:

$$\frac{v_1}{v_0} = e^{\frac{\mu}{2}\left(\frac{T_1 - T_0}{T_1 T_0}\right)},$$

where e is the base of the Napierian logarithms, v_1 and v_0 are the velocities corresponding to absolute temperatures T_1 and T_0 and μ is a constant, in this instance 10,570. This is the law which is characteristic of the influence of temperature upon other chemical reactions, and its possible significance, when it is found applicable to protein systems, has been commented upon in Chap. VII.

The influence of temperature upon the rate of digestion of thymol-gelatin by pepsin is also capable of representation by the above formula (Arrhenius, loc. cit., p. 71). In this case μ = 10,750. The velocity both of pepsin and trypsin-digestion is, therefore, doubled by a rise in temperature from 20 to 30 degrees.

Using different substrates it is found that, as might be expected,

the value of μ for hydrolysis by a given enzyme varies with the nature of the substrate. (Cf. table in Arrhenius' work, loc. cit., pp. 99–98.)

The influence of temperature upon the coagulative power of rennet has been studied by Fuld (32); here also the van't Hoff-Arrhenius' rule holds good; μ is 20,650.

The interesting observation has been made by Cesana (17) in studying the influence of temperature upon the ultramicroscopic appearance of trypsin solutions, that the optimum temperature for its proteolytic activity corresponds with that at which there is a minimum of visible granules in the ultramicroscopic field and a maximum dispersion of the trypsin leading to a maximum frequency of collisions between trypsin particles or molecules and the molecules of the substrate.

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CHAPTER XVII

THE ENZYMATIC SYNTHESIS OF PROTEINS

1. The Reversion of the Hydrolysis of Proteins by Pepsin and Trypsin. — In "typical" catalysed reactions (Cf. previous Chapter, section **1**), since the equilibrium of the system is not shifted by the catalysor and the point of equilibrium is determined by the ratio of the velocity-constants of the forward and the opposed reactions, both velocity-constants must be multiplied to an equal degree by the catalysor; in other words, if under given conditions a point of equilibrium exists at which an appreciable proportion of the substrate remains unaltered, then, under such conditions, the pure products (unmixed with substrate) are not in equilibrium and must be tending to restore the substrate, and this tendency must be increased by the presence of the catalysor in question.

As this fact became generally appreciated, following its experimental verification in a large number of non-fermentative catalytic reactions, and as the belief gained ground that the ferments were to be regarded as "typical" catalysors, it was anticipated that fermentative *reversions* might be accomplished, that is the *synthesis* of substances which are normally hydrolysed (or otherwise altered) by enzymes, through acting upon the products of hydrolysis with the same enzymes. This theoretical anticipation was first experimentally realized by Croft Hill (31) (32) in the fermentative synthesis of isomaltose (16) (2) (3) from glucose, and has since been realized in the fermentative synthesis of polysaccharides, fats, and proteins by various investigators (7) (34) (26) (8) (66) (67) (69) (70) (47) (48) (9) (51) (52).

In order to make clear the conditions under which such reversions occur it will be well to consider in some detail the fundamental experiment performed by Croft Hill.

The hydrolysis of maltose (or of isomaltose) is represented by the equation:

(A) $$C_{12}H_{22}O_{11} + H_2O \rightarrow 2\,C_6H_{12}O_6;$$

the reversion is represented by the equation:

(B) $$2\,C_6H_{12}O_6 \rightarrow C_{12}H_{22}O_{11} + H_2O.$$

The condition of "balance" at equilibrium is therefore represented by the equation:

(C) $$C_{12}H_{22}O_{11} + H_2O \rightleftarrows 2\,C_6H_{12}O_6.$$

If m_1 be the mass of maltose, m_2 that of glucose and W that of water, then at equilibrium we shall have, in accordance with the mass-law:

$$m_1W = \frac{k_2}{k_1} m_2^2, \qquad \text{(i)}$$

in which k and k_2 are the velocity-constants of the forward and the reverse reactions, respectively. If maltase is indeed a "typical" catalysor, then we must assume that although k_1 and k_2 are each multiplied by the presence of the catalysor their *ratio* remains unaffected and, consequently, the relative proportions between m_1 and m_2 and W at equilibrium are not affected by the presence of maltase.*

Now the value of the ratio $\frac{k_2}{k_1}$ for this reaction is such that in dilute solutions (i.e., when W in equation (i) is large) the point of equilibrium in equation (C) lies so far over towards the right that hydrolysis of the maltose, at the end of the reaction, is practically complete, no appreciable quantity of unhydrolysed maltose remaining in the system. This being the case we cannot expect, through the agency of any "typical" catalysor, to secure the synthesis of maltose in a dilute solution of glucose. A considerable increase in the concentration of the system, however, although it does not bring about any appreciable alteration either in k_1 or in k_2, nevertheless alters the proportion of maltose to glucose at equilibrium very profoundly. The reason for this is apparent upon inspection of equation (i). Increasing the concentration of the system is equivalent to reducing the magnitude of W and hence inducing a compensating increase in m_1 (the mass of maltose) at equilibrium. In a *concentrated* system we find, accordingly, that the hydrolysis of maltose is never complete, whether maltase or any other catalysor be employed to accelerate the process. A *concentrated* solution of glucose is therefore not in a condition of equilibrium until a proportion of maltose has been restored to the system by the occurrence of

* It should be clearly borne in mind that the verity of this statement has never been proven.

reaction (B). In the absence of any catalysors this reaction takes place very slowly, but if any of the catalysors be present which are capable of accelerating the *hydrolysis* of maltose, for example maltase, then the synthesis of maltose or of isomaltose occurs with measurable rapidity. It was by adding maltase to *concentrated* glucose that Croft Hill obtained a fermentative synthesis of isomaltose.

In satisfactory accord with the above account of the chemical mechanics of fermentative reversions, the majority of observers have found that reversion of a fermentative hydrolysis is only attainable when the enzyme is made to act upon the *concentrated* products of the hydrolysis. Nevertheless reversion does not always occur when it might be expected to occur; thus Bradley (9) has shown that in the presence of 50 per cent of water no appreciable synthesis of triolein from glycerin and oleic acid can be brought about through the agency of lipase, although in the presence of 50 per cent of water an appreciable proportion of triolein remains unhydrolysed. The converse is also true, reversion occurs when it might not be anticipated. Thus I find (53) that it is possible to completely hydrolyse paranuclein to products which have lost its characteristic properties and yet, without any previous concentration, to bring about, through simply altering the temperature and the enzyme concentration, a fermentative synthesis of this substance. These apparent anomalies arise from the fact that these ferments do not belong to that restricted class of catalysors which, as explained in section 1 of the previous chapter, has come to be regarded as typical, but to what Stieglitz has shown to be the more general class of catalysors, namely those which appreciably participate in the reactions which they accelerate and therefore appreciably disturb the final equilibrium. The significance of these phenomena, in so far as the proteolytic enzymes are concerned, will be more fully discussed below.

The fermentative synthesis of protamin through the action of trypsin upon the concentrated products of its hydrolysis has been accomplished by Taylor (69) (70) and I have described the fermentative synthesis of paranuclein (51) (52) through the action of pepsin upon the products of its hydrolysis.

In Taylor's experiments the mixed products of the tryptic digestion of protamin sulphate were converted into carbonates

and then (the ferment having been destroyed by boiling) evaporated until precipitation (at room temperatures) just began. The solution which was thus prepared contained the products of the hydrolysis of 400 grams of protamin and was free from unhydrolysed protamin, since the original solution (before evaporation) was miscible with five volumes of acidulated alcohol without any resulting opacity. To this solution were then added 300 cc. of a glycerin extract of the livers of the large soft-shelled California clam (*Schizothærus nuttalli*), which contain a proteolytic ferment of the trypsin type which is very resistant to autodestruction and therefore especially adapted for employment in experiments of long duration. After the addition of this trypsin the solution was still miscible with alcohol without cloudiness. Excess of toluol was then added, and the flask (containing over 4 litres) was set aside at room temperature.

The mixture gradually became opalescent, then cloudy, and, finally, after the lapse of some months, a distinct precipitate had formed. After five months the flask was opened, the contents were heated to boiling to destroy the ferment, acidulated with sulphuric acid, which dissolved the precipitate, and filtered. Then four volumes of absolute alcohol were added, which resulted in the throwing down of a heavy white precipitate, which, after purification, was analysed. In all, about 2 grams were obtained.

The percentage formula of the protamin sulphate employed (salmin sulphate prepared from the spermatozoa of *Roccus lineatus*) had previously been determined by Taylor and found to be:

$$C_{30}H_{60}N_{17}O_6.2\ H_2SO_4.$$

The analysis of the synthetical preparation yielded the following results:

	Calculated for $C_{30}H_{60}N_{17}O_6.2\ H_2SO_4$, per cent	Found, per cent
C =	37.85	37.68
H =	6.72	6.89
N =	25.13	24.90
H_2SO_4 =	20.60	20.68

The substance was digestible by trypsin and not digestible by pepsin. Taylor concludes that it was salmin. A concentrated

solution of the products of the hydrolysis of protamin, to which no ferment had been added, yielded no trace of protamin after standing for several months.

My experiments were conducted as follows: Four hundred cc. of $N/10$ potassium hydroxide were neutralized (or rendered faintly acid) to litmus by the addition of casein and the solution was subjected to the action of pepsin for a considerable period, fresh pepsin ($\frac{1}{2}$ g. per litre) being added from time to time, at 40° C. The digest at the end of this period, contained no casein, but a considerable quantity of paranuclein had been precipitated and had not undergone hydrolysis. The digest was then heated to 100 degrees for about 10 or 15 minutes to destroy the ferment, and filtered while hot. The solution was then evaporated on a water-bath to about 70 cc. (the total concentration of the system having been thus multiplied by about 6). This concentrated solution of the products of the peptic digestion of casein (and its first product of hydrolysis, paranuclein) is a clear brown syrup which is strongly acid and gives no precipitate or opalescence upon the addition of acetic acid, or upon the addition of acetic acid in excess after previously rendering the solution alkaline by the addition of KOH or NaOH. Both casein and paranuclein are therefore absent from it. To 70 cc. of this solution were added 30 cc. of a concentrated (approximately 10 per cent) solution of Gruebler's pepsin *puriss. sicc.* which had previously been filtered through rapid-filtering paper. The mixture of the two solutions is a clear brown syrupy fluid which yields no trace of a precipitate with acetic acid either before or after neutralization with alkali. The mixture was set aside at 40° C. in the presence of excess of toluol to prevent bacterial infection. *Within two hours* a thick white precipitate had formed in the solution; after 48 hours the solution was filtered and the precipitate thus collected on the filter was dissolved in a minimal amount of sodium hydroxide and the filter so arranged that the alkaline solution dropped directly into water acidified with acetic acid. The precipitate thus obtained was reprecipitated twice and was washed by decantation several times, employing several litres of water at each decantation. Finally it was collected on a hardened filter, washed with several litres of alcohol and ether, and dried, first over $CaCl_2$ and then over H_2SO_4 at 60° C. In this way 1.02 grams of a friable greyish-white hygroscopic powder

were obtained. This substance gave every indication of being identical with paranuclein, the first product of the peptic hydrolysis of casein. Accordingly, for purposes of comparison, paranuclein was prepared by partly digesting sodium caseinate with pepsin, filtering off the precipitate, dissolving in sodium hydroxide and precipitating with acetic acid. The paranuclein was reprecipitated twice, washed with large volumes of water, alcohol, and ether, and dried over $CaCl_2$ and later over H_2SO_4 at 60 degrees. A powder exactly similar in appearance to the synthesized substance was thus obtained.

The synthesized substance and the paranuclein were both analysed for phosphorus by Neumann's method, with the following results:

Synthetic Substance	Paranuclein
P_2O_5 = 1.61 per cent	P_2O_5 = 4.18 per cent

Previous observers agree in stating that the percentage composition of paranuclein varies very greatly with the circumstances under which it is prepared, the percentages of phosphorus which have been found in various preparations varying from 0.88 to 6.86 (43). This fact leads us to suspect that the substance which has been termed paranuclein is, in reality, a mixture of at least two substances and the hypothesis which has suggested itself to me is that during the hydrolysis of casein by pepsin a substance of high phosphorus content, insoluble in dilute acids, is first formed; that this substance splits off a soluble phosphorus-containing moiety, leaving another insoluble substance of lower phosphorus content, and that this second substance is in its turn attacked and split up into soluble substances. That this explanation is probably the correct one, although, of course, several such steps may possibly be involved, is shown by the following experiment.

One gram of the paranuclein containing 4.18 per cent of P_2O_5 was dissolved in 400 cc. of 0.045 *N* $Ca(OH)_2$ and the mixture was allowed to stand at 40° C. for 12 hours; acetic acid was then added in excess and the precipitate purified, washed and dried in the manner described above. This substance, which we may designate paranuclein *A*, was analysed for phosphorus and found to contain 1.51 per cent of P_2O_5, thus agreeing closely with the synthetic substance. Only a little over 0.2 gram of this sub-

stance was obtained from the gram of paranuclein originally dissolved in the lime-water.

The splitting off of phosphorus from paranuclein in alkaline solution has been commented on by other observers (61).

The substance which is obtained synthetically, as described above, resembles paranuclein *A* very closely both in general properties and in its percentage-content of P_2O_5. The synthesized substance is insoluble in dilute weak acids, readily soluble in dilute alkali, precipitates protamin from a 1 per cent solution of the sulphate at a reaction just alkaline to phenolphthalein (at which reaction both the protamin and the paranuclein remain in stable solution when not mixed), and a 2 per cent solution in $N/10$ sodium hydroxide is precipitated by $m/10$ ferric ammonium sulphate; these being all well-known properties of paranuclein (61) (42).

The synthesized substance also resembles my preparation of paranuclein *A* in the following properties; in approximately 2 per cent solution in $N/100$ NaOH it gives the xanthoproteic, Millon's, Adamkiewicz and the biuret (violet) reactions; it is precipitated by cupric chloride (1 vol. of $N/1$ to 100) and by zinc chloride, but not by mercuric chloride (5 vols.; of $N/10$) it is precipitated by picric and tannic acids, but the precipitate redissolves on rendering the solution alkaline; it is not precipitated by the addition of five volumes of absolute alcohol; and the precipitate which is at first produced by the addition of acetic acid is soluble in considerable excess of the reagent.

The change in the refractive index of $N/50$ KOH which is brought about by the introduction of one per cent of the synthetic substance is identical with that which is brought about by the introduction of one gram of paranuclein or paranuclein *A* (54), namely, 0.00140 for sodium light.

If no pepsin be added to the concentrated solution of the products of the peptic digestion of casein, prepared as described above, the solution, after keeping for over a year at room-temperatures or for several months at 40° C. remains perfectly clear and homogeneous and gives no tests for paranuclein or for casein. A 10 per cent filtered solution of Gruebler's pepsin, on standing for many months at 40 degrees, also remains perfectly clear and homogeneous. Yet these two solutions, when mixed in the proportion of one volume of ferment-solution to five volumes of

the solution of the casein-products, give a voluminous precipitate which remains permanent for many weeks.

The fact that paranuclein *A*, rather than paranuclein or casein, is produced in the reversion of protein hydrolysis which presumably occurs in the experiments described above, is probably to be interpreted as follows:

In the initial stages of the hydrolysis of casein, paranuclein is produced, part of which undergoes further hydrolysis and part of which escapes further hydrolysis owing to the fact that it is thrown out of the sphere of action of the enzymes by precipitation (59) (60) (61). The proportion of the paranuclein which undergoes hydrolysis passes through the intermediate stage paranuclein *A* and then to the stage of proteoses and peptones. Only that proportion of the paranuclein which undergoes this further hydrolysis, therefore, yields to the filtered and concentrated digest all of the substances which are necessary for resynthesis. In event of synthesis occurring in a mixture such as that described above, the first substance which resulted which is insoluble in solutions of weak acids would necessarily be thrown out of the sphere of action and the reaction would terminate at this point. Hence, from what has been said above, it is evident that were a member of the paranuclein group produced in these mixtures it would be paranuclein *A*, rather than one of the other members of the group of higher phosphorus-content.

In the above experiments, in order to secure synthesis of the paranuclein, the products of its hydrolysis had to be concentrated to a considerable degree. From what has been said in the beginning of this section the rationale of this procedure will be evident. Later experiments, however (53), showed that previous concentration of the products of the complete peptic hydrolysis of paranuclein is not necessary if the synthesis be carried out in the presence of a considerable excess of pepsin and at a much higher temperature, namely from 60 to 70 degrees.

I made up the following mixtures in duplicate, having first ascertained that the unconcentrated products of the peptic hydrolysis of *N*/50 alkali caseinates and 10 per cent pepsin (Gruebler's *puriss. sicc.*) can be kept separately for weeks at 65 degrees without a trace of precipitate forming in either solution.

(*a*) 10 cc. of unconcentrated products + 0.5 cc. of 15 per cent pepsin.
(*b*) 10 cc. of unconcentrated products + 1.0 cc. of 15 per cent pepsin.
(*c*) 10 cc. of unconcentrated products + 1.5 cc. of 15 per cent pepsin.
(*d*) 10 cc. of unconcentrated products + 2.0 cc. of 15 per cent pepsin.
(*e*) 10 cc. of unconcentrated products + 3.0 cc. of 15 per cent pepsin.

The one set was kept at 65 degrees, while the other was kept at 36 degrees, both in tightly stoppered vessels containing excess of toluol. After 24 hours there was no sign of any precipitate or opalescence in the mixtures which had been kept at 36 degrees, while in the duplicate set, which had been kept at 65 degrees, (*e*) contained a heavy precipitate which left the supernatant fluid clear, (*c*) and (*d*) contained, also, heavy precipitates which, however, left the supernatant fluid strongly opalescent, and (*a*) and (*b*) both contained slight precipitates. After 24 hours more, no change had occurred in any of the solutions and those which had been kept at 65 degrees were now returned to 36 degrees. After a lapse of three weeks no trace of precipitate had appeared in any of those solutions which had been at 36 degrees throughout, while no further change had occurred in those which had been kept at 65 degrees for 48 hours.

The probable identity of the precipitate which is thus produced, with that which is produced by the action of pepsin upon the concentrated (= 6 times) products at 36 degrees was shown by the following experiments.

Thirty cubic centimeters of 15 per cent pepsin (Gruebler's *puriss. sicc.*) were added to 150 cc. of the unconcentrated products of the complete peptic hydrolysis of $N/50$ sodium caseinate and the mixture was kept at 65 degrees for 48 hours in the presence of excess of toluol. The resulting precipitate was collected on a hardened filter paper and washed with distilled water until the washings were colorless; it was then dissolved by allowing dilute sodium hydrate to pass through the filter and reprecipitated by allowing this filtrate to pass into a beaker containing excess of dilute acetic acid. The precipitate thus obtained was collected on a hardened filter paper, washed with large volumes of water, alcohol, and ether and dried over $CaCl_2$ and later over H_2SO_4. The product was a greyish-white, friable hygroscopic powder resembling in its physical properties and precipitation-reactions "paranuclein *A*." It was analysed for phosphorus by Neumann's method and found to contain 1.65

per cent of P_2O_5, thus agreeing closely with paranuclein *A* and with the substance synthesized at 36 degrees from the *concentrated* products of the hydrolysis of paranuclein.

Furthermore, the change in the refractive index of $N/50$ KOH which is brought about by the introduction of one per cent of this synthetic substance is identical with that which is brought about by the introduction of one per cent of paranuclein, paranuclein *A* or of the substance synthesized at 36 degrees, namely 0.00140 for sodium light (54).

A reversion of hydrolysis can be brought about, therefore, even in the *diluted* products of the complete peptic hydrolysis of $N/10$ solutions of the alkali caseinates (*diluted*, since varying amounts of pepsin solutions were *added* to the solution of products) by the addition of 0.5 cc. of 15 per cent pepsin to 10 cc. of products (final concentration of pepsin 0.75 per cent) and keeping the mixture for 24 hours at 65 degrees, while it requires 15 cc. of 10 per cent pepsin in 100 cc. of mixture (final concentration of pepsin 1.5 per cent) to bring about, in 24 hours, reversion of the hydrolysis at 36 degrees in a solution of products which has been concentrated four or five times.

It is an extremely significant fact that the synthesis which occurs at 65 degrees does so at a temperature from 10 to 15 degrees in excess of that at which, according to the majority of authors, pepsin is rapidly and completely deprived of its proteolytic activity.* True, the destruction even, at this temperature, must be a matter of time, and one might be inclined to believe that a short period of very intense action at 65 degrees produced, in the above experiments, a similar result to the much more prolonged but weaker action of pepsin at 36 degrees. The facts are not in favor of this view, however, since the appearance of the precipitate which marks a certain stage in the reversion does not occur (if the pepsin is not too concentrated) until the solution has been standing at 65 degrees for two or three hours, and it progressively increases in amount for over 24 hours. It appears that *the active agent in reversion is not identical with the active agent in hydrolysis.*

* Cf. Oppenheimer (45). From the determinations of Schwarz (64) it appears that concentrated (10 per cent) solutions of pepsin are deprived of their power of accelerating protein hydrolysis and at the same time acquire considerable power of inhibiting the activity of unaltered pepsin after having been heated to 60 degrees for 5 minutes.

Additional experiments were undertaken by myself and Biddle (55) with a view to further elucidating the relationship between the paranucleins and the synthetic substances which I have described. We determined their carbon, hydrogen and nitrogen content, with the results tabulated below *; for the purpose of comparison analyses of paranuclein by Lubavin (41) are also included in the table.

	Paranuclein (Lubavin), per cent	Paranuclein (Robertson and Biddle), per cent	Paranuclein *A* from paranuclein, per cent	Substance synthesized 36 degrees, per cent*	Substance synthesized 70 degrees, per cent
C	48.5	49.98	49.47	53.39	49.99
H	7.1	7.20	6.80	7.80	7.00
N	13.3	12.80	12.50	13.00	13.10

* Three separate preparations of this substance were made, the figures given (except that for N which was only determined in the first preparation) are those obtained with the preparation which had been subjected to the most rigorous purification.

It is evident that the carbon, hydrogen, and nitrogen-contents of paranuclein, paranuclein *A* and the substance synthesized at 70 degrees, are so closely similar that they may be considered identical; they also agree well with the original analyses of Lubavin. The product obtained at 36 degrees, however, contains a very appreciably higher percentage of carbon. Since the carbon-content of this preparation was found to sink somewhat during successive purifications it appears possible that the high carbon is due to contamination with some impurity, possibly a coagulose (Cf. below). The product which is formed at 36 degrees is deposited from the mixture more slowly than that which is formed at 70 degrees, hence it would be more liable to carry down contaminations.

The objection has been urged by Bayliss (5) and by Rohonyi (57) that it is impossible to adequately identify a protein by purely analytical similarities. They point out that Gruebler's pepsin contains a substance which is coagulable by heating and that after this substance has been removed the pepsin solution no longer retains the power of causing the appearance of a precipitate when mixed with a solution of the products of the peptic hydrolysis of casein. Merck's pepsin, on the other hand, yields

* For quantitive details of the methods employed in preparing these substances in bulk consult the communication just cited.

no coagulum on heating and yields no precipitate upon admixture with a concentrated solution of the products of the peptic hydrolysis of casein. From these facts they infer that the substance which I believed to have been enzymatically synthesized paranuclein was, in reality, a compound of the heat-coagulable protein contained in Gruebler's pepsin with the caseoses contained in the solution of the products of the peptic hydrolysis of casein.

While the analytical characterization and identification of a protein substance is extremely difficult and inconclusive, the phenomena of specific immunization, on the other hand, afford us a method of identifying protein substances which, so far as our knowledge at present extends, is decisive and extremely sensitive. The investigations of Wells (75) and of Wells and Osborne (76) have especially demonstrated the high degree of specificity displayed by the immune-bodies which appear in the circulation of animals as a result of repeated administration of foreign proteins. It would appear to be thoroughly established that only protein substances are antigenic, that protein split-products below a certain degree of complexity are non-antigenic, and that the immune-bodies which are developed in response to immunization against a given protein will react only with that protein or with a protein which contains, as an integral portion of its molecule, a large fraction of the molecule of the protein employed in immunization.

Advantage was taken of these facts by Gay and Robertson (20) to investigate more fully the question of the identity or non-identity of the enzymatically synthesized paranuclein with the paranuclein which results from the partial hydrolysis of casein. They found, employing both anaphylaxis and alexin-fixation as indicators of the development of immune-bodies, that the products of the complete peptic hydrolysis of casein are toxic for normal animals. They have, however, no antigenic property, nor any specific intoxicating effect upon animals sensitized to themselves or to paranuclein. Gruebler's pepsin itself is likewise non-antigenic. Paranuclein and the enzymatically synthesized paranuclein, however, both yield specific antibodies and these antibodies react interchangeably with either substance. These results would appear to yield unequivocal evidence of the occurrence of a genuine protein synthesis under the conditions outlined above.

The question remains as to why this synthesis is not achieved, or, at least, does not result in the production of a visible precipitate, when Merck's pepsin is substituted for Gruebler's preparation. While a positive answer to this question cannot as yet be advanced with confidence, it would appear to offer no very valid reason, especially in view of the experiments of Gay and Robertson, for deciding that enzymatic synthesis does occur under defined conditions in mixtures of Gruebler's pepsin and solutions of the products of the complete peptic hydrolysis of casein. It would appear, on the contrary, to afford additional justification for the view, expressed above, that the active agent in the reversion is not identical with the active agent in hydrolysis. The former substance is present in Gruebler's pepsin, but is absent from Merck's preparation.

The occurrence of synthetic processes (i.e., the reverse of hydrolyses) in mixtures of pepsin and concentrated solutions of the peptic split-products of proteins has also been demonstrated, in quite a different manner, by Henriques and Gjaldbaek (27). These observers have shown that if pepsin be added to an acid and concentrated solution of the peptic split-products of a protein, a synthetic process (union of COOH and H_2N groups) occurs, as indicated (*a*) by a progressive increase in the substances precipitable by tannic acid, and (*b*) by a decrease in the groups (H_2N-) which are capable of reacting with formaldehyde. The process occurs at all temperatures lying between 5 and 70 degrees. The more completely split the material in the digest is to begin with the less complex are the products which result, but at the same time their mass is greater. The most complex substances which are formed in consequence of this reversion do not contain much more formol-titratable nitrogen than the original proteins. They have, moreover, shown (28) that the synthesis is reversible, more dilution leading to a progressive increase in the formol-titre. Furthermore, a decrease in the formol-titre also occurs when trypsin is added to a concentrated solution of the products of the peptic hydrolysis of protein.

2. The Probable Nature of the Coaguloses and Plasteins. — It will be observed, on referring to the analytical data obtained by Robertson and Biddle (55), that the carbon content of the paranucleins (49–50 per cent) is considerably lower than that of the proteins (51–55 per cent). In this, and in other respects,

paranuclein differs very fundamentally from the coaguloses, for the carbon-content of the coaguloses, so far from being abnormally low, is abnormally high (58–59 per cent, Kurajev). The syntheses described above are therefore not merely examples of "plastein" or "coagulose" formation.

Kühne and Chittenden (35) observed that if the portion of a protein which is not acted upon by prolonged digestion with pepsin be subsequently subjected to tryptic digestion a jelly separates out which they termed the "anti-albumid coagulum" and which is very slowly attacked by trypsin. Okunev, Lavrov, Savjalov, Kurajev and Umber (44) (38) (39) (62) (63) (36) (50) have prepared similar substances by acting upon peptic digests with rennet, or with salts (ammonium sulphate, Cf. Umber, loc. cit.), or with finely divided insoluble powders (lycopodium), and have termed them "plasteins" or "coaguloses." These substances are albumoses and not proteins, since they are digested by erepsin (Lavrov and Salaskin). Savjalov finds that the amount of plastein which is formed runs parallel with the quantity of hetero-albumose which the protein yields, that is, to the quantity of the difficultly-digestible anti-fraction.

All the circumstances of plastein formation point toward a modified hydrolysis as the condition of their formation. No preliminary concentration of the system is requisite, and no rise in temperature. It appears probable, therefore, that plastein formation is not a reversion of protein hydrolysis, as some authors have suggested, but rather a coagulation due to partial hydrolysis induced by the rennet-like action of ferments upon certain albumoses or peptids,* these playing the part which is played, in our more familiar experience, by calcium caseinate.

In satisfactory harmony with this view Herrmann and Chain (30) have found that the antisera from rabbits immunized with plastein derived from Witte's peptone, tested by the precipitin reaction, react with plastein, not only from Witte peptone but from other proteins, for example, edestin, serum albumin, egg-albumin and the globulin from almonds. They do not react, however, with Witte peptone itself nor with casein. In this respect the plasteins differ very decisively from enzymatically synthesized paranuclein, for the antiserum to synthetic paranuclein reacts not only with normal paranuclein but also with

* Regarding the probable mode of action of rennet (Cf. previous chapter, 6).

the protein, casein, from which paranuclein is derived and of which it forms a large and integral proportion. The plasteins, on the other hand, would appear to form, antigenically speaking, a group of substances which are very closely related to one another and probably contain a large common fraction.

3. The Chemical Mechanics of the Fermentative Synthesis of Proteins. — We have seen in section **1** that in instances of "typical" catalytic hydrolysis, although the degree of hydrolysis in dilute systems, under the influence of a catalysor, may be practically complete, yet, if the solution of the products be concentrated, the station of equilibrium will be shifted and may be shifted to such an extent that the pure products are not in equilibrium and an appreciable quantity of substrate may be restored to the system under the influence of a catalysor. Hence it was natural to suppose, as Taylor did (loc. cit.) that the synthesis of protamin, accomplished through the action of trypsin upon the concentrated products of its hydrolysis, is an example of the reversion of a "typical" catalysed reaction in which the catalysor plays no part in determining the final equilibrium. Nevertheless it must be recollected that the validity of this view can by no means be regarded as proven until it has been shown that the station of equilibrium in the presence of the catalysor is definitely the same as it is in its absence, and this has not been done; indeed, the great technical difficulty of the problem, as it at present appears to us, discourages the attempt. Moreover, there are many facts, a number of which have been alluded to in the previous chapter, which speak very decidedly against the view that the fermentative syntheses of proteins are instances of "typical" catalytic reversion. To those which have already been dwelt upon may be added the following.

We have seen that the velocity of protein hydrolysis is, under certain conditions, directly proportional to the concentration of the ferment, under others proportional to the square root of the ferment-concentration (Chap. XVI, section **3**). If the proteolytic ferments act as "typical" catalysors and do not in any way affect the final equilibrium in the system, then the velocity of synthesis must also vary directly or as the square root of the ferment-concentration, for otherwise the ratio between the velocity constants of the forward and opposed reactions would be a function of the ferment-concentration and, since the station of equilibrium

is determined by this ratio, the equilibrium of the system would also be a function of the ferment-concentration. The synthesis of paranuclein *A* through the agency of pepsin affords us an opportunity of determining the influence of the ferment-concentration upon the velocity of protein synthesis, since the product is rapidly formed and can readily be determined quantitatively. The following was the experimental procedure:

The products of the complete peptic hydrolysis of $N/10$ potassium hydrate neutralized to litmus by casein * were evaporated to one-sixth of their volume and filtered. Seventy-five cubic centimeters of the clear, deep yellow filtrate were placed in each of six flasks and to each, respectively 0, 5, 10, 15, 20 and 25 cc. of 10 per cent pepsin (*puriss. sicc.* Gruebler's) were added, and the total volume in each flask was made up to 100 cc. by the addition of distilled water. After the addition of toluol, the tightly-stoppered flasks were set aside at 36 degrees for 22 hours. At the end of this time the flasks containing 25 and 20 cc. of 10 per cent pepsin, respectively, contained heavy precipitates, while that containing 15 cc. contained a precipitate and those containing 5 and 10 cc. had undergone no change other than a slight increase in opacity. The contents of the flasks were now filtered through rapid-filtering papers and the precipitates were washed with distilled water until colorless filtrates were obtained; in all cases the filtrates gave no precipitates or increase in opalescence upon the addition of acetic acid. The filters were then washed with 10 cc. of $N/10$ potassium hydrate and the filtrates were collected in water containing 20 cc. of $N/10$ acetic acid.

* Prepared as follows: To 6 litres of $N/50$ sodium or potassium caseinate, neutral or faintly acid to litmus, were added two gram of Gruebler's pepsin *puriss. sicc.* which had previously been dissolved in a little water. This solution, after thorough mixing, was allowed to stand at 36 degrees for 10 days, 2 more grams of pepsin being added after the first 4 days (in the presence of toluol), and was then sterilized by steam at 100 degrees and filtered through hardened filter paper. To the filtrate were then added two more grams of pepsin, dissolved, as in the previous cases, in a little water, toluol introduced, and the solution was again allowed to stand at 36 degrees for 7 to 8 days; it was then again sterilized by steam at 100 degrees and filtered through hardened filter paper. The filtrate thus obtained is of a clear yellow color with little or no opalescence and gives no trace of a precipitate or opalescence upon the addition of acetic acid either before or after neutralization with alkali; hence both casein and paranuclein are completely absent from the solution.

The filter papers were then thoroughly macerated in dilute potassium hydrate and the magma thus prepared was filtered and washed with distilled water, the filtrate and washings being collected in the beaker which received the first washings with $N/10$ potassium hydrate. Care was taken to prevent more than a slight excess of acetic acid from being finally present in this beaker. The precipitate which formed in the beaker settled rapidly in small flocculi, was collected upon a soft rapid-filtering paper and thoroughly washed with distilled water until the washings were neutral to litmus. In all cases the filtrates and washings were perfectly clear and free from protein. The papers and precipitates thus obtained were macerated in water containing a known quantity (10 cc.) of $N/10$ potassium hydrate, the magma thus obtained was diluted to about 200 cc., phenolphthalein (4 drops of 2 per cent alcoholic solution) was added and the solutions thus prepared were titrated to neutrality with $N/10$ hydrochloric acid. A weighed amount (227 milligrams) of paranuclein A prepared in the manner described in section **1** was dissolved in about 100 cc. of distilled water containing exactly 10 cc. of $N/10$ potassium hydroxide, and the solution was titrated to neutrality with $N/10$ hydrochloric acid and phenolphthalein indicator; in this way it was found that 1 gram of paranuclein A neutralizes 4.8 cc. of $N/10$ potassium hydroxide*; hence 1 cc. of $N/10$ alkali = 0.208 gram of paranuclein A and we can estimate from the determinations described above the amount of paranuclein A produced in each of the mixtures by varying amounts of pepsin. The following were the results obtained:

Amount of pepsin in 100 cc. of solution	Milligrams of paranuclein A produced at the end of 22 hours
25 cc. of 10 per cent	296
20 cc. of 10 per cent	210
15 cc. of 10 per cent	162
10 cc. of 10 per cent	0
5 cc. of 10 per cent	0
0 cc. of 10 per cent	0

* I have found that upon standing in the presence of excess of alkali, the amount of alkali neutralized by a given quantity of paranuclein A increases slightly. I have been unable to determine whether this phenomenon is due to the slow dissolving of microscopic suspended particles or whether it is due to

From these results it is clear that the velocity of reversion is by no means directly proportional to the concentration of pepsin, nor even proportional to the square root of the ferment-concentration. While the velocity of synthesis in the most concentrated solutions (25 to 15 cc. of 10 per cent pepsin in 100 cc.) roughly approximates to direct proportionality to the concentration of ferment, in the more dilute solutions (10 to 5 cc. of 10 per cent pepsin in 100 cc.) the velocity of synthesis falls off with extraordinary rapidity as the concentration of the ferment diminishes. Making every possible allowance for experimental error arising out of loss of material during the estimation, an increase in pepsin-concentration from 1.0 per cent to 1.5 per cent multiplies the velocity of the synthesis over 10 times, while an increase in pepsin concentration from 1.5 per cent to 2.5 per cent only doubles it; these facts are obviously irreconcilable alike with direct proportionality between the velocity of synthesis and the concentration of ferment, and with the Schütz rule of proportionality to the square root of the concentration of the ferment. The velocity of reversion does not bear the same relation to the concentration of ferment that the velocity of hydrolysis does; hence the ratio of the velocity constants of hydrolysis and reversion must be dependent upon the concentration of the ferment, or, in other words, the *equilibrium between protein and its products must, to some extent, be altered by pepsin.*

We have seen that a reversion of the hydrolysis of paranuclein can be brought about without any previous concentration of the products of its hydrolysis, provided the temperature be raised to 60–70 degrees. In the light of this fact there can be little doubt that a shift in the station of equilibrium between paranuclein *A* and its products occurs as a result of the addition of pepsin and that this shift in equilibrium is favored by a rise in temperature.

That a shift in equilibrium is involved even at lower tempera-

hydrolysis (56). The number of cubic centimeters neutralized by one gram given above is the lower figure, obtained directly after complete solution, judged by the disappearance of obvious particles within the solution. I have obtained figures as high as 5.2 after allowing the solution to stand for about an hour at a warm temperature before titration. The titrations in the experiment described were performed immediately after the complete disappearance of obvious particles.

tures is shown by the fact that if a sufficiency of *dry* pepsin* be added to the *unconcentrated* products of the complete peptic hydrolysis of $N/10$ sodium caseinate synthesis will occur at 36 degrees. I have found about 2 grams of pepsin (Gruebler's *puriss. sicc.*) per hundred cubic centimeters sufficient for this purpose. That the equilibrium is still further shifted by a rise in temperature is shown by the following experiment:

To 300 cc. of the unconcentrated products of the peptic hydrolysis of $N/10$ sodium caseinate were added 6 grams of dry pepsin. After 48 hours at 36 degrees (in the presence of excess of toluol) a precipitate had formed, while the supernatant fluid remained somewhat opalescent; after 6 days the supernatant fluid was quite clear and a bulky precipitate lay at the bottom of the flask; the clear fluid was now decanted from the precipitate and divided into two parts; the one was kept at 36 degrees and the other at 65 degrees; within 8 hours a precipitate had formed in the latter, the supernatant fluid being strongly opalescent, while the part of the solution which remained at 36 degrees had developed no trace of precipitate or opalescence after a period of two weeks. It is clear, therefore, that the system had arrived at equilibrium at 36 degrees before the fluid was decanted, since this fluid must have been "saturated" with paranuclein *A* (soluble in these acid solutions only to an undetectable extent) and any further formation of paranuclein *A* would have resulted in an increase in opalescence if not in actual precipitation. This did not occur, however, even during a period of two weeks. Yet at 65 degrees a fairly abundant precipitate was produced within 8 hours.

It is clear, therefore, that in the enzymatic synthesis of paranuclein *A* a marked shift in equilibrium is involved, and we must therefore definitely abandon the view that pepsin is a "typical" catalysor which does not, to an appreciable extent, participate in the reactions which it catalyses.

The existence of a similar influence of trypsin upon the equilibrium in protein solutions is possibly indicated by the investigations of Walters (73), who has shown that whereas the time-relations of the hydrolysis of casein by trypsin are very accurately

* *Dry*, that is to say, not in solution, for otherwise water is added to the system and the solution of products is actually diluted by the addition of the ferment.

defined by the mono-molecular formula, terminating only when hydrolysis is practically complete, the *autohydrolysis*, on the contrary, which caseinates undergo in aqueous solution in the absence of enzymes falls off in velocity with time much more rapidly than would be indicated by the monomolecular formula, so that the process comes to a stop when a very large proportion of the protein is still unhydrolysed. The products of the tryptic hydrolysis of casein exert, moreover, an almost negligible effect upon the velocity of autohydrolysis, even when added in very high concentration. We must infer, therefore, either that trypsin, in dilute solutions, shifts the equilibrium between protein $\rightleftarrows$ products towards the right, or else that the rapid slowing-down of the autohydrolysis is due to the attainment of a "false equilibrium," i.e., an indefinite delay in the attainment of equilibrium due to the interposition of a specifically slow reaction in a series of catenary reactions, each one of which utilizes the products of the preceding reaction.

4. Reciprocal Catalysis. — We have seen that enzymatic synthesis of paranuclein *A* may occur at temperatures 10 to 15 degrees in excess of that at which the hydrolysing activity of pepsin is rapidly and completely destroyed. This invites adoption of the view, previously expressed by Euler (17) (18) that the synthesizing and hydrolysing forms of the enzyme are not identical. Euler believes that two varieties of every hydrolysing enzyme exist, the one accelerating, primarily, hydrolysis, the other accelerating, primarily, synthesis, and that under definite conditions a definite equilibrium exists between the two enzymes. He bases this view, in the main, upon the non-constancy of the velocity-constant of certain enzyme-accelerated reactions with varying substrate-concentration, and upon the non-identity of the synthesized product (isomaltose) derived by the action of maltase upon concentrated glucose with the maltose from which the glucose was derived by hydrolysis, and of the product (isolactose) obtained by the action of lactase upon a concentrated solution of galactose and glucose with the lactose from which the galactose and glucose are derived.* Euler believes that the "anti-trypsins" found in serum and in egg-white are simply the synthesizing form of the enzyme and that the anti-pepsin and

* It is stated, however, that even sulphuric acid, acting upon a concentrated solution of glucose, gives rise, not to maltose but to isomaltose (19).

anti-trypsin which appear in the circulation after the injection of the enzymes do so as a result of the production of the synthesizing form in restoration of the equilibrium between the synthesizing enzyme and the hydrolysing form.

The hypothesis which I have put forward (53) in explanation of the phenomena of protein hydrolysis and synthesis is a modification and extension of Euler's hypothesis, and involves the view that these processes are examples of which I have termed "reciprocal catalysis." The essential features of this hypothesis have already been embodied in equations (A) to (D) in Chap. XV, section **1.** For convenience of reference they may be recapitulated here.

We know that during or preceding the hydrolysis of proteins by proteolytic enzymes the ferment combines with the substrate (previous chapter, **2**). Furthermore, the reaction consists in the introduction of the elements of water into $-COH.N-$ bonds of the protein molecule, each bond which is attacked yielding two molecules. We may therefore represent the reaction of hydrolysis as follows:

$$\text{(A)} \qquad -COH.N- + HFFOH \rightarrow -COH(HF).N(FOH)-$$

$$\text{(B)} \qquad -COH(HF).N(FOH)- \rightarrow -COOH + H_2N- + FF$$

while, subsequently, the dehydrated form of the enzyme (FF) reacts with water to regenerate the hydrated form:

$$\text{(D)} \qquad FF + H_2O \rightleftarrows HFFOH$$

enzymatic *synthesis* of a protein consisting in the reverse of these reactions.

The net result of the first two reactions is the transference of the elements of water from the ferment to the substrate molecule, while, in the third reaction, the ferment recoups itself from the medium. Provided the station of equilibrium in the reaction (D) lay far enough to the right and the velocity of this reaction

measured from left to right were great compared with that of either of the reactions (A) and (B) measured from right to left, and provided, also, the quantity of enzyme combined at any instant with the substrate were unappreciable we would obtain, for the kinetics of the reaction of hydrolysis, the monomolecular equation, in which the velocity constant of hydrolysis would be proportional to the ferment-mass. If these conditions were not fulfilled we would obtain, provided the proportion of the dehydrated to the hydrated enzyme did not alter very appreciably during the reaction, the differential equation

$$\frac{dx}{dt} = \frac{k_1 F (A - x)}{1 + \alpha (A - x) - \beta x^2}, \qquad \text{(i)}$$

or its integrated form:

$$\log \frac{A}{A - x} + \frac{\alpha + \beta A}{1 - \beta A^2} x + \frac{\beta}{2 (1 - \beta A^2)} x^2 = \frac{k_1 F}{1 - \beta A^2} t. \qquad \text{(ii)}$$

(Cf. Chap. XVI.) For the reversion we would obviously obtain the differential equation:

$$-\frac{dx}{dt} = \frac{k_2 F x^2}{1 - \alpha (A - x) + \beta x^2}. \qquad \text{(iii)}$$

From equations (A) to (D), applying the mass-law, it will be obvious that the quantity of enzyme dehydrated by unit mass of substrate will be dependent upon the mass of the hydrated form which is present, consequently, the constant α (and similarly the constant β) will be dependent upon the mass of ferment present, and will also, if an appreciable shift in equilibrium represented by equation (D) occurs as a result of the hydrolysis, alter somewhat in value as hydrolysis proceeds *; hence the final position of equilibrium, when the velocity of reversion is equal to the velocity of hydrolysis, must be dependent upon the mass of ferment.

Quite independently of the mathematical formulation of the time-relations involved in this process, it will be clearly seen that the presence of the enzyme must result in a greater or smaller shifting of the point of equilibrium between the protein and its products when we reflect that the hydrated form HFFOH, according to the above scheme, only accelerates the hydrolysis, while the anhydrous form FF only accelerates the synthesis, and since

* It is for this reason that we cannot employ equations (i) and (iii) to determine the station of equilibrium in these systems.

these are, in general, present in unequal concentrations, the equilibrium between the forward and reverse reactions of protein hydrolysis must be shifted. Since, however, for every shift in equilibrium, there must be a corresponding expenditure of energy, the equilibrium between the anhydrous and hydrated forms of the enzyme must also be shifted by the protein; just as the *enzyme* accelerates the hydrolysis of the *protein* more than its synthesis when the hydrated form of the enzyme is initially present in considerable excess of its anhydrous form, so the *protein* accelerates the dehydration of the *enzyme* more than its hydration because it is initially present in great excess of its products of hydrolysis. This latter fact will itself lead to a slowing of the hydrolysis of the protein since the hydrated (and hydrolysis accelerating) form of the enzyme is thereby diminished in concentration; as the hydrolysis proceeds, however, this effect will diminish; the products of the protein hydrolysis will tend to increase the proportion of the hydrated form of the enzyme to the anhydrous form, and the rate of hydrolysis will increase at the expense of the rate of synthesis. Ultimately, it is evident that a condition of equilibrium must be reached in which the station of equilibrium between the protein and its products is shifted further in the direction protein → products than its position in the absence of the enzyme, while the station of equilibrium between the hydrated and anhydrous forms of the enzyme is shifted further in the direction hydrated form → anhydrous form than its position in the absence of the protein. This position of equilibrium will depend, obviously, upon the total concentration of the enzyme and of the substrate, respectively, and once attained, a further addition of substrate would reinaugurate the hydrolysis of protein, it is true, because the active mass of hydrolysable protein would thus be increased, but it would shift the point of equilibrium in the direction products → protein; addition of enzyme would shift the station of equilibrium in the direction protein → products, as has been found by Bayliss and others (4) (68). This latter statement, however, holds good only while the water in the system is in great excess of the enzyme, so that varying concentration of the enzyme does not appreciably affect the proportion subsisting between the hydrated and anhydrous forms at equilibrium in the absence of proteins. If, however, the enzyme be very concentrated, then the proportion of water to enzyme (Cf. Eq. (D)) will appreciably affect the equilibrium be-

tween the hydrated and anhydrous forms of the enzyme, and there will be present a relatively greater proportion of the anhydrous (synthesis accelerating) form FF; hence, under these conditions, a further addition of enzyme will shift the equilibrium of the protein in the direction products → protein.

A remarkable feature of the syntheses of protein through enzyme agency which have been accomplished by Taylor and myself is the high concentration of enzyme which has to be employed; the reason for this is now clear; the highly concentrated enzyme contains a greater proportion of the anhydrous form and it shifts the equilibrium of the protein in the direction of synthesis. It is now clear, also, why a sufficiently high concentration of enzyme will actually bring about synthesis of protein in the *unconcentrated* products of the complete hydrolysis of a solution of protein, brought about by the agency of *dilute* enzyme. The dependence of the velocity-constant of hydrolysis, calculated from the monomolecular formula, upon the initial concentration of the substrate (Cf. Chap. XVI, section **3**) is also readily comprehended, since, as pointed out above, increase in the concentration of substrate must, in the initial stages of hydrolysis, lead to an increase in the proportion of the anhydrous to the hydrated form of the enzyme, and hence to a diminution of the concentration of the hydrolysis-accelerating form of the enzyme; hence the velocity-constant of hydrolysis diminishes with increasing substrate-concentration.

5. The Influence of Temperature upon the Enzymatic Synthesis of Proteins. — The action of high temperatures in enhancing the synthesizing power of the enzyme while diminishing or abolishing its power of accelerating hydrolysis may be interpreted in either of two ways. Either the high temperature destroys the hydrated form by accelerating its hydrolysis, while leaving the anhydrous form unaffected, so that during the period that the anhydrous is changing into the hydrated form only the synthesis of the protein is being accelerated, and not its hydrolysis, or, more probably, the high temperature actually shifts the equilibrium of reaction (D) in the direction HFFOH → FF + H_2O, a conception which, if we regard the proteolytic enzymes as being substances analogous to proteins, is in good accord with our knowledge of the influence of heat upon these bodies (Chap. XII). The fact that heating a proteolytic enzyme leads to the formation of substances

which strongly retard protein *hydrolysis* has been observed by a number of investigators. Thus Schwarz (64) has shown that if solutions of pepsin be heated to 80 degrees for some time and then added to peptic digests the digestion is greatly retarded, while Pollack (46) had previously obtained, by heating pancreas extracts to 70 degrees, a substance which greatly retards tryptic hydrolysis of proteins; he further observed that this substance is a colloid since it does not pass through the membrane of a dialysor. Hensel (29) has observed that if the mucous membrane of a stomach be treated with acidulated water at 50 degrees, the watery extract thus obtained contains an organic substance which greatly retards peptic hydrolysis of proteins. Bearn and Cramer (6) have found that solutions of rennet which have been heated to from 56 to 60 degrees exert an inhibitory influence on the activity of unheated rennet. Bayliss (4) has found that heated trypsin not only greatly delays the tryptic hydrolysis of casein but would appear to inaugurate a chemical reaction in the reverse sense, since mixtures of heated trypsin and a caseinate, instead of *increasing* in conductivity with time, at first *decrease* in conductivity, later slowly increasing. Similar phenomena have been observed with other enzymes (15) (6).

The anti-tryptic actions of such substances as egg-white (72) (4), normal blood serum (22) (17) (49) (37) (12) (24) and extracts of intestinal worms (13) and the anti-peptic and anti-tryptic actions of the bodies produced in the circulation by the injection of pepsin and trypsin into living animals (58) (1) (74) have usually been attributed to the formation of more or less stable compounds between the ferment and the anti-ferment. One would be inclined to similarly attribute the action of heated solutions of pepsin and trypsin in inhibiting these ferments to the formation of compounds between the heated and the normal ferment were it not that the heated ferment, although deprived of its power to accelerate hydrolysis of protein, markedly accelerates its synthesis. The inhibitory action of the heated ferment is thus clearly seen to consist in bringing about a change which is opposite in sense to that which is brought about by the normal ferment.* We see,

* Bayliss, regarding the phenomenon from the standpoint of Ehrlich's "side-chain" hypothesis, has advanced the opinion that the decrease in conductivity which is observed upon the addition of heated ferment to a solution of protein is to be attributed to the destruction of the "zymophore," or

also, that the loss of proteolytic activity which occurs on heating solutions of proteolytic ferments is to be attributed, not to autohydrolysis of the ferment, but to the partial or complete conversion of the hydrolysing enzyme into the synthesizing form. From the above account of the chemical mechanics of the enzymatic hydrolysis and synthesis of proteins it would appear that the "inactivation" of proteolytic ferments by heat (provided actual coagulation does not occur) should be a reversible process, if not in the pure enzyme-solution, at any rate in the presence of products of protein hydrolysis.* Owing to the fact that the enzymatic activity of hydrolysing enzymes has in the past been identified with their power to accelerate hydrolysis, the application of heat to enzyme solutions has always been regarded as involving the "destruction" of the enzymes and restoration of their activity under varying conditions following a return to lower temperatures has not been anticipated and therefore has not been looked for with any especial care. Nevertheless, Bayliss (4) has observed that the power of heated trypsin to accelerate protein hydrolysis is to some extent regained after the heated enzyme has been mixed with protein for some time, and Howell (33) has observed that thrombin is not "destroyed" by moderate heating, but is *reversibly* thermolabile inasmuch as it regains its activity on treatment with alkali and subsequent neutralization at lower temperatures. Gramenisky (21) has also observed that oxidase inactivated by heating rapidly regains its activity on standing at lower temperatures.†

6. The Thermodynamics of the Enzymatic Hydrolysis and Synthesis of Proteins. — The thermodynamical aspect of the hypothesis developed above is of interest. As I have pointed out, the shift in equilibrium of the system protein $\rightleftarrows$ products towards

digesting group of the ferment while the "haptophore" group, by which the enzyme attaches itself to the protein molecule, is unaffected. The heated ferment he terms "zymoid," and he believes that the initial decrease in the conductivity of mixtures of casein and heated ferment is to be attributed to the formation of a compound between the "zymoid" and the protein. This view, of course, fails to account for the *reversion* of protein hydrolysis which is brought about by heated pepsin.

* Since, as we have seen in the preceding section, these will catalyse the reaction $FF + H_2O \rightarrow HFFOH$.

† In this connection it is of extreme significance that the oxidase employed by Gramenisky behaved, at 80 degrees, no longer as an *oxidase* but as a *reductase*, or reducing ferment.

the *right* which results from the introduction of a proteolytic enzyme into the system must result in a corresponding shift in the equilibrium of the system anhydrous enzyme ⇄ hydrated enzyme towards the *left* and vice versa. Since the enzyme is usually present in small concentrations compared with the protein, the shift in the equilibrium between the two forms of the enzyme must be large compared with that of the equilibrium between the protein and its products; or else *the energy expended in a shift of the enzyme-equilibrium must be great compared with the energy expended in a shift of the protein equilibrium.* The latter appears to be the more probable view; since, otherwise, the shift in the protein-equilibrium would probably be in all cases too small to be observed, and, moreover, we know that the reaction of protein hydrolysis is only very faintly exothermic (65) (23) (25) (40) so that the energy change involved in a shift in the equilibrium between protein and the products of its hydrolysis is probably very small, and a slight shift in the equilibrium of a relatively very small amount of enzyme might suffice, if the reaction of enzyme hydration involved a relatively considerable energy-change, to provide the energy for a considerable change in the equilibrium of a relatively large mass of protein.

It will be observed, therefore, that the processes of the enzymatic hydrolysis and synthesis of proteins do not necessarily involve any deviation from the laws of thermodynamics, in spite of the fact that the enzyme which catalyses these processes markedly influences the equilibrium between the protein and its products. In the analogous case which is afforded by the enzymatic synthesis and hydrolysis of fats Dietz (14) assumes that because the station of equilibrium is altered by the presence of the enzyme the second law of thermodynamics must be violated in these systems, for, he argues, since the catalysor (enzyme) can be withdrawn from the system without the expenditure of any work, it would only be necessary to allow the system to come to equilibrium in the presence of the enzyme and then withdraw the enzyme and allow it to come to its normal station of equilibrium and then reintroduce the enzyme, and so forth, to bring about an endless cycle of changes and to secure an unlimited quantity of energy without the expenditure of any work. But in this argument, it should be pointed out, Dietz assumes the answer to the very question at issue; he assumes in a word, that the enzyme in question is a "typical" catalysor,

not entering to any appreciable extent into the reaction, and therefore not requiring the expenditure of any energy to withdraw it from the system. Most investigators will prefer to assume, from Dietz' results, that lipase is not a "typical" catalysor, but enters appreciably into the reaction which it catalyses (10) (11).

7. Biological Applications. — It may here be noted that the hypothesis outlined above is not inconsistent with the generally expressed view that the enzyme may be recovered without appreciable loss of activity from a protein digest which has reached equilibrium. Even if, in consequence of the presence of protein, the station of equilibrium between the two modifications of the enzyme has been shifted in such a direction as to diminish its power of accelerating hydrolysis, yet upon removal from the system the enzyme would regain its normal equilibrium, although, of course, this process might involve a greater or a shorter period of time. The energy thus apparently gained would be *slowly* lost through the slow (because of the absence of catalysors) regaining of equilibrium between the protein and its products. The protein $\rightleftarrows$ products system would, in other words, be left "supersaturated" in respect to products, or, were the enzyme in the system highly concentrated, or did it, for any other reason, initially contain a high proportion of anhydrous (synthesis-accelerating) form so that it *gained* power of accelerating hydrolyses as a result of the presence of protein, then the protein $\rightleftarrows$ products system would be left, by removal of the enzyme, "supersaturated" with respect to protein. In the former case the anhydrous $\rightleftarrows$ hydrated enzyme system would be left supersaturated with respect to the anhydrous (synthesis-accelerating) form, in the latter, with respect to the hydrated (hydrolysis-accelerating) form. By mechanical separation of the enzyme and substrate at various periods in living tissues it is obvious that a cycle of such operations could occur, the enzyme being utilized over and over again, now for hydrolysis and now for synthesis. It is obvious that if such reciprocal relations as that outlined above find a place in living material, the organism may be enabled thereby to temporarily and locally store up large quantities of energy. The significance of this possibility in the general interpretation of life-phenomena is patent.

Finally, it may be pointed out that the conceptions which I have developed above enable us to understand how the living organism, through temporary mechanical or chemical separation and localiza-

tion of the synthesizing forms of these enzymes can bring about protein-synthesis even in the presence of the considerable quantity of water which living tissues contain. If we regard the enzymes as "typical" catalysors then, as Bradley has pointed out (9), enzymatic synthesis of fat (and the same may be said of the enzymatic synthesis of protein) could only occur under conditions approximating to dessication, conditions which it is very difficult to picture as occurring in living tissues.

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APPENDIX

THE TECHNIQUE OF ELECTROCHEMICAL MEASUREMENTS IN PROTEIN SYSTEMS

The carrying out of accurate electrochemical measurements in solutions which contain proteins is attended by a number of difficulties which result in part from the physical characteristics of such solutions and in part from the instability of the proteins and their tendency to undergo hydrolysis. The following is a description of the technique which I have found it best to employ when obtaining such measurements in solutions of casein especially* but also of ovomucoid and serum globulin. My experimental results cited in Chaps. VIII to XI, inclusive, were obtained by these methods; they are obviously applicable, with necessary or advisable modifications, to aqueous solutions of the majority of proteins.†

In the gas-chain determinations two platinized platinum electrodes saturated with hydrogen are used, the one being dipped in the solution of unknown hydrogen- or hydroxyl-concentration, the other in a solution of known hydrogen- or hydroxyl-concentration, the latter being, as a rule, the solution of acid or base in which the protein is dissolved and the former the solution of protein. The electrodes which I employ are of a design due to my colleague,

* Robertson, T. Brailsford, Journ. of Physical Chem., 14 (1910), p. 528.

† For the technique employed in determining H^+ and OH' concentrations and methods used in the computation of results, consult also

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Dr. F. G. Cottrell; they have been described in detail by Schmidt and Finger.*

The hydrogen is generated by the electrolysis of 6 per cent (by volume) sulphuric acid, in an apparatus which has previously been described by Schmidt and Finger in the paper cited above. To guard against the possibility of any oxygen, ozone or hydrogen peroxide being carried over with the hydrogen from the generator, the gas is passed through a heated glass tube which is loosely filled with platinized asbestos and which has wrapped around it for a distance of about 20 cm. a coil of fine resistance-wire, the internal diameter of the tube being about 0.5 cm. The hydrogen is completely cooled before it reaches the electrodes, because, after leaving the heater, it passes through a narrow glass tube, partly filled with glass wool, about 70 cm. long, leading to the water-bath; since a considerable portion of this tube is within the incubator which holds the water-bath, the temperature of the hydrogen, when it reaches the electrodes, may be considered as that of the incubator itself. The coil of the heater attached to the hydrogen-generator is heated by a portion of the same current (110-volt circuit) which generates the hydrogen, the current being led into the generator through 4 lamps in parallel, one of the lamps being connected in series with the coil. In order to maintain the pressure of hydrogen which is necessary to drive it through the electrodes, the oxygen which comes off from the outer cylinder of the generator is carried off by a tube which dips into a column of water, the depth of the opening of the tube in the water being adjusted until the levels of the fluid in the inner and outer cylinders of the generator are approximately equal.

The complete chain is arranged as follows: The syphon-tube of the "half-element" containing the fluid into which the electrode dips is immersed in a beaker filled with the same fluid. Thus the "half-element" containing the solution of unknown hydrogen- or hydroxyl-concentration is in fluid connection with a beaker which contains the same solution and the "half-element" containing the solution of known hydrogen- or hydroxyl-concentration is in fluid connection with a beaker filled with that solution. The two beakers are then connected by a U-tube filled with agar saturated

* C. L. A. Schmidt and C. P. Finger, Journ. of Physical Chem., 12 (1908), p. 406. For improvements in the original design, Cf. C. L. A. Schmidt, Univ. of Calif. Publ. Pathol., 2 (1916), p. 157.

with KCl, thus effectively preventing any mixing of the two solutions and diminishing any contact-difference of potential between them.* The gas is passed through the electrode which dips into the solution containing protein (the solution, that is, of unknown hydrogen- or hydroxyl-concentration) at the rate of from one to two large bubbles per second, and the excess of gas is allowed to pass through the other electrode. The whole chain is immersed in a small water-bath which is placed inside an incubator maintained at a temperature lying between 31 and 32 degrees (Cf. below). It was thought necessary, at first, not to permit the hydrogen to escape into the incubator, lest it should be ignited, on opening the door of the incubator, by the flame beneath; consequently the electrodes were inserted into the half-elements through tightly-fitting rubber stoppers, and rubber tubes were attached to the side-tubes of the half-element and carried outside the incubator and the cupboard within which the incubator is set up. For reasons which will shortly be described, however, this procedure was, of necessity, abandoned and the gas was permitted to escape into the incubator. There appears to be no danger involved in this procedure. The incubator is of the usual double-walled type employed by bacteriologists; its internal dimensions are 45 cm. wide by 24 cm. deep by 48 cm. high. It is provided with two doors, the outer of the usual double-walled type, the inner a glass door through which thermometers, etc., can be read without disturbing the apparatus or causing fluctuations of temperature by currents of air. The inner chamber is provided at the top with two small air-exits.

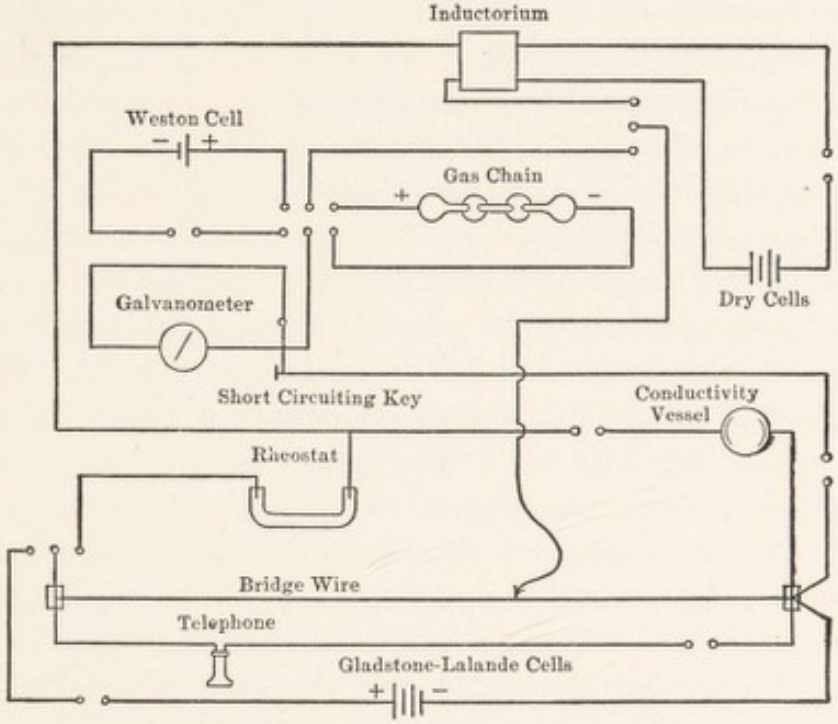
The potentials between the electrodes of the chain are measured on a 100-cm. potentiometer bridge-wire (previously standardized).† For the detection of the zero-point on the bridge-wire I have employed a D'Arsonval galvanometer provided with a damping coil; this instrument gave a decided deflection with the potential corresponding to 1 mm. displacement on the bridge in all of the experiments described in Chap. IX. The constant fall in potential from end to end of the potentiometer wire is best supplied by a good storage-cell; but I have found an arrangement of four Gladstone-Lalande cells (Model G-50), two in parallel and two

* N. Bjerrum, Zeit. f. physik. Chem., 53 (1905), p. 428.

† The potentiometer manufactured by the Leeds & Northrup Co. is, however, more convenient to use, and also more accurate.

in series, very satisfactory. The potential derived from these is measured against a standard Weston cell just before and just after every reading. The potential provided by the Gladstone-Lalande cells is quite sufficiently constant during the progress of an observation, provided they are always short-circuited for 15 to 20 minutes beforehand.

The electrodes are platinized with Lummer and Kurlbaum's solution. They are very carefully washed, both within and without, between the observations, first in a stream of distilled water and then in the solution in which they are about to be immersed; every few days, if they are in constant use, they should also be washed in chromic and sulphuric acid solution and then, after thorough washing in a stream of distilled water, allowed to soak for 12 hours in distilled water. From time to time they should be replatinized.



The conductivity-vessel which I employ is of the Kohlrausch-Holborn type, with a thermometer dipping into the fluid between the electrodes. This is immersed in the same water-bath as the gas-chain, and the conductivities of the solutions are measured at exactly 30 degrees. The electrodes are platinized with Lummer and Kurlbaum's solution. The same bridge-wire is employed for determining the conductivities and for determining potentials. A telephone is employed to detect the zero-point and the alternating

current is supplied by an inductorium of the Ostwald type. The rheostat reads correctly to within 0.01 per cent and supplies any desired resistance between 0.1 ohm and 1000 ohms. The resistance in the rheostat is always adjusted until the zero-point is exactly in the middle of the bridge; the resistance in the rheostat is then, of course, exactly equal to that of the conductivity-vessel filled with the fluid under investigation. The arrangement of the wiring is represented in the preceding diagram.

The wires of the conductivity circuit are all of "bell-wire" so that their resistance can be neglected. The wires of the potentiometer-circuit are somewhat thinner. All of the wires are insulated and carefully supported on glass, and are never allowed to touch the table. Where it is necessary to carry wires through the table, for example, they are run through glass tubes. The wire connected with the slider on the bridge is encased in rubber tubing.*

I have pointed out in Chaps. V and XII that it is difficult to obtain solutions of caseinates of much higher acidity than neutrality to litmus by merely shaking up casein in solutions of bases, — not because casein will not form such solutions, but because, although it dissolves at first with considerable rapidity, after the excess of alkali is neutralized, further casein dissolves with extreme slowness. Now the conductivities of solutions containing proteins must be measured as soon as possible after the complete solution of the protein, for, otherwise, the hydrolysis which the protein undergoes, the more rapidly the more alkaline its solution, introduces a serious error into the determination. Hence it is imperative, not only that the conductivities of these solutions should be determined as soon as possible after complete solution of the protein, but, also, that the preparation of the solution, after the introduction of the protein, should consume as little time as possible. Hence all of the solutions of casein which are acid to phenolphthalein are best prepared by dissolving the casein in excess of alkali and then neutralizing this excess with acid. This procedure, if gas-chain measurements are being made at the same time, has the further advantage that the absolute conductivities of the solutions thus obtained being higher, the detection of the zero-point on the bridge-wire by means of a gal-

* More accurate conductance measurements may be made by employing the method of E. W. Washburn. Cf. "The Measurement of Conductivity of Electrolytes," Leeds and Northrup Co., Philadelphia, 1916.

vanometer is rendered easier and a less sensitive galvanometer can be employed than would otherwise be required. It should not be forgotten, however, that although a small concentration of KCl, for example, does not alter the dissociation of caseinates of the alkalies nor the combining-capacity of casein for bases (Chap. VIII) yet this is certainly not the case with many other proteins.

In this connection it is to be carefully noted that if the solution employed to dissolve the casein be too alkaline, little or nothing is gained by the rapidity of its solution, because the rapidity of its hydrolysis is also great. On the other hand, as I have said, if too small a proportion of free alkali is present solution is so slow that hydrolysis is extensive. Evidently an avoidance of both of these extremes will yield the most satisfactory results. I have found the proportion 10 cc. of $N/10$ KOH to 1 gram of casein to be the most satisfactory solvent for this protein. In the experiments described in Chaps. IX and X, save in the formation of solutions containing more than 100×10^{-5} equivalents of base per gram of casein, therefore, the procedure was as follows: Part of the KOH was neutralized until the portion unneutralized stood in this proportion to the mass of casein undergoing solution, the casein was dissolved therein, and then the desired final proportion of KOH to casein was attained by the further addition of HCl. For example, it was desired to obtain a solution of 1 per cent casein in 0.005 N KOH. Accordingly, to 75 cc. of 0.1 N KOH were added 50 cc. of 0.1 N HCl and in this were dissolved 2.5 grams of casein; upon the attainment of complete solution and while stirring, 12.5 cc. of 0.1 N HCl were added and the whole solution was made up to 250 cc. with distilled water; another solution was made up in precisely the same way but without the introduction of the casein; the conductivities of both solutions were than determined and their difference ($= \lambda$) estimated.* The two solutions were arranged in the gas-chain in the manner described above and the potential between the gas-electrodes immersed in them determined. Hence, of course, the OH' concentration of the solution not containing casein being known, that of the solution containing casein was

* Since the concentration of KCl is the same in both solutions it adds the same amount to both conductivities and this disappears in their difference; that is, presuming that the caseinate does not combine with or decompose the KCl and that the presence of excess of K^+ ions does not depress the dissociation of the potassium caseinate; Cf. Chap. VIII.

determined. The effect of the presence of KCl upon the dissociation of the KOH is negligible provided the KOH and KCl are always sufficiently dilute to be practically completely dissociated.

The extent of the error which is introduced into the determination of λ by dissolving the casein in the first instance in a solution of too high alkalinity may be gauged from the following results:

FINAL SOLUTION 3 PER CENT CASEIN IN 0.015 *N* KOH

Amount of unneutralized KOH employed to dissolve 7.5 grams casein	$\lambda \times 10^5$.
100 cc.	305.0
75 cc.	296.9

I have mentioned that the desired concentration of the KOH unneutralized by HCl in the solutions containing casein is attained by the addition, to the solutions of the casein in excess of KOH, of HCl *while stirring*. This is a matter of some importance. If acid be poured into a solution of a caseinate which is imperfectly mixed, the casein which is precipitated in the acid portions of the fluid forms bulky coagula and is only with difficulty redissolved, even if the quantity of alkali still unneutralized by the acid is more than sufficient to hold in solution all of the casein that may be present. Consequently, the solution of the caseinate must be rapidly stirred while the acid is being added. The same procedure, of course, considerably enhances the rapidity with which the casein dissolves in the alkali employed for its solution. I place the fluid in which the casein is to be dissolved, together with the casein,* in a beaker of squat form and 400 cc. capacity. The mixture is then agitated by a flattened glass rod bent at right angles so that the horizontal arm is about $2\frac{1}{2}$ cm. long and as near as possible to the bottom of the beaker; this is rotated at the rate of about 1600 revolutions per minute by a small motor. As soon as the casein is completely dissolved the acid is delivered into solution, a few drops at a time, from a pipette, the opening of which is held *at some depth below the surface of the liquid*. In some of the earlier experiments the acid was poured upon the surface of the solution, but all of these solutions foam to a certain extent and the foam is not agitated by the

* Regarding the procedure to be followed in adding the casein to the solvent Cf. Chap. XII, **1**.

stirrer with the same rapidity as the fluid which lies below it, consequently, if the acid is poured upon the surface, casein is precipitated within the foam and is only with great difficulty redissolved, hence solutions prepared in this manner yielded very irregular results both in the gas-chain and in the conductivity determinations. In delivering the acid into the solution it is very necessary to avoid holding the opening of the pipette too close to the side of the beaker, as in that case a film of casein is precipitated on the glass and this film redissolves with great difficulty.

Since the conductivities of solutions of the caseinates must be determined as soon as possible after the introduction of casein into the solution employed to dissolve it, for otherwise hydrolysis introduces a considerable error, it is evident that as little time as possible must be consumed in bringing the temperature of the solution to that at which its conductivity is to be determined. This could be achieved in either of two ways: either a small volume of fluid could be employed, so disposed as to take up the temperature of the water-bath very quickly; or the water-bath may be maintained at a somewhat higher temperature than that actually desired, and the conductivity of the fluid can be measured at the moment when it reaches the desired temperature. For reasons which will be sufficiently obvious the latter procedure is found to be more convenient. The water-bath is kept at a temperature lying between 31.5 and 32.5 degrees, a preliminary measurement of the conductivity of the solution is made at a temperature between 29 and 29.5 degrees and this preliminary determination is corrected at precisely 30 degrees.*

Solution of 1 per cent casein in 0.03 *N* KOH:

$\lambda \times 10^5$ determined immediately	348.8
$\lambda \times 10^5$ determined after allowing solution to stand at about 30 degrees for 20 minutes	356.3

It is obvious, however, that in this procedure the gas-chain measurements are made at a temperature some 2 degrees higher than the conductivity measurements, and it may be inquired to

* For maintaining constant temperatures, W. M. Clark employs an air-bath; Cf. Journ. Amer. Chem. Soc., 35 (1913), p. 1889; Journ. Biol. Chem., 23 (1915), p. 475. C. L. A. Schmidt, Univ. of Calif. Publ. Pathol., 2 (1916), p. 157, employs an oil-bath similar in construction to that described by G. A. Hulett, Physical Review, 32 (1911), p. 257.

what extent this invalidates the comparison of the two sets of determinations. The error which is thus introduced, for the small difference of temperature concerned is, however, negligible, since it was found by actual trial that the difference between the potentials measured at 30 degrees and those measured at 34 degrees could barely, with certainty, be detected upon my potentiometer-bridge. Nor is this fact surprising for, exclusive of any possible change in the equilibrium between protein and alkali, the potential, according to the Nernst formula, varies directly as the absolute temperature, and, therefore, only increases by 1/300th per degree centigrade at 30 degrees. It has been observed by W. A. Osborne* and myself† that there is no evidence of an appreciable shift in the equilibrium between casein and alkali, as the temperature rises, until the temperature of 36 degrees is reached.

I have mentioned in describing the apparatus employed, that difficulty was encountered in leading off the hydrogen, after it had bubbled through the fluids, through the exit-tubes of the half-element to the outside of the incubator. The difficulty was this, — all of the solutions containing casein foam upon passing the hydrogen through them, and to a greater extent the less the excess of alkali in the solutions. This foam, collecting in the exit and connected rubber tubes, gave rise to a pressure which drove the fluid out of the half-element and thus interrupted the continuity of the chain. Accordingly, it was found necessary, not only to abandon the idea of conveying the waste hydrogen out of the incubator, but also to cut off short the exit-tube of the half-element and permit the foam to escape freely into the water-bath (the lower end of the exit-tube was, of course, well above the surface of the water in the bath). After this plan had been adopted no further trouble was encountered from this source. Foaming, may, however, be prevented or greatly reduced by the addition of a few drops of octyl or amyl alcohol.

All observers who have endeavored to measure the conductivities of solutions containing proteins have encountered the difficulty involved in the precipitation which occurs at the electrodes, particularly in the case of casein, in neutral or very faintly acid solutions. Whetham and Hardy‡ in order to minimize the error

* W. A. Osborne, Journ. Physiol., 27 (1901), p. 398.

† T. Brailsford Robertson, Journ. Biol. Chem., 5 (1908), p. 147.

‡ W. B. Hardy, Journ. of Physiol., 33 (1905), p. 251.

arising from this source, adopted the plan of heating the electrode, after platinization, to a dull red, thus clumping the platinum black and reducing the total surface of the electrode.

I formerly tacitly assumed this phenomenon to be connected in some way with the passage of the alternating current through the solution. In my own experiments, however, I speedily found that in neutral or faintly acid solutions of potassium caseinate marked precipitation of casein occurs at the gas electrode, without the passage of any current, usually after but sometimes even before the passage of the hydrogen. At first I was inclined to attribute this to gases collected, in the interval between experiments, in the tubes conveying the hydrogen, to impurities in the hydrogen, etc., — but after very careful exclusion of all of these possibilities the precipitation still took place, and it occurred to me that it might be due to the hydrogen ions dissolved in the platinum itself. When hydrogen is passed through a platinized platinum electrode, the potential measured across the chain represents, at first, a higher acidity of this electrode than it does later on, when the electrode has come into equilibrium with the solution; in other words, the hydrogen ions dissolved in the platinum have not yet come into equilibrium with those in the solution; an excess of hydrogen ions is still present in the platinum. It therefore appeared possible that this initial acidity of the platinum itself might be responsible for the precipitation of casein at its surface, and this idea found confirmation in the fact that on prolonged passage of hydrogen the film of protein deposited on the electrode slowly redissolved. It occurred to me that it might be possible to avoid this precipitation altogether by bringing the electrode nearly to equilibrium with a low concentration of hydrogen ions before introducing it into the solution at all; accordingly, before making a gas-chain determination with a caseinate solution of very low hydroxyl concentration (neutral or acid to litmus) the electrode which was to be dipped into the protein solution was immersed in distilled water and gas was passed through it for an hour or more — it was then immediately washed in the protein solution and used. The device was found to work excellently, and in all of the experiments described herein precipitation at the electrodes was avoided entirely, at least so far as the eye could perceive. A similar device was found effective in avoiding precipitation at the electrodes of the conductivity-vessel. The vessel was simply filled with dis-

tilled water and allowed to stand in the water-bath for some hours before making a determination.

In order to ensure a correct determination of the potential of the chain in these experiments it was always found necessary to pass the gas for three hours and, in the neutral or faintly acid solutions, for as much as six hours. If two successive readings on the bridge, taken an hour apart, did not differ by more than 1 mm. the result was considered correct. Difficulty in obtaining constant readings is, of course, only encountered in the chains yielding the highest potentials, in which, as the apparatus is arranged, 1 mm. on the bridge-wire makes a difference of only about one-half per cent to the calculated value of the potential. In the chains of low potential greater accuracy is desired, but no difficulty is encountered in attaining it, since, in these, successive readings are nearly always identical, or if they are not, the difference can invariably be traced to some obvious source of error which may be eliminated in a repetition of the experiment.

Since, however, prolonged exposure of the protein solution in the gas-chain to the temperature of the water-bath is essential, the question arises whether the accuracy of the determinations is not invalidated by hydrolysis of the casein. The answer to this question is in the negative, the change in the hydrogen- or hydroxyl-concentration of a protein solution, due to hydrolysis, is negligible in comparison with the change in its conductivity. Moreover, it has been shown* that the displacement of the neutral point on the potentiometer-wire, due to hydrolysis of the casein, in an alkaline solution of a caseinate is opposed in sense to the displacement due to the coming to equilibrium of the electrode with the solution in which it is dipped. Were appreciable change in the hydroxyl concentration of alkaline solutions of casein due to hydrolysis occurring, therefore, the position of the neutral point on the bridge should indicate at first a diminishing and later increasing potential. I have discovered no trace of this save in one or two of the most alkaline solutions which I have employed (1 per cent casein in 0.03 *N* KOH; 3 per cent casein in 0.05 *N* KOH), in these the displacement due to hydrolysis was never more than 1 mm. and the minimum value of the potential was taken as the true one. It is, however, to be recollected that the hydrolysis was probably

* T. Brailsford Robertson and C. L. A. Schmidt, Journ. Biol. Chem., 5 (1908), p. 31.

most rapid in the period of time preceding the attainment of this minimum, so that even the minimum potential may be considerably in error, especially as in the chains containing these solutions the potential was low and 1 mm. displacement on the bridge introduced a considerable error into the determination of the potential. This error is diminished in its percentage magnitude in the calculation therefrom of m, the amount of alkali neutralized by the protein, but, nevertheless, the determination of m in the solutions mentioned is not to be considered trustworthy. In less alkaline solutions no effect upon the hydroxyl-concentration of the caseinate solutions, due to hydrolysis, could be discovered; had such an effect been present to any appreciable extent it would, of course, have been detected in these solutions much more readily than in those in which such an effect was detected, since the potentials of the chains containing these solutions were higher, so that a smaller change in hydroxyl-concentration of the solution would have produced a greater (absolute) displacement of the neutral point upon the bridge-wire.

In concluding these remarks, it may be stated that unless all of the precautions which have been described are observed with the utmost fidelity the results of experiments such as these, and conducted in this manner, will be found to be wholly irregular, and, save in a qualitative sense, untrustworthy.

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