THE CRYSTALLIZATION, DENATURATION, AND FLOCCULATION OF PROTEINS, WITH SPECIAL REFERENCE TO ALBUMIN AND HEMOGLOBIN; TOGETHER WITH AN APPENDIX ON THE PHYSICOCHEMICAL BEHAVIOR OF GLYCINE

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Certain aspects of the behavior of denaturable proteins, in particular egg albumin and hemoglobin, in respect to crystallization, denaturation and flocculation, are reviewed, a number of closely related problems necessarily coming under consideration at the same time.

The various topics discussed are grouped under the following heads:

I. CRYSTALLIZATION

The possible significance of the electric moment of certain groupings; the work of Miss Chick and Martin upon the composition of crystalline egg albumin precipitated by ammonium sulfate (prior to the subsequent washing process); the results of Sörensen on the composition of the finally purified egg albumin.

II. DENATURATION

1. The general features of denaturation by heat and by acid or alkali

The probable unimolecular character of the process; the lack of precise knowledge regarding the nature of the chemical and physical changes involved; the identity in titratability (by acid) of denatured and undenatured egg albumin; the reopening of the question of the reversibility of denaturation by the results of Anson and Mirsky.

2. The extent of interaction of undenatured protein with acid and alkali

The evidence that over a considerable pH range (from the iso-electric point outwards) protein salts (e.g., albumin chloride (or sulfate) and sodium albuminate) are practically completely ionized, but, that beyond certain pH limits appreciable quantities of unionized salt exist in solution; the chemical union versus adsorption problem; the hydrolysis of albumin chloride and sodium albuminate, and the average net charge on the protein ions at different pH values.

3. Behavior of denatured protein with respect to acid and alkali

4. The question of the extent of union of undenatured and denatured protein with neutral salts

The work of Pauli and his collaborators, and of Northrop and Kunitz; the conclusion that both ions of an inorganic salt simultaneously combine to a slight extent (and reversibly) with protein, but that the ions are combined in general in non-equivalent amounts; the difference in behavior of denatured and undenatured serum albumin towards silver ion (Pauli and Matula); the problem of the positions of attachment of inorganic ions with protein.

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The critical increment of flocculation: observations of Miss Chick and Martin; influence of temperature on rate of flocculation of protein and of a colloidal metal; the nature of the problem raised by the observations of Miss Chick and Martin upon the rapid fall of the critical increment with rise in temperature.

APPENDIX

The physicochemical behavior of glycine in solution.

INTRODUCTION

In the first place it is necessary to recall the definitions attaching to the three terms, crystallization, denaturation, and flocculation.

Crystallization is the precipitation in the solid crystalline form of *un*denatured protein. This material is obtained as a rule by

salting out with ammonium sulfate of appropriate concentration and subsequent washing. According to the careful work of Sörensen on egg albumin, referred to later, the crystalline material is best obtained not at the iso-electric point (pH = 4.8) but at pH = 4.58. It consists therefore of protein sulfate having a sulfate content of 0.43 per cent. The crystalline protein (i.e., the sulfate) is easily soluble in the pure solvent water.

Denaturation is a change of a kind not yet understood which the fresh (undenatured) protein is capable of undergoing in the presence of water. It is frequently brought about by heating the protein solution. It may likewise be brought about at ordinary temperature by addition of sufficient acid or alkali. The denaturation process is unimolecular in nature so far as the protein itself is concerned, and appears to be associated with some internal physical or chemical change generally regarded as being of an irreversible nature within the structure of each protein unit. Denaturation appears to be a definite step in the transition of the fresh undenatured (and crystallizable) protein molecules to the amorphous state represented by flocculation.

Flocculation is the precipitation of protein in the neighborhood of the iso-electric point in an amorphous form, this form not dissolving in the pure solvent (water). Flocculation is possible only provided the original protein has suffered the change known as denaturation. The two consecutive changes—namely, denaturation plus flocculation—taken together are frequently referred to as *coagulation*. In order that flocculation may occur with measurable speed a small amount of an electrolyte (neutral salt) must be present even at the iso-electric pH. In the total absence of such electrolyte the protein may be denatured but it will not flocculate, except on boiling and then only provided the pH be adjusted close to the iso-electric point.

The coagulum, formed as a consequence of flocculation, although insoluble in the pure solvent, will dissolve when sufficient acid or alkali is added. This, however, does not entail reversal of the denaturation step itself.

So much for the terms crystallization, denaturation and flocculation; we have now to consider in greater detail certain points in connection with the phenomena associated with these terms.

I. CRYSTALLIZATION

In the first place, the fact that crystalline undenatured protein can be obtained at all, demonstrates that mere proximity of such molecules is not in itself sufficient to bring about the coagulation change—i.e., denaturation plus flocculation. Since, however, crystallization is itself brought about in the presence of (much) salt, and since we know that even a small amount of salt is sufficient to bring about flocculation of material already denatured, it follows that neither close approximation of protein molecules nor the presence of much ammonium sulfate at ordinary temperatures is capable of bringing about denaturation. One concludes that the undenatured protein molecules are relatively inert. They are however, titratable—i.e., the acidic and basic portions are capable of interacting with an added acid or alkali without undergoing denaturation, as has been shown conclusively by Booth (1) in the light of his own data and of the data of P. S. Lewis and of Cubin. An excess of such reagent will, however, bring about denaturation even at room temperature.

To return to crystallization. The function of the (concentrated) ammonium sulfate in bringing about crystallization of undenatured protein is usually regarded as that of a dehydrating agent. The dehydration view implies that there is a greater tendency for either ion of the salt to associate itself with a water molecule dipole than with the amino or carboxyl dipoles situated on the protein molecule. This tendency will be directly proportional to the electric moment of the dipole of the water molecule or of the protein group respectively. Williams (2) has given a table for the characteristic moments of certain groups based upon the dielectric capacity of solutions (in benzene) of benzene derivatives of different types. Benzene itself is taken as non-polar. It is thus found that the electric moment, μ, for the NH_2 group is 1.5×10^{-18} e.s.u. For gaseous alkyl amines it is known that $\mu = 1.31 \times 10^{-18}$. For the carboxyl group $\mu = 0.9 \times 10^{-18}$. On the other hand, for water molecules in the gaseous state (monohydrol) the electric moment is distinctly higher, namely, 1.87×10^{-18}.

So far as these values go they are in agreement with the pre-

ferential attraction of the salt for the water molecules. It is necessary however to draw attention to the fact that μ for liquid (polymerized) water is only 0.5×10^{-18} (cf. Fowler (3)). If water were entirely composed of stable H_4O_2 molecules we would therefore expect the salt to adhere to the protein in preference to the water. It is probably right to infer that in hydration we have actually to do mainly with oriented unpolymerized H_2O molecules.

In the foregoing considerations it has been tacitly assumed that the amino and carboxyl groups of the protein molecule are in the undissociated or unionized form. Certain of these groups, however, taken in pairs, are very likely to give rise to the zwitterion structure usually represented by $^{+}NH_3 \cdot R \cdot COO^{-}$. (Actually in proteins it is more probable that the zwitterion formation arises from the mutual influence of contiguous branches thus, $R \cdot \overset{+}{N}H_3$ and $^{-}OOC \cdot R$, the positively charged portion corresponding to the terminal (ϵ) amino group of a diamino unit such as lysine, or the analogous group in arginine, or histidine, while the negatively charged portion belongs to the terminal carboxyl in a dicarboxylic unit such as aspartic or glutaminic acid). Although represented in the above manner the positively and negatively charged groups do not function as free and independent ions but are so placed as to cause a partial mutual saturation or neutralization of their electric fields. In fact, if this mutual interference did not occur, the zwitterion formation would be scarcely likely to persist, union with the H^+ ion and OH^- ion in the solvent transforming the zwitterion to the "classical" form. So far as attractive forces are concerned, therefore, the type and extent of interaction between salt, water, and protein would not be expected to be much affected whether we assume an undissociated structure or a zwitterion structure.

In view of the fact that the dipole moments of an amino group, a carboxyl group, and a water molecule, although not the same in magnitude are at least of the same order of magnitude, it would be expected that the ions of a salt such as ammonium salt, while exhibiting preferential affinity for the water molecule, would also attach themselves to a certain extent to the appropriate groups of the protein. It would be anticipated therefore,

that the precipitate obtained by salting out a protein such as egg albumin by means of ammonium sulfate would contain (prior to further washing and purification) not only protein but salt and water as well. This is shown by the classical work of Chick and Martin (4), who determined the composition of the solution in equilibrium with the precipitate under various conditions, as well as the composition of the precipitate freed as far as possible from adhering mother liquor by pressure, the pressure employed in this case being 3 tons per square inch. In the table of results given by these authors the percentage of protein in the pressed precipitate varies from 64 per cent to 73 per cent. At the same time the $(NH_4)_2SO_4$ varies from 30 per cent to 22 per cent, and the water from 8.5 per cent to 6.4 per cent. (These are rounded values given simply by way of illustration.) It is evident that the amount of water remaining in the pressed precipitate is determined by the inorganic salt present therein. This points to the water being associated principally with the salt, in agreement with the Hofmeister concept of the dehydrating action exerted by the salt upon the protein prior to precipitate formation.

It is of interest to look at the matter briefly from the point of view of relative dipole moments. The problem is essentially the distribution of the ions of the salt between the water dipoles and protein dipoles present simultaneously. The probability of attachment between salt ion and water dipole would be expected to be given by $(1 - e^{-E_{iw}/RT})$ where E_{iw} is the energy required to break the ion-water union. A similar expression involving E_{ip} would be expected for the ion-protein complex. Since the two energy terms are of the same order of magnitude we would expect to find appreciable quantities of both ion-water and ion-protein complexes in the system, as in fact we do find. It is to be observed that the condition contemplated in the salting-out process is that in which there is a high concentration of salt, that is, the condition is favorable to *partial* as distinct from complete hydration of the salt. The ions of the inorganic salt present possess consequently a considerable stray field wherewith to compete for further water dipoles and for protein dipoles. On increasing the active mass of the water (as by washing), the condi-

tion becomes favorable for the preferential union of salt with water rather than of salt with protein, so that the protein is obtained salt-free. It would be interesting to know what sort of effects would be produced by the addition of, say, a non-electrolyte such as sugar which would further compete for the water dipoles.

In the case of flocculation of iso-electric protein on the other hand, the electrolyte required is present usually in small concentration. There is abundant water present to saturate the ions of the salt, with the result that although the ions function locally to favor aggregation of the denatured protein units (compare the section on flocculation), the conditions are not favorable for permanent chemical union between protein and salt ions. The protein aggregates are therefore formed practically free from the precipitating salt. If this view be correct it follows that the degree of hydration of the ions of the salt plays an important part. Thus it is implied that if excess salt be used in connection with flocculation, union of salt and protein should now be possible, not because there is much salt but because of the partial dehydration of the ions. This would conceivably result, not in flocculation, but in peptization (compare section on solvent action of neutral salts); or, alternatively, if flocculation occur, it would be expected that the salt should form a definite part of the material thrown out of solution. Experimental evidence of this nature seems to be lacking.

To return to crystallization. Since the function of the ammonium sulfate is taken to be that of a dehydrating agent, the question arises as to whether its place can be taken by another salt with strong affinity for water. So far as present experience goes (cf. Sörensen (5) and Sörensen and Palitzsch (6)), sulfates stand out as by far the best precipitating agents, although their place may be taken by phosphate, arsenate, and citrate. These authors themselves leave the matter in some doubt. Further information is obviously required in this connection. As regards alcohol, Preyer (7) uses this in small quantities in his method of obtaining crystalline hemoglobin. If this material be in fact undenatured crystalline protein it would indicate that ions of

salts such as $(NH_4)_2SO_4$, even in small quantities, do not necessarily constitute a characteristic feature of the crystalline state. It has to be borne in mind, however, that alcohol in appreciable amount brings about denaturation of protein.

The most careful experimental investigation on the production of pure protein by crystallization is that of Sörensen (*loc. cit.*) on crystalline egg albumin, using ammonium sulfate as the precipitating salt.

As already mentioned, Sörensen finds that precipitation occurs best at pH = 4.58. The crystalline material was washed several times with sodium chloride solution until it was impossible to detect any sulfate in the filtrate. The material was finally treated with water, the greater part of it dissolving and some of it, apparently denatured, remaining undissolved. Sörensen remarks that "the results of our experiments must be interpreted as showing that it is not possible from such washing experiments to draw any conclusions as to whether the crystals contain ammonium sulfate or not." In the light of the results obtained by Sörensen using his "proportionality method," it becomes almost certain, however, that ammonium sulfate as such is not a necessary constituent of the crystalline protein material finally obtained. At the pH employed, however, the crystalline material contains the sulfate radical. Sörensen expresses this by saying that there is approximately 1 equivalent of sulfuric acid to 125 equivalents of (total) protein nitrogen. Booth has calculated from this, on taking the most recent and apparently most reliable value for the molecular weight of (undenatured) egg albumin, namely 43,000 (Marrack and Hewitt (8)), and also making use of the fact that the mass of nitrogen in egg albumin is 15.51 per cent of the total mass (Mathews (9)), that at the pH = 4.58 there are four equivalents of sulfate combined with one molecule of albumin. Thus the egg albumin sulfate obtained on precipitation has the formula, albumin $-(SO_4)_2$.[1]

[1] To attempt to ascribe a stoichiometric formula to a protein salt even in the "solid" state is naturally a doubtful procedure in view of the tendency of such material to produce micelles (i.e., aggregates of numerous molecules with occluded solvent and ions) when dissolved. It may, however, be pointed out that such

A point arises here. At the above pH, when this precipitate is dissolved the ionization may be regarded as complete. That is, in solution we will have albumin in the form in which its molecule carries four positive charges. As we shall see later at a much lower pH range the albumin sulfate is far from being completely ionized. This suggests that the albumin cation would conceivably react with the OH^- of the solvent to give some "undissociated" base which would in fact be iso-electric undenatured albumin itself. If this occurs it would not be permissible to regard the crystalline albumin sulfate of Sörensen as a single substance; it should be regarded as a mass of mixed crystals of sulfate and iso-electric crystalline albumin. As will be shown later, however, the pH range over which hydrolysis in the above sense is appreciable is so minute that even at pH = 4.58 it is already virtually absent. On the whole, it is probably safe to regard the washed crystalline material obtained at pH = 4.58 as a single entity.

II. DENATURATION

1. General considerations

The first question which naturally arises here is whether denaturation is a general phenomenon in the transition of a protein molecule or unit from the fresh natural dissolved state to the flocculated state. In this connection it is to be remembered that simple denaturation is a process which can be brought about with relative ease by heating or at ordinary temperatures, by the action of chemical reagents such as acid or alkali in sufficient amount, or by alcohol and probably by other organic solvents.

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evidence as exists, e.g., the magnitude of viscosity, suggests that while micelle formation is the outstanding feature of certain proteins, notably gelatin, in the dissolved state, molecules as such, but in the hydrated form, appear to predominate in the case of egg albumin solutions. It is probable that this is true in particular for solutions of fresh undenatured egg albumin. After denaturation has taken place and the pH adjusted within certain limits, the conditions for incipient flocculation thus produced may lead likewise to micelle formation even for this protein.

Consequently, if the actual preparation or isolation of a given protein involves such conditions or reagents the resulting material, if it had originally been capable of denaturation, would be likely to have become denatured prior to any systematic investigation of its physicochemical behavior. In such cases it is impossible to say whether the intramolecular change denoted by denaturation has occurred at any stage in the history of the protein or whether such change is incapable of occurring, i.e., whether the phenomenon of denaturation is non-existent in certain proteins. In other words, it is impossible to state with certainty whether denaturation is a general feature of protein reactivity or not. Among the simple proteins which are "heat coagulable" and therefore presumably denaturable by heat are the albumins, globulins and possibly glutelin. In the group of conjugated proteins hemoglobin stands out as definitely denaturable. It is very doubtful, however, whether casein (caseinogen) is denaturable; probably it is not. Incidentally, the change of casein to paracasein by means of rennet has been shown by Palmer and Richardson (10) to involve marked increase in acid and alkali binding capacities on the part of the protein over the entire range of pH examined, and we may consequently infer that this change is of a much more profound character than would be demanded by denaturation alone. Probably no protein has been so intensively examined from a physicochemical point of view as gelatin. From its mode of preparation, however, which involves relatively drastic hydrolytic conditions, it may be concluded with certainty that gelatin is already denatured or more exactly, for this protein, denaturation may be regarded as having no meaning. Further, aqueous "solutions" of gelatin are apparently much more complex than are solutions of albumin. Of the proteins the methods of preparation of which do not bring about denaturation, crystalline hemoglobin and crystalline egg albumin are the most prominent examples. For this reason, in discussing denaturation and the properties of solutions of undenatured and denatured protein we are forced to confine ourselves very largely to these two proteins.

The unimolecular velocity constants of denaturation in the case of these two proteins have been determined by Chick and

Martin (11), and later in the writer's laboratory by P. S. Lewis (12), Cubin (13) and Booth (unpublished work) over a wide range of pH and at different temperatures. It has been shown for both egg albumin and hemoglobin that the velocity of denaturation is a minimum not at the iso-electric point of the protein but at the neutrality point of water. The iso-electric point has no special significance whatever for the act of heat denaturation.

The fact that denaturation is a strictly unimolecular process in respect to the protein affords *prima facie* evidence that the process is not one of aggregation of protein units. A review of the literature on osmotic pressure measurements tends to confirm this, although at the present time no systematic work upon protein deliberately denatured has been carried out, by either the osmometer or centrifuge method. It is evidently desirable that such measurements be carried out by someone familiar with specialized technique of this kind.

As already mentioned, the precise nature of the chemical change, if any, which occurs in denaturation is quite unknown. The fact that the process is catalyzed to approximately the same extent by H^+ ion and OH^- ion, but that at the same time no change in the H^+ ion concentration accompanies it, serves to eliminate a number of plausible chemical changes. Thus the absence of change in the H^+ ion concentration and the identity in titratability of denatured and undenatured proteins (Booth (1)) rule out the possibility of associating denaturation with hydrolysis of a peptide linkage, unless indeed there be a simultaneous and *exactly equivalent* change in the opposite sense elsewhere in the molecule.[1a] Even if hydrolysis of some kind were shown to occur it would not in itself be particularly helpful, unless it could be shown that this change was accompanied by a change in configuration rendering the contiguous amino and carboxyl groups more easily available for the subsequent flocculation process, for which denaturation is regarded as a favorable preliminary step.

It is to be remembered that the observed critical increment or energy of activation in denaturation is excessively great—of the

[1a] That denaturation may involve hydrolysis of a peptide link and simultaneously union of amino and carboxyl elsewhere was suggested to the writer by Professor Ramsden.

order of 100,000 calories per mole in the neighborhood of the neutrality point. An energy term of this magnitude suggests as the most significant alteration in the protein unit an actual physical distension or opening up of the structure, possibly accompanied by the breaking of bridge-like linkages in the original undenatured unit. The act of denaturation on the part of a single protein molecule requires the presence of an appreciable amount of water. This is shown by the well-known experiments of Chick and Martin (*loc. cit.*) in which proteins "freed as much as possible from moisture by squeezing in a press between filter papers, but still containing 20 per cent of water" could be heated in a current of air at 120°C. for five hours without becoming denatured. Denaturation obviously is not brought about by a small amount of water, but appears to require water in bulk or at least amounts of water vapor such as are produced from a heated water bath (cf. Chick and Martin, *loc. cit.*).

The suggestion that denaturation may be accompanied by physical distension of the protein molecule in solution is likewise in agreement with the increase in viscosity attributed to the change (Chick (14)). Further, the fact that denatured protein is more easily attacked than is undenatured protein by proteolytic enzymes[2] in the act of digestion points to a more penetrable and less tightly bound structure. Also the denaturing effect ascribed by Chick to lecithin may be associated with this substance's well known peptizing capacity, an effect also likely to open up a molecular structure. The fact, however, that globulins are brought into solution, i.e., peptized by neutral salts and that nevertheless the resulting peptized globulin, e.g. edestin, is stated to be denaturable, indicates that denaturation and pepti-

[2] Talarico (15) has shown this fairly conclusively for the action of trypsin on egg albumin. His experiments likewise indicate that when coagulation has been carried far by prolonged heating the resulting material, as one would expect, becomes less digestible. Lin, Wu and Chen (16) also find that the denatured material is on the whole the more digestible, although protein denatured by alcohol is more digestible by trypsin but less digestible by pepsin than is undenatured protein. Linossier (17) finds that egg albumin denatured by heat is less digestible by pepsin than is the unheated material. In general apparently no great effort has been made to allow for the interfering effect of flocculation.

zation *per se* are not necessarily closely related phenomena. This is emphasized by the fact that while iodide ion and sulfocyanide ion are good peptizing agencies they both markedly raise the "temperature of denaturation," i.e., they inhibit denaturation (Pauli and Handovsky). It does not appear to be quite certain, however, how far the effect of these ions is exerted upon the denaturation process as distinct from the subsequent flocculation process.

The influence of salts upon the rate of denaturation is considerable; the mechanism however remains obscure. Lepeschkin (18) has investigated the phenomenon fairly extensively. As an example of the marked accelerating effect of salts of the "heavy" metals, one may cite the behavior of Ba^{++}. Using egg albumin (Kahlbaum) dialyzed for 6 days, Lepeschkin observed that "in presence of 0.1 *N* KCl the average time of denaturation was found at 75°C. to be 2095 seconds, while in the presence of 0.1 *N* $BaCl_2$ denaturation proceeded instantaneously at the same temperature; in the presence of 0.003 *N* $BaCl_2$ it required 95 seconds, 0.003 *N* $MgCl_2$ 120 seconds, 0.0001 *N* HCl 52 seconds, while it proceeded instantaneously in the presence of 0.003 *N* HCl." Lepeschkin points out that the effect of salts is so marked as "to make necessary the suggestion that the salts in question first enter into chemical unions with proteins which are soluble in water and react with it more easily than the free proteins." While union is highly probable, there is as yet no clear reason why the denaturation is thereby facilitated. That the influence of salt upon the rate of denaturation is of a dual nature is shown by the results of P. S. Lewis, who found in certain cases that the velocity passed through a maximum with increase in salt content. The same dual effect has been observed by Pauli and Handovsky in connection with the socalled "temperature of heat denaturation," a mode of examining denaturation which has been superseded by the method of velocities and velocity constants. The phenomenon of the influence of salts upon denaturation on the whole has not been systematically examined with adequate pH control. Little can therefore be said about it at the present time.

Owing to the great variation in the rate of denaturation with

hydrogen ion and hydroxyl ion concentration, it is impracticable to work over a wide pH range at a single temperature. Measurements are made at different (suitable) temperatures, and with the aid of the critical increments (which are themselves a function of pH and become enormously great as we approach neutrality from either side) it is possible to calculate at least approximately what the velocity constants would be at a given single temperature over a wide pH range. In table 1 the temperature chosen is 25° and as a comparative measure of the rate of denaturation the

TABLE 1

Time required for 50 per cent denaturation of proteins at 25°C.

pH	TIME		
	Days	Hours	Minutes
Crystalline egg albumin			
1.02		12	6
1.35		23	30
1.99	3	20	—
2.55	9	3	—
3.83	139	—	—
5.13 to 8.29	Time varies between 560 and 29600 years		
Crystalline hemoglobin (from ox blood)			
4.08	—	—	6.5
4.41	—	—	31
4.77	—	3	46
5.10	—	17	6
5.50	4	14	—
7.0	291	—	—

times for half denaturation have been computed. These results are of practical value as giving some idea of the time that may safely be spent in dialyzing etc. without danger of appreciable denaturation. It is evident that the rate of denaturation of egg albumin at ordinary temperatures is exceedingly slow compared with that of hemoglobin. The enormous critical increment of denaturation in the pH region in the neighborhood of neutrality is the cause of the almost unbelievably slow rate given in table 1.

As there is apparently some difference of opinion regarding the

feature or features which characterize and accompany denaturation it is necessary to state here the sense in which the term is employed by the present writer. It may be put simply thus. A protein dissolved in water may be said to be undenatured if no flocculation occurs when the pH is adjusted to the iso-electric point of the protein and if at the same time a small amount of neutral salt be present in the system. Denaturation is most conveniently brought about by heating in the presence of water. It may also be brought about by addition of sufficient acid or alkali, but in such cases it is essential that excess of the reagent be avoided in order that no unnecessary chemical changes be brought about at the same time. The statement not infrequently made that denaturation is accompanied by production of acid, i.e., by definite hydrolysis, is undoubtedly true, but such changes are unnecessarily violent and are not essential to the denaturation process itself.

As already mentioned denaturation is regarded as irreversible, the process going to completion under all ordinary circumstances. The whole question of the reversibility of denaturation has recently been raised, however, chiefly by the striking results obtained by Anson and Mirsky (19), who believe that they have obtained fairly conclusive evidence that denaturation may be reversed in the case of hemoglobin and hence, by inference, for other proteins. The method of obtaining soluble undenatured hemoglobin from coagulated hemoglobin is as follows. "If an acid or alkaline solution of completely coagulated hemoglobin is neutralized all the protein is precipitated. If, however, before complete neutralization the solution of denatured hemoglobin is allowed to stand in slightly alkaline solution under conditions to be described, then, on complete neutralization only a part of the protein is precipitated. The remaining soluble part is the apparently reversed hemoglobin. How much of this soluble, apparently reversed hemoglobin is obtained depends, other factors being constant, on the state of the prosthetic group, heme, and on the species of the hemoglobin."

The features of denaturation and the question of its reversibility bear some analogy to the hydrolysis of sucrose. Under all

ordinary conditions the hydrolysis of sucrose by acid to glucose and fructose is complete. There is no reliable evidence of combination of the resultants, not because of absence of collision, for which the conditions are favorable, but because of the fact that one of the hexose moieties (the fructose portion) as it exists in the intact sucrose molecule is in a five-membered ring form.[3] On hydrolysis this form changes almost entirely to a six-membered ring form, and interaction between this and the glucose cannot produce sucrose though it might well produce another disaccharide. Of course in the case of denaturation the "resultants" are not separated from the parent molecular structure, but, by the setting up of a new configuration within the original structure involving new spatial arrangements of atomic groups and distribution of valency forces, the probability of reproducing all the initial conditions simultaneously is so small as to be negligible. On this basis, for all practical purposes denaturation would be irreversible; it does not eliminate the possibility of reversal however. The surprising thing is that a change in environmental conditions relatively as minor as that described by Anson and Mirsky should have any appreciable influence on reversal at all. The whole problem is obviously now under revision and at the present stage it would be premature to speculate on the outcome.[4]

2. Behavior of undenatured protein with respect to acid and alkali

In connection with the mode of union of a strong acid, HCl, with egg albumin, reference may be made in the first place to experiments carried out a long time ago by Bugarsky and Liebermann (22). These authors do not state definitely whether the albumin was denatured or not, but it may be inferred from their paper that it was almost certainly undenatured to begin with, although prolonged contact with the most concentrated acid solution employed

[3] For an examination of the significance of this structure in relation to the critical increment of hydrolysis of sucrose and other glucosides, cf. Moelwyn-Hughes (20).

[4] In connection with the solvent action of certain salts (e.g., sulfocyanides and salicylates) upon coagulated albumin (cf. Willheim (21)) it is not clear whether denaturation is here reversed or simply prevented.

would probably bring about denaturation. Measurements with hydrogen ion concentration cells and chloride ion concentration cells were carried out, and their results show definitely that in the presence of HCl both H^+ and Cl^- combine with the protein. The initial concentration of HCl, to which various amounts of albumin are added, was 0.05 *N*. With 6.4 grams albumin in 100 cc., 96.56 per cent of the original H^+ is combined, while 76 per cent of the Cl^- is combined. In view of the general reactivity of H^+ (and OH^-) it is not surprising that the union of H^+ with the protein should be as extensive as these data show. It is somewhat surprising, however, to find evidence of marked union of Cl^- to form unionized protein chloride. With the concentrations involved one would have expected the protein chloride to be very largely ionized. The protein salt, in fact, appears to be much more sensitive (in respect to ionization) to the presence of a strong electrolyte with a common ion than would be the case with sodium chloride, for example. Although the general behavior of egg albumin with respect to acid is illustrated qualitatively by the results of Bugarsky and Liebermann, the extent of union with the chloride ion thus indicated is somewhat misleading. Manabe and Matula (23) were the first to show definitely that Cl^- is in general less combined with the protein than is H^+, for HCl concentrations not exceeding 0.05 *N*. With very dilute HCl the protein salt is practically completely ionized. Actually Manabe and Matula examined serum albumin, but there is no reason to doubt that their conclusion is applicable in principle to egg albumin as well. Using a 1 per cent solution of serum albumin in the presence of HCl originally 0.02 *N*, the H^+ bound is 0.014 while the Cl^- bound is 0.005. The latter figure corresponds to the concentration of unionized salt, whence it may be concluded that the degree of ionization under these conditions is of the order 64 per cent. In the presence of HCl originally 0.04 *N*, the degree of ionization is only of the order 25 per cent.

Reference may likewise be made to electrometric measurements by Rohonyi (24), who employed Merck's presumably undenatured crystallized egg albumin, as well as other material. With a 1.9 per cent egg albumin solution in HCl, initially 0.05 *N*,

Rohonyi finds that the concentration of bound H^+ ions in equivalents per liter is 0.0183, the concentration of bound Cl^- ions being almost the same, *viz.* 0.0170. It follows that the concentration of free H^+ ions in equilibrium with this amount of albumin is 0.0317 N (pH = 1.5), and the apparent equivalent of albumin at this pH is 1040. The "effective" concentration of the protein as salt is 0.0183 N, and from the figures given the degree of ionization in respect to Cl is only about 7 per cent. Incidentally albumose (Witte peptone) behaves very similarly to the egg albumin, while alanine under similar conditions is ionized considerably more (43 per cent), but by no means completely. Attention may also be drawn to the results obtained by Chick and Martin (25) upon the union of undenatured crystalline egg albumin (in 0.95 – 2.0 per cent solutions) with sulfuric acid. The hydrogen electrode was employed, thus giving information regarding the union of hydrogen ion with the protein. Similar electrode measurements with the SO_4^{--} or HSO_4^- ion are of course impossible,[5] so that no information is available as regards the degree of ionization of the sulfate. These authors find a general resemblance between their results and those of Bugarsky and Leibermann, although apparently considerably greater union occurred in the case of sulfuric than in the case of hydrochloric acid. On the other hand Loeb (Proteins and the Theory of Colloidal Behavior, p. 49 (1922)) finds that over the pH range 4.2 – 2.4 (the entire range quoted in Loeb's table), the union of 0.1 N sulfuric and 0.1 N hydrochloric acids with 1 per cent crystalline egg albumin is the same, as one would expect on simple stoichiometric grounds. In Loeb's table just referred to, the largest amount of sulfuric acid recorded as combined with one gram of albumin happens to be 7.0×10^{-4} equivalents. (Due allowance has been made in the titration experiments for the titration capacity of the solvent in arriving at this figure.) The pH corresponding to this degree of combination is 2.4. If this were the limiting value the apparent equivalent weight would be 1430. In Chick and Martin's experiments the recorded number of equivalents of sulfuric acid combined is 1.79×10^{-3}, this occurring at a pH = 1.65.

[5] Northrop and Kunitz (26) have suggested and employed a method for such cases as this based upon Donnan's Membrane Theory.

On this basis the apparent equivalent weight of albumin is as low as 560. The number of titratable amino groups per gram of egg albumin is 64.9×10^{-5}. On this basis the effective equivalent weight of albumin is 1540. This corresponds on the basis of Loeb's data to the titratability of egg albumin at a pH = 2.6. It was suggested long ago by Brailsford Robertson that the titratability of a protein was greater, in general considerably greater, than would be expected from the free amino groups, and for this reason he proposed his well-known concept of the titratability of the —CONH— grouping[6] in addition to that of the free amino groups themselves. Whether the actual "additional" titration is to be ascribed to the —CONH— grouping or not, it would appear very probable that groupings of some kind would be involved in a stoichiometric sense. It would probably lead to clearness if it were generally agreed to regard the equivalent weight arbitrarily as that which corresponds to the number of free amino groups, i.e., the value would be about 1540 in the case of albumin.

As a matter of fact, the above stoichiometric view of the "additional" titratability (assuming this to be real) have been subjected to considerable criticism, particularly by those who draw a sharp distinction between mass action combination and adsorption. The distinction is supposed to be emphasized by ascribing mass action binding to primary valency, adsorption to secondary valency. Thus Gortner (cf. Hoffman and Gortner, (27)), who has investigated a considerable number of vegetable proteins as well as casein, concludes that "the amount of acid bound at acidities lying between the iso-electric point of the protein and pH 2.5 was definitely correlated with the free amino nitrogen of the protein. . . . Accordingly, in this region the acid binding was different for the various proteins and was dependent upon the chemical composition of the proteins. At greater acidities the further acid binding was independent of the

[6] The "titratability" of the peptide grouping is here attributed to addition of acid to the *intact* grouping. There is no evidence for Robertson's idea of fission of this linkage merely by the act of titration, much more drastic conditions being required for this purpose. Cf. Escolme and Lewis (28).

chemical composition of the proteins and was related only to protein concentration. At acidities greater than pH = 2.5 all proteins bound gram for gram the same amount of acid." Presumably "all" is not to be taken quite literally. It may be pointed out, however, that even on an adsorption basis the same amount of binding need not necessarily occur.

As Hitchcock has pointed out, it is impossible to distinguish between a true mass action combination and adsorption which obeys Langmuir's equation, that is, adsorption at a certain fraction of a finite number of "places" on a surface.[7] Incidentally, some of the criticism levelled against Loeb's conclusions is for this reason pointless. Considering in the first place the reactivity of the protein from the iso-electric point out to a pH = 2.6, we undoubtedly have a finite number of groups situated on any single protein molecule, these groups being titratable by the appropriate reagent. To regard such interaction as a chemical process, as does Loeb for example (and over this pH range Gortner is in agreement with Loeb), would seem to be a not unreasonable way of looking at the matter. If we prefer to call such interaction adsorption, we imply adsorption in the Langmuir sense. Apparently those who emphasize the view of adsorption as sharply distinguished from mass action have in mind an effect, which they ascribe to secondary valency as distinct from primary valency, in which the adsorbed molecules or ions are not located at definite positions in or on the protein molecule structure, but are distributed statistically over the "surface" of the molecule. Such an effect, if it occurs, would be likely to be minor compared with the localized and specific adsorption at definite groups; nor is such a view essential to the production of the electrokinetic p.d. (potential difference). At the present time it is probably unnecessary to appeal to such a general type of adsorption in the case of a

[7] In connection with the union of Cl^- ion with dissolved glycine (aminoacetic acid) examined by Oryng and Pauli (29), these authors point out that the curve obtained by plotting extent of combination against the free chloride ion content of the system gives rise to a curve which "has the typical form for an adsorption process." As Oryng and Pauli themselves point out, the term adsorption in this connection has no physical meaning. The general shape of the curve cannot therefore be taken as a criterion.

complex molecule such as that of a protein wherein there are numerous groupings having marked specific reactivity.

Returning to the work already considered upon the union of acid with dissolved undenatured albumin to produce, in solution, protein salt of variable composition—the composition being a function of the pH—it may be pointed out that when such salt is obtained in the solid form it is probably crystalline. We have in mind a degree of acidity not sufficient of itself to bring about denaturation even at ordinary temperatures (25°C.). If, however, the concentration of acid is greater than this, i.e., greater than 0.1 N in the case of egg albumin and greater than 10^{-4} N in the case of hemoglobin, denaturation could occur. In such a case it does not seem to be known with certainty whether the salt precipitated would be crystalline or amorphous. Presumably an appeal would have to be made to the x-ray method of investigation.

Returning to the question of the degree of ionization of protein chloride in solution, reference may be made to two investigations of relatively recent date—namely, that of Hitchcock and that of Frisch, Pauli and Valko. Measurements on the interaction of hydrochloric acid (up to 0.1 M) with the proteins, gelatin, egg albumin, casein, edestin and serum globulin (in solutions containing one gram of protein in 100 cc.) have been carried out by Hitchcock (30) using the electromotive force method. The electrodes are the hydrogen and silver-silver chloride electrodes, respectively. The data cover a considerable range and are apparently of a high degree of accuracy. The results have been calculated simply on a concentration basis, not on an activity basis, and consequently would require recalculation to estimate with precision the degree of ionization at different pH values. The conclusion reached by Hitchcock is that the protein chlorides are highly ionized except in the most acid regions studied.

Electrometric determinations of the behavior of egg albumin and other proteins towards strong acid—employing the hydrogen and calomel electrodes respectively—have been carried out by Frisch, Pauli and Valko (31). The range of acid employed is approximately that covered by Bugarsky and Liebermann,

but the data are more numerous and presumably very much more accurate. The theoretical treatment involves activity considerations instead of the earlier concentration relations. The method of calculation is not particularly clear and the writer is unable to follow certain of the steps. It is possible, however, to make a partial use of the data presented in order to check the conclusions already drawn as regards the degree of ionization of the albumin chloride. Thus using 1 per cent albumin solution dissolved in HCl initially approximately 0.05 N (accurately 0.0492 N), the activity of the uncombined Cl^- expressed as a "corrected concentration" is found to be 0.0353 N. Employing the activity coefficient obtained by Pauli and Wit (32) for Cl^- ion in a 0.05 N HCl solution, namely $\alpha = 0.831$, it follows that the concentration of free Cl^- ion is $0.0353/0.831 = 0.0425$ N. Consequently the quantity of Cl^- bound to the protein in the form of unionized albumin chloride is (0.0492 − 0.0425) or .0067 equivalents per liter. The authors give 0.0103 expressed in equivalents per liter as the quantity of H^+ ion bound by protein. If the protein chloride at the pH in question (namely, 1.41 corresponding to the equilibrium (free) H^+ ion 0.0389 N) were completely unionized, the concentration of bound Cl^- would likewise be 0.0103. It follows that the fraction of albumin chloride ionized is $\frac{0.0103 - 0.0067}{0.0103}$, or 35 per cent.

Again, with 1 per cent albumin in the presence of HCl of initial concentration 0.03 N (accurately 0.0295 N) the activity of the free Cl^- is found to be 0.0220. Taking the Pauli-Wit α value 0.850 for Cl^- in HCl of this concentration, it follows that the concentration of free Cl^- ion is 0.0259 N, whence the concentration of bound Cl^- is 0.0036. Since the concentration of bound H^+ is 0.010, it follows that the degree of ionization with respect to Cl^- ion is $\frac{0.10 - 0.0036}{0.010}$, or 64 per cent. A similar calculation for 1 per cent albumin in 0.0197 N HCl gives a dissociation of 75 per cent, while in 0.00984 N HCl the dissociation is practically complete. That is to say, between pH = 2 and the iso-electric point, pH = 4.8, the albumin chloride (containing

1 gram protein in 100 cc.) may be regarded as completely ionized.[8]

As regards union of undenatured albumin with alkali, the usual titration experiments indicate the capacity of the protein to combine with hydroxyl ion. This is borne out also by the e.m.f.

[8] It might, of course, be argued in general that as albumin chloride is a salt—its composition being a function of pH—it will act as a strong electrolyte and be completely ionized at least over the entire range of concentration contemplated in the measurements of Pauli and his collaborators. As regards the likelihood of attachment of Cl^- ion, i.e., the existence of undissociated salt molecules, it would seem that localized adsorption in the Langmuir sense upon certain fixed charged places situated on a solid surface would be more clearly analogous to the conditions obtaining here than is the case of attachment of Na^+ to Cl^-, for example, to produce an isolated molecule of NaCl. If the albumin were present as a porous solid carrying local positive charges, the possibility of attachment of the Cl^- ions would scarcely be denied. There does not appear to be any essential difference between the opportunity afforded for attachment in this condition and that represented by dissolved protein units. The question has to remain open as the experimental tests which suggest themselves may be equally well accounted for, either by combination, or by alteration (decrease) in the activity of the Cl^- ion treated as otherwise uncombined. Pauli, Frisch and Valko assume complete ionization of the albumin chloride over the entire range of concentration of HCl ($0 - 0.05\ N$) examined. On this basis they calculate the activity coefficient of the Cl^- ion which apparently falls very rapidly with increase in concentration. This they ascribe to a marked depressing effect exerted upon the activity of the Cl^- ion by the presence of the albumin as cation: they assume that the activity of the H^+ ion is not affected by the albumin cation. In the account given above I have preferred to regard the albumin ion as not exercising any special influence upon the activity of the chloride ion. Instead it has been assumed that actual combination and consequently removal of a certain fraction of the Cl^- ions takes place. The activity of the free Cl^- ions is taken to be approximately the same as would obtain in a hydrochloric acid solution of similar concentration. The argument that the albumin ion by virtue of its very high valency would exert a depressing effect on the Cl^- ion activity may not be valid, owing to the circumstance that the protein ion is extremely large and the localized electrical effects may not exceed those exerted by a univalent ion. The view that the effective valency, so far as influence upon the activity of other ions is concerned, lies between one and two has been put forward by Simms (33) for the case of gelatin. Similarly Cohn and Prentiss (34) have concluded that crystalline hemoglobin acts as though it were divalent.

In the long run there may not be a very fundamental physical difference between the two views referred to since a marked decrease in activity is due to the operation of large interionic forces of attraction. When these are large enough one would speak of chemical combination in the ordinary sense. Some advantage, however, is probably gained by maintaining the distinction.

measurements of Bugarsky and Liebermann using hydrogen electrodes. No one, so far as the writer is aware, has as yet employed the sodium amalgam electrode to determine to what extent the sodium ion is combined at the same time. In the absence of such data one is forced to a consideration of the cryoscopic measurements of the above investigators, although it is recognized that in such a case as that considered the method is somewhat insensitive. Employing the data of Bugarsky and Liebermann we may proceed to make the following rough calculation. When 6.4 grams of albumin are dissolved in 100 cc. of NaOH which was originally 0.05 N the observed depression of freezing point, δ, is 0.097°. The albumin itself accounts for a depression (due almost certainly to electrolytic impurities) of 0.022°. Hence $\delta_{corr} = 0.075°$. This is due to the free Na^+ and OH^- remaining in the solution. From the e.m.f. measurements with the hydrogen electrode, Bugarsky and Liebermann calculate that with this quantity of albumin in alkali originally 0.05 N the hydroxyl ion is bound to the extent of 97 per cent of its original concentration. That is, the concentration of free OH^- ion is $0.03 \times 5 \times 10^{-2}$ equivalents per liter. Denoting the concentration of free Na^+ ion by x it follows that

$$\delta_{corr.} = 0.075° = k \times 0.03 \times 5 \times 10^{-2} + kx$$

The factor k is obtained from the observation of Bugarsky and Liebermann that the 0.05 N NaOH alone gives a depression of 0.181°. Hence

$$k = 0.091/\ 5 \times 10^{-2}$$

and

$$x = 3.95 \times 10^{-2} \text{ equivalents of } Na^+ \text{ ion per liter.}$$

Hence 21 per cent of the original Na^+ is combined with the protein. The same result is obtained for the case in which 12.8 grams of albumin were dissolved in 100 cc. of alkali. Although no stress can be laid upon the actual numerical value obtained which (in view of the fact that the depressions observed are

somewhat larger than would have been anticipated) may well be a minimal value, we are justified in concluding that an appreciable quantity of sodium ion is combined in the form of unionized protein salt at an OH^- ion concentration of 1.5×10^{-3} N (i.e., pH = 11.2).

Hydrolysis of albumin chloride and sodium albuminate prepared from crystalline egg albumin, and the net average charge on the protein units at different pH values

The simplest type of hydrolysis of, say, albumin chloride, can be represented thus:

$$Alb^+ \rightleftarrows Alb + H^+$$

where Alb^+ denotes the cation form of albumin carrying the least possible charge, namely one; the symbol Alb denotes the iso-electric form of albumin. We are concerned with conditions not far removed from the iso-electric point. The existence of hydrolysis would thus be demonstrated by a calculation of the net charge on the albumin units at a given pH, a net value of less than unity denoting the existence of a certain number of uncharged (i.e., iso-electric) individuals present. If the net charge comes out greater than unity, the hydrolysis may be regarded as inappreciable.

From the titration data given by Loeb (35), one may calculate the equivalents of strong acid, i.e., of H^+ ion, combined with one gram of albumin in a 1 per cent solution of the latter existing initially at the iso-electric pH. Taking the molecular weight of albumin as 43,000, it follows that a 1 per cent solution is 1/4300 or 2.3×10^{-4} M. Taking a particular case, Loeb finds that 1.15 cc. of 0.1 N HCl are required to bring the albumin from the iso-electric point (pH = 4.8) to pH = 4.2. This means that 1.15×10^{-4} equivalents of H^+ ion are bound. In other words, 1000 cc. of the albumin solution would bind 1.15×10^{-3} equivalents of H^+ ion. The ratio of equivalents of H^+ ion bound to the gram-molecules of albumin present is $\frac{1.15 \times 10^{-3}}{2.3 \times 10^{-4}} = 5$. That is, there are on the average 5 single positive charges upon each

albumin unit. Hence even at pH = 4.2 the hydrolysis to the iso-electric form is probably negligible. Similarly it may be shown that at pH = 4.4, the average charge is 3.3. Even at this pH, therefore, the quantity of iso-electric albumin is negligible. An analogous result is obtained from the titration data with alkali. Thus from Loeb's curve (*loc. cit.*, p. 60) at pH = 5, the net (negative) charge per molecule of albumin is 2.2.

It seems legitimate to conclude therefore that the concentration of iso-electric albumin is quite negligible compared with the ionic form over any pH range except in the immediate neighborhood of the iso-electric point, a range so small—namely of the order pH = 0.02—that for most purposes it could be regarded as sensibly identical with the iso-electric point itself.

It is of some interest to collect in tabular form the pH values, on either side of the iso-electric point, at which the electrical charges on egg albumin—in one case positive, in the other negative—are the same. See table 2.

It may be mentioned that the variation in the electric charge with pH given in table 2 is similar to the variation in the rate of electrophoresis exhibited by egg albumin according to the measurements (by the ultra-violet absorption method) of Svedberg and Tiselius (36), as would be expected if the magnitude of the net or "free" charge is the main factor influencing the rate of motion of the particles in the field.

3. Behavior of denatured protein with respect to acid and alkali

In the first place, it has been shown by Booth for egg albumin that the act of denaturation is not necessarily accompanied by any change in the number of titratable acidic and basic groups in the protein molecule. The essential matter in such titration experiments is to be certain that during the time taken for titration to the different pH values, the protein shall remain undenatured. This is possible by making use of the data of Cubin upon the rate of denaturation at various temperatures and at various pH values. The point is considered at length in the paper by Booth referred to above. The conclusion regarding the behavior of egg albumin is almost certainly true also for hemoglobin.

In the absence of neutral salt, the denatured proteins referred to remain in solution even in the neighborhood of the iso-electric point. When the solution is made distinctly acid or alkaline the denatured protein exists in solution in the form of protein salt. Probably the same relations hold for the ionization of this salt—the composition of which is a function of pH—as hold for the corresponding salt of undenatured protein. The experiment has not actually been carried out.

As will be pointed out later we have reason to believe that gelatin and caseinogen—in view of their method of preparation—more closely resemble a denatured than an undenatured protein. In view of this, it is pertinent to recall the behavior of caseinogen in the pH region 6–7, that is, on the alkaline side of its iso-electric point, where it exists in the form of caseinogenate anion.

TABLE 2

Positions of equal net charge (+ or −) for egg albumin

pH (POSITIVELY CHARGED UNITS)	pH (NEGATIVELY CHARGED UNITS)	MAGNITUDE OF MEAN NET CHARGE
4.8	4.8	0
4.2	5.4	5
3.8	6.3	10
3.65	7.0	12
3.4	8.0	15
3.1	9.2	20
2.8	9.9	25

Actually in the experiment about to be referred to, the protein system was examined in the presence of NaCl and $CaCl_2$, respectively. We are not here concerned with the question of union of both ions of the inorganic salt with the caseinogen individual,[9] but with the degree of ionization of sodium caseinogenate and calcium caseinogenate respectively.

The investigation referred to is that of Wright (37), who investigated the Donnan distribution equilibrium (across a cellophane membrane permeable to simple ions but impermeable to caseino-

[9] The effect of neutral salts upon proteins is considered in the next section. The experimental conditions referred to here were not adapted (nor were they so intended) to determine the union of chloride ion with caseinogenate ion, which at best is not an effect of large magnitude.

gen and caseinogenate anion) for the systems sodium caseinogenate-sodium chloride, and calcium caseinogenate-calcium chloride. The pH of the solutions remained between 6 and 7. The concentration of caseinogen was 0.5 per cent, this being dissolved in 0.025 *N* NaOH. On the other side of the membrane the initial concentration of NaCl was varied from 0.0125 *N* to 0.1 *N*. In the experiments with calcium ion, the concentration of caseinogen was varied from 2 per cent to 7 per cent in the presence of $Ca(OH)_2$, which in turn was varied from 0.025 *N* to 0.125 *N*, the $CaCl_2$ on the other side of the membrane being varied initially from 0.0125 *N* to 0.025 *N*. Wright concludes that, in order to make the Donnan theory apply quantitatively as it should do in a case like this, it is necessary to assume that the sodium caseinogenate is ionized incompletely, namely to the extent of about 68 per cent, while calcium caseinogenate is ionized only to about 30 per cent. The results recall those of Bugarsky and Liebermann for undenatured protein in alkali. Wright's calculations are based on "concentration" considerations. How far these figures would be affected by substituting activities for concentrations (as should theoretically be done) it is difficult to say.

4. On the question of the extent of union of undenatured and denatured protein with neutral salts

We are considering in the first place the behavior of solutions of egg albumin believed to be undenatured. Again reference may be made to the work of Bugarsky and Liebermann (*loc. cit.*). Sodium chloride was added to dialyzed albumin solution so as to make the sodium chloride 0.05 *N*. The quantity of albumin was varied from 0.4 to 6.4 grams per 100 cc. No change either in the freezing point, or in the e.m.f. of a chloride ion concentration cell was produced by addition of the protein. It is to be concluded, therefore, that under the above conditions no detectable union of Cl^- or Na^+ with albumin takes place. (Again, the cryoscopic measurements are to be regarded as less accurate than the e.m.f. measurements.) Unfortunately the pH was not actually determined, but we may probably assume it to be in the neighborhood

of 6—the value for distilled water as ordinarily prepared.[10] Since these authors do not refer to any coagulation of the albumin occurring under these conditions of pH and neutral salt content, we may infer with some degree of certainty that the protein was in fact undenatured.

The results obtained by Bugarsky and Liebermann for egg albumin are contradicted by those of Oryng and Pauli (29), who, working with evidently greater care, conclude that there is union of Cl^- ion with serum albumin. The precipitate obtained by the use of saturated ammonium sulfate was pressed, redissolved and dialyzed against distilled water for eleven weeks; no mention is made of bacterial decomposition. As the temperature was that of the laboratory, we may infer with some confidence that the material was undenatured, although the actual rate of denaturation of serum albumin at different pH values has not yet been effected. To detect the union of Cl^- with albumin, Oryng and Pauli employ the e.m.f. method, using calomel electrodes to detect the free Cl^- ion concentration. Readings are taken with and without the addition of serum albumin to a calomel-KCl solution—as in fact was done by Bugarsky and Liebermann with egg albumin. Oryng and Pauli find—as is to be expected if the pH of the solution is slightly on the alkaline side of the iso-electric point—that a fraction of the mercuro ions unites with the protein thereby releasing Cl^- ions, which introduces a correction term (treated as a constant but actually a variable, being a function of the Cl^- ion content) into the final estimates of the chloride ion bound by the protein. Inspection of the data shows that the e.m.f. differences produced by addition of albumin amount to 1–2 millivolts, which is not much to go on particularly in the very complicated system employed. Nevertheless the e.m.f. readings with albumin present are consistently higher than those obtained in its absence. The KCl content is varied over the range 0.01 N to 0.05 N. The corresponding quantities of Cl^- ion bound by 1 per cent albumin are 4×10^{-5} to 2.7×10^{-4} equivalents of Cl^-

[10] Kendall (38) found for distilled water which had been allowed to come into equilibrium with the CO_2 of the air, that at 18° the pH = 5.67, and at 25°, the pH = 5.69.

per gram of albumin. It is evident that the upper value recorded does not necessarily represent the maximum combination possible for Cl^- ion. No attempt is made to determine the amount of alkali metal bound, if any. Unfortunately no pH determinations are recorded. The prolonged dialysis of the protein against distilled water possibly brought this material to pH = 6 approximately, but it is uncertain whether this remains unchanged in the final system containing protein, calomel and potassium chloride. If the pH is on the alkaline side of the iso-electric point, as it certainly appears to be, union of an anion is a fact of profound importance. On making the solution definitely acid, it is found that the Cl^- ion bound is increased. This is to be expected, however, in view of the positively charged protein units thereby brought into existence. It is of interest to remark here that Oryng and Pauli find that glycine combines with Cl^- ion while urea does not. The limiting combination for glycine is in round numbers 5×10^{-3} equivalents of Cl^- per mole of glycine at the pH of the solution—presumably about the iso-electric point (pH = 6.08) of this amino acid. It is known that glycine under these circumstances is very largely in the zwitterion form, which thus evidently possesses combining capacity presumably for both anions and cations.

Limiting our consideration to the simple salts of the alkali metals, the conclusion which appears to be justified is that while undenatured albumin in the form of cation, i.e. at a pH definitely on the acid side of the iso-electric point, is capable of uniting with, say, chloride ion to an extent which varies with the pH and the same is true for albumin as anion in respect to, say, sodium ion, union of sodium ion and/or chloride ion with dissolved undenatured albumin, when the latter is in the near neighborhood of the iso-electric point, is less certain, though it probably occurs to a slight extent.

A question which suggests itself at this point is whether undenatured iso-electric albumin has a greater or less tendency to unite with the ions of neutral salts[11] than has denatured albumin.

[11] In general the union here contemplated would not necessarily mean the binding of salt anion and cation in equivalent amounts.

No data bearing upon this appear to be available so far as the alkali salts are concerned. Fortunately, however, Pauli and Matula (39) have examined the behavior of both undenatured and denatured serum albumin towards silver ion. The cell employed is:

Ag	AgCl, $AgNO_3$ 0.1 N (constant concentration)	KNO_3 saturated	$AgNO_3$ + protein	AgCl	Ag

It is not clear why the simple silver-silver nitrate electrode was not employed. The silver nitrate of variable concentration to which the protein could be added covered the range 0.0025 N to 0.05 N. The (undenatured) albumin concentration was 0.7 per cent. The differences in e.m.f. readings produced by the presence of the protein are larger than those obtained in the KCl experiments of Oryng and Pauli. The solutions remained clear until a concentration of 0.04 N $AgNO_3$ was attained. With 0.07 N $AgNO_3$ the precipitation of the albumin was complete, "a heavy white precipitate" being obtained. Presumably this is silver albuminate; it does not appear to have been analyzed, nor is it stated whether it was crystalline or not.

As regards union of silver ion with the dissolved protein, Pauli and Matula point out that the same limiting degree of combination—amounting to 5×10^{-4} gram equivalents (? 6×10^{-4}) in round numbers per gram of albumin—is attained both by the undenatured and denatured, but still dissolved, protein. Nevertheless, there is marked difference in behavior between the two, for it is found that the denatured material reacts much more readily than the undenatured, *the denatured protein approximating towards the limiting degree of combination at a much lower concentration of silver nitrate than is the case with the undenatured protein.* This is in agreement with the view that at the pH obtaining in the experiments—once more its precise value is uncertain, but it is evidently on the alkaline side of the iso-electric point as indicated by cataphoresis experiments referred to below—the undenatured molecular units present a certain degree of obstruction to the

salt ions not exhibited by the denatured units. The obstruction slows down the rate of interaction without influencing its ultimate extent. It almost looks as though the undenatured molecule had a number of closed rings forming part of its structure, which are absent in the denatured form. The behavior of serum albumin appears to find a parallel in the behavior of gelatin and casein, two proteins which are not denaturable but which probably exhibit certain resemblances to a denatured protein. In these cases heating has absolutely no effect upon the extent of combination with silver, which, judging from the curves given in the paper of Pauli and Matula approximates to the maximum value for the individual protein at a lower concentration of silver nitrate than is the case with undenatured serum albumin.

Pauli and Matula point out that the limiting extent of combination with silver ion in equivalents is the same as that found by Oryng and Pauli for chloride ion in the experiments with KCl in the presence of calomel.[12] Evidently it is assumed that the pH is the same approximately in the two cases. That it lies on the alkaline side of the iso-electric point is shown by cataphoresis experiments, which indicate a negative charge on the "pure proteins" (gelatin and serum albumin) as well as on the silver compounds.

Measurements with undenatured egg albumin and $ZnCl_2$ (Pauli and Schön (40)) showed similar behavior to the silver nitrate case. The pH which was determined in this case indicates a H^+ ion concentration of the order 10^{-6}, corresponding to the alkaline side of the iso-electric point. Cataphoresis experiments showed the protein material to be negatively charged. The Cl^- ion, which is the important one in view of the pH value, is shown to be bound and evidently to a greater extent than in the case of KCl. We must conclude, I think, that Pauli has justified his contention regarding the capacity of a salt anion to combine with negatively charged protein. This being the case, the claim that no union occurs at the iso-electric point falls to the ground. Evidently

[12] This statement appears to be fairly exact for gelatin; the evidence is less certain for serum albumin. On the whole the Cl^- ion bound seems to be little less than the Ag^+ ion bound.

some union is possible for both ions at the iso-electric point and in the neighborhood on both sides thereof. Further confirmation along the lines suggested by Northrop and Kunitz (41) and carried out by them for gelatin would however be desirable for denaturable protein. Incidentally, the work of Pfeiffer (42) upon the isolation of compounds in the solid form between simple amino acids and neutral salts, i.e., both ions involved simultaneously, may be taken at least as evidence of the possibility of similar compounds with protein in solution.

The conclusion that both ions of inorganic neutral salts are capable of uniting simultaneously with protein in solution at or near the iso-electric point does not require of course that undenatured protein when crystallized out in the presence of such salt and subsequently washed should still contain the salt, for as Pauli has shown these compounds are reversible and the salt may therefore be removed beyond the limits of detection by washing. The conclusions of Pauli are therefore quite consonant with the finding of Sörensen already referred to regarding his inability to detect $(NH_4)_2SO_4$ in the washed crystalline egg albumin.

It has been tacitly assumed in the above that the Na^+ and Cl^- attach themselves to carboxyl and amino groupings respectively. There is no conclusive evidence that such is the case. Thus the experimental results obtained by Northrop and Kunitz (43), using a membrane p.d. method, regarding the union of various anions and cations with gelatin are for the most part difficult of interpretation as regards the position at which the addition takes place. For example, the conclusion, based upon Hitchcock's observation (44) to the effect that, since the deaminization of gelatin diminishes the amount of hydrogen ion combined (from 0.00089 moles of acid per gram of untreated gelatin to 0.00044 moles per gram of deaminized gelatin), the hydrogen ion therefore combines at the amino group, is not necessarily true. Thus deaminization by destroying a certain number of the free amino groups would cause, if the zwitterion form exists, a corresponding number of the "partner" $-COO^-$ groups to become practically unionized $-COOH$ groups, no longer titratable by acid of which a smaller amount is now required, corresponding, in fact, to the

zwitterion $-COO^-$ groups unaffected (indirectly) by the deamination. Obviously this way of regarding the matter entails the assumption that deamination does not remove all the free amino groups. Simms (45), who repeated Hitchcock's measurements, concludes that "deamination removes a large part of the lysine group and does not remove the pre-arginine, the arginine or the histidine groups."

An important observation made by Northrop and Kunitz is that the position of attachment of hydrogen ion is likewise the position of attachment of copper ion. As to the location of other ions the evidence is uncertain. In the case of Cl^- ion Northrop and Kunitz show that when $CuCl_2$ is employed the amount of Cl^- ion combined is the same whether the gelatin be deaminized or not. Of the two types of groups, amino and carboxyl, it is evident that Cl^- would only combine at an amino group. We might possibly account for the above result by asserting that the Cl^- unites at the pre-arginine, arginine or histidine portions and not at the lysine. This would be purely an *ad hoc* assumption at this stage. Failing this, we would have to assume union at some other group, such as the peptide linkage or the "oxonium" oxygen of the carboxyl group. If this be assumed in this case, it would be only logical to take such positions into consideration when union with cations is considered. This would greatly enlarge the variety and therefore complexity of the problem presented by union of protein with simple ions. The position is therefore very obscure at present.

As will have been realized the real difficulty in demonstrating beyond any doubt the fact of union and the extent of union of both ions of a neutral salt with a protein at its iso-electric point lies in the minuteness of the extent of union. This is particularly the case with salts of the alkali metals. Parenthetically it may be well to point out that the union of either or both ions of a neutral salt with iso-electric protein even on the assumption (which may not be true) that union takes place at the amino and carboxyl groups is not at variance with the conclusion already drawn regarding the behavior of protein towards dilute acid alone or dilute alkali alone, resulting in the production of a largely ionized

protein chloride and sodium proteinate. These are true salts, and as such are largely ionized over a fairly wide pH range and completely ionized in the neighborhood of the iso-electric point, i.e., no union of protein ion with oppositely charged inorganic ion. The amount of the latter present from the acid or alkali is small in the neighborhood of the iso-electric point (of the order $10^{-4.7}$ N) compared with the concentrations of the ions of neutral salt (of the order 0.01 to 0.1 N) employed in the experiments dealt with above.

Ito and Pauli (46) have recently investigated the effect of neutral salts upon a number of proteins on the alkaline side of the iso-electric point. They find that with salts of the NaCl type even up to 0.02 M the direction of motion (of egg albumin and serum albumin) is unchanged—namely, towards the anode. With chlorides of Ba^{++}, Ca^{++} and Mg^{++} at 0.05 M, migration occurs in both directions. In the case of gelatin the chlorides of the alkaline earths reverse the direction of motion of the protein particles even at a concentration of 0.004 M.

Ito and Pauli have likewise examined the behavior of pseudo globulin on the acid side of the iso-electric point. They find that potassium salts containing the following anions: Cl^-, SO_4^{--}, $Fe(CN)_6^{---}$, $Fe(CN)_6^{----}$, change the cathodic migration, reversal of the direction being produced by $Fe(CN)_6^{----}$ at 5×10^{-5} N, by $Fe(CN)_6^{---}$ at 1×10^{-4} N, by SO_4^{--} at 7.8×10^{-4} N, while Cl^- at 0.05 N causes movement in both directions. These effects are in general agreement with expectation.

Although Pauli has succeeded in comparing the interaction of silver nitrate with undenatured and denatured serum albumin under concentration conditions at which flocculation did not occur even in the case of the denatured protein, similar experiments do not appear to have been attempted in other cases, e.g., with denatured hemoglobin or egg albumin and alkali salts. Under ordinary circumstances on addition of neutral electrolyte even in small amounts, the outstanding phenomenon is flocculation, which is a maximum in the neighborhood of the iso-electric point and diminishes as the pH diverges from this region. This effect, therefore, seriously interferes with an examination of the

possible union of the ions of the salt with protein denatured but still dissolved. We are justified probably in inferring some degree of chemical interaction even here in the light of the behavior (already briefly discussed) of a protein such as gelatin, which we have already suggested to be analogous to a denatured protein rather than an undenatured one. Casein may also be cited as a protein which behaves in a like manner.

5. Alteration in pH as a consequence of union of protein with neutral salt

The conclusion that the ions of neutral salts (not necessarily in exactly equivalent amounts) can combine to a slight extent with protein at or near the iso-electric point raises the question as to the possibility of alteration in pH as a consequence of such union. We shall deal with this first on the assumption that the protein unit contains a certain number of zwitterion forms, and secondly from the point of view of the unionized form. If the zwitterion form is present, addition of ions of, say, NaCl in equivalent amounts will obviously leave the pH of the system unaffected. If the Na^+ is more combined than the Cl^- ion this will mean that in addition to a number of symmetric structures represented by $Cl \cdot NH_3 \cdot R \cdot COONa$, there will be produced on certain of the protein units one or more structures of the type $^+NH_3 \cdot R \cdot COONa$. This involves a destruction of the balance of the original zwitterion. If these structures remain intact they will not alter the pH, but will incidentally give a positive charge to the protein units even at the iso-electric pH. If, however, such units combine with OH^- ion, as it seems likely they would do in view of the weakness of the isolated charged amino group, the pH of the systems would be expected to decrease, and this should be observable in spite of the buffering action of the protein which has not interacted at all with the salt.

Incidentally, the differential effect should become more pronounced the greater the difference in valency possessed by the salt ions involved. If the protein be denatured, flocculation would of course occur, and this possibly by the union of the positively charged individuals (as the case may be) with free

protein molecules. This point is considered in the section on flocculation and will not be discussed here. On the other hand, if the basic-acidic groups of the protein do not give rise to the zwitterion structure, interaction of Na^+ and Cl^- would entail the splitting off of H^+ and OH^-, respectively, from the carboxyl and amino groups. Once more, if exactly equivalent amounts of Na^+ and Cl^- are united, the pH will remain unaffected. If there is an excess of Na^+ combined, the solution should become slightly acid. As might have been anticipated, it is not possible therefore to decide on such evidence as this whether the zwitterion form is present or not. The only experimental work bearing upon the question of production of acidity or alkalinity as a consequence of union with neutral salts which is known to the writer is that of Chick and Martin[13] (47), who have found, for example, that addi-

TABLE 3

Effect of addition of NaCl to a solution of crystalline egg albumin

CONCENTRATION OF NaCl	H^+ ION CONCENTRATION (DETERMINED ELECTRICALLY)
0	$10^{-4.88}$ N (132×10^{-7} N)
0.1 N	$10^{-4.88}$ N (132×10^{-7} N)
1.0 N	$10^{-5.30}$ N (50×10^{-7} N)
2.0 N	$10^{-5.73}$ N (19×10^{-7} N)

tion of considerable NaCl to a 1 per cent solution of crystalline egg albumin (practically at the iso-electric point initially) causes the solution to become alkaline (see table 3). Unfortunately the pH of the original NaCl stock solution itself is not recorded. As this may have been of the order 5.9, corresponding to aqueous solutions in equilibrium with the atmosphere, it is possible that the changes in pH indicated in the table were the natural consequence of increasing the NaCl content. On the other hand if the effect is a real one due to interaction of the protein and salt, the direction of the change indicates that the Cl^- ion is preferentially bound.

[13] Chick and Martin themselves point out that the earlier work by Hardy and by Pauli and Handovsky upon alteration in pH by addition of neutral salts is rendered somewhat uncertain by the fact that the indicator method was employed. Chick and Martin use the e.m.f. method.

If we assume for the time being that the Cl^- ion attaches itself to an amino group of a zwitterion structure, such union will render the carboxyl ion free to unite with H^+ (from the water) with consequent formation of OH^-. If the amino and carboxyl groups are both in the classical form, addition of Cl^- at the amino $-NH_3OH$ group would necessarily liberate OH^- from that position directly.

It may be pointed out that from Loeb's titration data for albumin with caustic soda it follows that approximately 1.0 cc. of 0.1 N NaOH is required to bring 1 gram of albumin (dissolved in 100 cc.) from the iso-electric point to pH = 5.3. That is, 1×10^{-4} equivalents of OH^- are involved. This ought to represent in the sodium chloride case the excess of Cl^- ion bound by 1 gram of albumin over the Na^+ ion bound at this pH. It gives us therefore a lower limit for the quantity of Cl^- bound. The result is at least consonant with the finding of Pauli and Matula (already referred to), who estimate the upper limit of Cl^- bound by 1 gram of albumin as 5×10^{-4} equivalents, at a somewhat uncertain pH on the alkaline side of the iso-electric point.

In the second of the papers referred to, Chick and Martin have recorded the behavior of sodium citrate with respect to 0.057 per cent denatured egg white. The protein material is dispersed in acid to begin with. The concentration of sodium citrate employed was varied from 0.0002 N to 0.006 N. Flocculation manifested itself when the concentration of sodium citrate reached 0.0006 N. On raising the concentration to 0.004 N, dispersion once more took place. The initial pH of the system (sodium citrate 0.0006 N) was found to be 3.59; the final pH (sodium citrate 0.006 N) was 6.21. In the first solution the protein particles were positively charged, as would be expected in view of the appreciable acidity. In the final solution the protein is negatively charged. As the final pH is as high as 6.21, this of itself would cause the protein portion which has not reacted with the salt at all to take on a negative charge. The production of OH^- ion is evidently occasioned by preferential union of citrate ion. If, again, this is regarded as taking place at the amino group, an equivalent of OH^- will be produced, thereby increasing the pH as in the NaCl case. It is evident, however, from the magnitude

of the change in pH that citrate ion—as would be expected from its valency—is much more readily bound than is Cl^- ion. This capacity for union, coupled with the fact of a valency greater than unity, is also probably the cause of the initial flocculation at the iso-electric pH, followed by dispersion owing to the existence of numerous mutually repelling structures of the type $\overline{\overline{\text{Citrate}}} - NH_3 \cdot R \cdot COOH$, i.e., divalent units. Owing to the probability of such units it is impossible to say whether the final negative charge is to be ascribed mainly to such or mainly to protein anions resulting from interaction with hydroxyl ion. Experiments in buffered systems would seem to be necessary in this connection.

Results have likewise been obtained by Chick and Martin for the salts KCl, K_2SO_4 and $CaCl_2$ acting on 1 per cent pure crystalline egg albumin, starting from a definitely alkaline point, pH = 8.88. Addition of these salts increases the H^+ ion concentration under the above circumstances. It would therefore be expected that preferential union with the cation of the salt occurs with consequent production of H^+. Thus, with KCl at 0.05 *N* the pH alters to 8.42; with $CaCl_2$ at the same normality the pH alters to 7.87. It may be inferred that higher concentrations of $CaCl_2$ would further affect the hydrogen ion, i.e., increase it still more. It may be anticipated that a pH identical with that of the iso-electric pH of the pure protein could thereby be attained. Under such conditions, however, the protein would not be simple iso-electric material represented by the structure $NH_2 \cdot R \cdot COOH$ or $^+NH_3 \cdot R \cdot COO^-$, but material containing in addition a considerable number of structures of the type $^+NH_3 \cdot R \cdot COOCa^+$ as well as of the type $Cl \cdot NH_3 \cdot R \cdot COOCa^+$, the pH being no longer that corresponding to electric neutrality of the protein material as a whole. On further addition of $CaCl_2$ with alteration of pH to the acid side of the iso-electric point of the parent protein, it may be expected that preferential union with Ca^{++} will more or less rapidly diminish owing to the cation form of the normal protein coming into existence. Thus Northrop and Kunitz (*loc. cit.*) have measured by the membrane p.d. method the amount of Ca^{++} bound at different pH values in the case of gelatin, and have found that at pH values lower than 3 the Ca^{++}

is completely displaced by the H^+ ion. A similar behavior may be anticipated with albumin. The corresponding membrane p.d. measurements have not yet been carried out.

We may at this point draw attention to the apparently "antagonistic" effects produced by NaCl and KCl, respectively, on crystalline egg albumin as recorded by Chick and Martin. The alteration in pH in the two cases is such that we have inferred preferential union of Cl^- ion over Na^+ in the case of NaCl, but preferential union of K^+ over Cl^- ion in the case of KCl. It is highly improbable, however, that there can be any distinction of this sort as between potassium and sodium. The explanation would appear to be that a different pH range was employed in the two cases. Thus in the KCl experiments the initial pH, namely 8.88, is distinctly on the alkaline side. This condition will naturally be very favorable to union of a metallic cation since the protein units here carry a considerable negative charge. Consequently the preferential union of K^+ which is observed decreases the alkalinity, i.e., decreases the pH. It seems likely that at a pH not far from the iso-electric point but on the alkaline side thereof, the union of either K^+ or Na^+ would be just about the same as that of Cl^- ion. Such a position should correspond to union of both ions of salt in equivalent quantities without alteration in pH. This position has not yet been found experimentally, so far as I am aware. For Ca^{++} a similar position corresponding to equal union of both ions would be expected to lie on the acid side of the iso-electric point.

It follows further that the presence of a suitable inorganic salt with multivalent anion may cause part at least of the protein to carry a negative charge even on the acid side of the iso-electric point. This has been shown to be the case by direct electrophoresis measurements by Chick and Martin in the case of denatured serum protein in the presence of 0.07 N Na_2SO_4 at a pH = 4.01, the protein being in the dispersed state under these conditions. The excess union of SO_4^{--} thus indicated, naturally demands that prior to addition of the salt the pH of the solution must have been more acid still. This is the case, the initial pH being 3.24, at which point it was shown by experiment that the protein was dispersed and positively charged.

In the light of the above considerations we are probably justified in concluding that observed alterations in pH—a quantity susceptible of very accurate determination—afford the most exact means at present available for determining the difference in the extent of union of the salt anion and cation respectively. If one of these ions can be determined with accuracy by an independent method, say the Na^+ ion by means of the sodium amalgam electrode, the problem of the extent of union of both ions would conceivably be brought in such a case to a much more satisfactory position than exists at present.

Returning to the results obtained by Chick and Martin for the alteration in pH on addition of $CaCl_2$, it is of interest to calculate the extent of the differential effect indicated thereby under the condition of the experiment. Thus, if x is the number of equivalents of Ca^{++} bound by 1 gram of protein in excess of the amount of Cl^- bound simultaneously, then x is likewise the net number of equivalents of H^+ which are thereby produced, and if y is the number of equivalents of H^+ taken up by the residual protein to reduce the negative charge, then $x - y$ is the number of equivalents of H^+ effectively added to the solution and remaining as such. In the experiments referred to it is found that the H^+ ion concentration increases from $10^{-8.88}$ N to $10^{-7.87}$ N by the addition of $CaCl_2$ in such quantity as to make the solution 0.05 N with respect to this salt. The increase in H^+ ion concentration observed is 0.1217×10^{-7} gram-equivalents per liter. Since the volume of the solution employed is 100 cc. (this volume containing 1 gram of crystalline egg albumin), the increase in mass of H^+ in equivalents is 0.1217×10^{-8}. That is $x - y = 1.2 \times 10^{-9}$ equivalents. From Loeb's titration curve it follows that approximately 0.9 cc. of 0.1 N NaOH is taken up by 1 gram of albumin in 100 cc., when the pH of the solution is changed from 7.87 to 8.88. Hence this quantity of 0.1 N acid would be taken up by the albumin in reversing the titration. That is, $y = 9 \times 10^{-5}$ equivalents. It follows that x and y are practically identical, *viz.* 9×10^{-5} equivalents. Hence when 5×10^{-3} equivalents of Ca^{++} are present in 100 cc., 9×10^{-5} equivalents of Ca^{++} are bound by 1 gram of the protein at pH = 7.87, in excess of the amount of Ca^{++}

bound simultaneously with and balanced by Cl^- ion. If the latter be very small, as would be expected at a pH so alkaline as 7.87, then the above figure gives the (minimum) value of Ca^{++} bound under these conditions. It may be mentioned in this connection that, according to Northrop and Kunitz, in the case of gelatin the amount of Ca^{++} bound attains a roughly constant value on the alkaline side of the iso-electric point, amounting to 3.5×10^{-4} moles per gram.

As already mentioned, addition of KCl to crystallized egg albumin in the distinctly alkaline region reduces the alkalinity. In view of this behavior it might be thought that the function of the inorganic salt in bringing about flocculation of the protein is that of adjusting the pH of the system towards the iso-electric point. It will be recalled, however, that in the case of NaCl, addition of the salt at pH = 4.88 altered the pH away from the iso-electric point. Such alteration therefore cannot account for the flocculating action. As Chick and Martin express it, "Whatever may be the nature of the process involved, it is clear that the change in reaction of protein solutions caused by electrolytes is inadequate to explain the influence of the latter upon agglutination." A possible mode of interaction in this connection is considered in the section dealing with flocculation.

A striking illustration of the effect of alteration in pH produced by a salt is afforded by the results of Thomas and Norris (48) in connection with the effect of heavy metal salts, namely, zinc chloride and thorium chloride, upon egg albumin. In this case, however, the increase in H^+ ion is ascribed entirely to hydrolysis of the inorganic salt itself. The very large variation in H^+ ion thus obtained is shown by the fact that with $ZnCl_2$ at 0.0008 *M* the pH = 7.07, at 1.715 *M* the pH = 2.5. Thomas and Norris, as a matter of fact, are concerned with the precipitating action of such salts, especially with the existence of the "tolerance region" between the first and second zones of precipitation. It is concluded that the effects to be expected are a function of the H^+ ion concentration, the first precipitation (with low salt content) being due to union of the heavy metal cation with albumin in the form of anion to produce a reversible precipitate. With further

addition of inorganic salt, the H^+ ion passes to the acid side of the iso-electric point with the result that the metal-albumin salt is no longer formed. On greatly increasing the salt concentration the albumin is denatured and is apparently regarded as being precipitated as such, though it may well have been denatured albumin chloride. (Compare Chick and Martin's results with denatured albumin sulfate, considered in the section on flocculation.) It is concluded that a "heavy metal ion is intrinsically an albumin denaturant." In general, the explanation offered for the tolerance zone is in agreement with the general line of thought pursued in the present section. As to why or how a heavy metal ion is capable of bringing about denaturation nothing is known. On the supposition that it is really a hydrogen ion effect it would be necessary to assume interaction of heavy metal even with the positively charged protein unit with local increase in H^+ ion. For this to occur it would be necessary to show actual union with the metal by means of cataphoresis. This was not done in the above case.

6. Solvent action of neutral salts and of non-electrolytes upon undenatured and denatured protein

We are not dealing here in the first instance only with proteins such as albumin and hemoglobin which possess an appreciable solubility in water, but with proteins of the globulin type which are insoluble in water but "soluble" in neutral salt solution. When much salt is present it acts as a precipitant, e.g., ammonium sulfate.

This striking, and as yet, imperfectly understood behavior has been the subject of considerable investigation at the hands, amongst others, of Hardy (49), Mellanby (50), Sörensen (51), Osborne and Harris (52), Cohn and Prentiss (53). The amount of serum globulin "dissolved" by a neutral salt depends not only upon the nature and concentration of salt but likewise upon the amount of protein present in the original globulin suspension. This has been ascribed to the existence of more than one protein

in the material employed.[14] It should perhaps be emphasized that the concentration of salt here contemplated is large compared with that required to bring about flocculation of a denatured protein of the egg albumin type. The phenomenon, in fact, suggests peptization rather than solution proper. So far as I can gather, the concentration of salt which possesses solvent action is comparable with the upper limits of concentration which have been employed in connection with experiments on the union of neutral salts with proteins of the albumin, hemoglobin and gelatin types.

Hardy (*loc. cit.*) has drawn attention to a striking quality of globulin when dissolved in neutral salt. "The solution contains no ionic, no electrically charged globulin, as is shown by the absence of all movement in an electric field, while solutions in dilute acids and alkalies are ionic." It would seem that both ions of the salt are in combination with the globulin to equivalent extents.

To give an idea of the extent of solvent action produced by salts, reference may be made to some of Mellanby's experiments with KCl acting upon globulin, from horse serum, at 20°, the globulin being initially in the form of a suspension in water containing approximately 1.4 grams of protein in 100 cc. The solvent action is expressed as "per cent of original globulin dissolved" by the salt. With KCl at concentrations 0.047 N and 0.0806 N respectively, the "per cent of original globulin dissolved" increased from 40.7 to 84.0. With NaCl the increase in solubility expressed in the same manner rose from 35.7 to 91.5 on altering the concentration of NaCl from 0.0427 N to 0.0853 N. Incidentally, Mellanby finds that the solvent action of potassium iodide is greater than that of potassium bromide and this is in turn greater than that of potassium chloride, but the differences are not large.

In the case of the vegetable globulin edestin, Osborne and Harris (*loc. cit.*) give a considerable number of solubility deter-

[14] The effect of the mass of globulin upon the composition of the fluid phase was first detected by Hardy (54) who, suspecting it to be due to a second component, repeated the experiments with "pure crystals of edestin washed with 20 per cent alcohol." The phenomenon was again observed. It is evidently a real characteristic of globulin behavior.

minations with salts of different valency and concentration. With NaCl at concentration 0.4 *N*, the solubility of edestin is 0.5 gram in 100 cc.; with 0.8 *N* NaCl, the solubility is 7.5 grams in 100 cc., the increase in solvent action being very great in the case of edestin. Bivalent salts are more potent agents than univalent salts.

That the solvent action is concerned with some kind of excessive disaggregation of the "solid" globulin is indicated by the finding of Gortner, Hoffman and Sinclair (55) in connection with certain vegetable globulins to the effect that on dissolving the protein with the aid of KBr (a very effective agent) and subsequently dialyzing so as to obtain the solid once more, this solid is now much more readily peptizable by KCl than it was in the first instance.

While it is almost certainly correct to describe the solvent action as peptization, this of itself does not carry us very far as regards the mechanism of the process, which still remains one of the least understood in the field of the physical chemistry of the proteins.

As regards the mechanism of the peptizing or solvent action of neutral salts in appreciable concentration upon globulins it appears to be generally assumed that the effect is a "direct" one due to the interaction of the salt (ions) with the protein. That peptization may be "indirect", that is, may have its origin in an alteration in the relative amounts of monohydrol and dihydrol of the water, has been suggested by Bancroft (56).

It should be mentioned that, according to Mellanby, "globulin is precipitated from solution in neutral salts by minute quantities of salts of the heavy metals."

Mellanby has concluded that "solution of globulin by a neutral salt is due to forces exerted by its free ions. Ions with equal valencies, whether positive or negative, are equally efficient and the efficiencies of ions of different valencies are directly proportional to the square of the valencies." The latter statement does not as yet rest upon a very certain experimental foundation. The position of the problem up to 1925 is well described by Cohn (57). Regarding the concentration of salt

involved, Cohn writes, "Many times as much salt as acid or alkali is required to dissolve a globulin, and solubility bears a logarithmic rather than an arithmetic relation to concentration." Clearly we are no longer dealing with stoichiometric effects alone, such as have come under review in previous sections.

Such stoichiometric unions must of course be presumed in the present case in the light of the behavior of the water-soluble proteins albumin and gelatin with respect to neutral salts. The acceptance of this, however, does not afford any real explanation of the solvent action itself, which has to be sought on a non-stoichiometric basis as esssentially due to a change in environ-

TABLE 4

Solvent action of salts upon proteins

(Cohn and Prentiss)

pH = 6.8

CONCENTRATION OF PHOSPHATE $KH_2PO_4 + K_2HPO_4$	SOLUBILITY OF HEMOGLOBIN PER 100 CC.
	grams
0.0213	3.32
0.062	3.81
0.121	4.85
0.293	6.66

ment. Incidentally, one would expect that the conditions for stoichiometric union would be most favorable subsequent to the disaggregating or peptizing effect itself.

Although the solvent action of salts upon proteins has been most clearly recognized in the case of the globulins, owing to the fact that these are practically insoluble in water, a similar effect would be expected even in the case of substances such as albumin and hemoglobin which are soluble to some extent in water, provided they be undenatured. This has been observed by Cohn and Prentiss (*loc. cit.*) for crystalline oxyhemoglobin from the horse, using a buffer to fix the pH. Table 4 illustrates the order of magnitude of the effect, which is much greater than would have been anticipated, especially as the concentrations of salts employed are by no means great. We are

evidently dealing with a phenomenon essentially similar to that observed in the case of the globulins. The solubility at pH = 6.6 was less than at either 6.4 or 6.8. Cohn and Prentiss treat the effect in terms of the Debye theory, according to which the activity coefficient of a given ionic species is diminished by the presence of all the ions existing in the solution. If the solubilities of hemoglobin in water alone and in a salt solution are S_0 and S respectively, it follows on the basis of the theory of activity that $\alpha_0 S_0 = \alpha S$, where α_0 and α stand for the activity coefficient of the protein in water and in the salt solution respectively. Since the activity coefficient α_0 can be set equal to unity in such a case, it follows that

$$\log_{10} \frac{S}{S_0} = - \log_{10} \alpha$$

Denoting by c the concentration of any single ionic species (say the Na^+ ion *or* Cl^- ion in the case of NaCl solution) and by ν the valency of this ionic species, then $\Sigma\, c\nu^2$ stands for the sum of the $c\nu^2$ terms for all the ionic species present. Cohn and Prentiss employ the equation of Debye, which expresses the logarithm of the activity coefficient of a given ionic species—the hemoglobin in the present case, here treated as an electrolyte with mean valency z—in terms of $\Sigma\, c\nu^2$, which measures the influence exerted by the ions upon the protein activity coefficient. The equation is

$$- \log_{10} \alpha = \log_{10} \frac{S}{S_0} = \frac{0.5\, z^2 \sqrt{\Sigma\, c\, \nu^2}}{1 + 1.5 \sqrt{\Sigma\, c\, \nu^2}}$$

Cohn and Prentiss show the general applicability of this expression to dissolved horse hemoglobin by obtaining with its aid a reasonably constant value for S_0, the magnitude of which is in agreement with the somewhat approximate but directly observed value in water alone. To make the equation fit in this satisfactory manner, it is necessary to conclude that hemoglobin acts like a bivalent or uni-quadrivalent compound, i.e., $z^2 = 4$.[15]

[15] The authors suggest that the second alternative is physically the more probable in the present case. It may be added that the measurements referred to apply to a pH region near the iso-electric point. It is probable that the valency is a function of pH.

With the aid of the equation the values of S_0 are calculated to be 12.2, 11.2, and 13.1 grams per liter at pH 6.4, 6.6, and 6.8, respectively. The authors point out that the effect of salt on hemoglobin, though very considerable, is much less than upon a globulin. They regard the latter type of protein as presumably of higher valence type. In the case of edestin $\sqrt{z^2}$ would have to lie between 5 and 6 to account for the solubility data of Osborne and Harris on the basis of the Debye theory of ionic interaction.

Incidentally, the change in solubility of hemoglobin with pH serves to indicate that this is a factor the possible significance of which has been overlooked hitherto in investigations of the solvent action of neutral salts upon globulin suspensions. It may be pointed out that the fact that hemoglobin shows minimum solubility—which on the above mode of treatment one would take as indicating minimum valency, i.e., an iso-electric state—not at the true iso-electric point but at a pH on the acid side thereof, is probably to be ascribed to preferential union of protein with the phosphate ion present in the buffer employed, thereby giving the protein unit an effective negative charge which is balanced by an alteration to a more acid environment.

It must be pointed out that to ascribe to z, the valency of the protein, a value greater than zero implies that the protein has certain groups effectively electrically charged even at the iso-electric point. This point has already been made by Bjerrum himself (58) in connection with simple amino acids. Bjerrum suggests that a mode of demonstrating the existence of zwitter-ions, as distinct from the classical form, would be to determine the influence of electrolytes on the activity coefficient of the amphoteric body. To quote from Bjerrum's paper: "For the form $NH_2 \cdot R \cdot COOH$ it is to be expected that the solubility will not be influenced by addition of neutral salt to a greater extent than the solubility of a non-electrolyte in general would be influenced. It is otherwise for the form $^+NH_3 \cdot R \cdot COO^-$. In view of the electric charges one would expect that an addition of neutral salt would here diminish the activity and consequently increase the solubility, as is always the case with salts not having an ion in common. If the two charges are far apart, the

increase in solubility ought to be twice as great as for an ordinary univalent salt. The closer the charges are together the smaller must be the effect, since the charges partly annul one another's influence. Preliminary solubility measurements of methyl orange in potassium chloride solutions . . . . indicate an effect which is about one-third of that expected on the basis of distant charges." Under the conditions of the experiments referred to methyl orange exists almost entirely in the zwitterion form. Bjerrum likewise points out, "the zwitterion of an amphoteric electrolyte like all other ions must likewise diminish the activity of other ions present." He finds this borne out by experiment. Further, "mixtures of amino acids are much more soluble in water than would be expected from the solubilities of the single acids. Not only do the acids crystallize with greater difficulty, but they are in fact more soluble. Thus the unexpected increase in solubility on the part of difficultly soluble substances of this type can be explained by their zwitterion nature as arising from attractive forces between the electric charges." The mutual effect here referred to by Bjerrum may possibly be operative in biological fluids, for example in blood, where globulin and albumin are present simultaneously.

In spite, however, of the apparent cogency of Bjerrum's argument, the possibility still presents itself that the strongly dipolar groups represented by unionized amino and carboxyl might be as effective as zwitterions in respect to the influence of salt and other substances on solubility.

To return to the question of the effective valency of a protein as estimated from the influence of neutral salt upon the solubility of the protein, it may be recalled that in the work referred to undenatured protein was employed. It would be of considerable interest to attempt to make similar measurements with denatured material as a step towards understanding the peptizing effect to which appeal has to be made in considering the solvent action of salts upon insoluble proteins, e.g., the globulins.

A further point arises in connection with the application of the Debye equation to the solvent action of salts upon a protein. The dielectric capacity D appears in the numerator and in the

denominator of the Debye expression, the numerical factor 0.5 (in the form of the equation quoted above) including a group of terms having $D^{3/2}$ in the denominator thereof, the factor 1.5 likewise having $D^{1/2}$ in the denominator of the group of terms covered by this numeral.

As ordinarily employed the value used for D is the dielectric constant for water at the temperature to which the measurements refer. With the concentrations of salts present in protein solubility determinations referred to, the D term may differ somewhat from that of pure water. Dielectric capacity measurements of aqueous salt solutions carried out by different observers show very great discrepancies. On the whole there is agreement as to the fact that on addition of small quantities of salt the dielectric capacity falls. On further addition of salt, beyond the concentration $0.01M$ approximately, according to the observations of Schmidt (cf. Carman and Schmidt (59)), the dielectric capacity in the case of NaCl and KCl rises and soon exceeds that of water. In the latter region (which may correspond with that employed in the experiments under consideration) to make the Debye equation apply it would be necessary to adopt higher values of z (the valency of the protein) than that suggested by Cohn and Prentiss. The initial fall in the dielectric capacity on addition of very small quantities of salt would, if the Debye equation be applicable, be expected to increase the solvent action more rapidly than that calculated in the ordinary way. Actually this anticipation does not appear to be realized. Thus Osborne and Harris (*loc. cit.*) state that "edestin, dissolved in water with a minimal quantity of alkali, forms a solution which is abundantly precipitated by minute quantities of sodium chloride, in just the same way as solutions similarly made with acid are precipitated, the precipitate in each case being dissolved by a larger proportion of salt."

If this behavior is general in character[16] it suggests that before the Debye effect becomes appreciable the effect of salts, in minute

[16] Analogous results for the effect of salts on albumin have been obtained by Mathews (61).

amounts, is essentially that of inducing colloid precipitation.[17] In other words, the presence of small amounts of salt acting by chemical union or by local decrease in dielectric capacity, or by the operation of both functions, "sensitizes" the colloidal particles or suspension thereby increasing the tendency towards aggregation.

In discussing the presumed alteration in dielectric constant attributed to the presence of added salt it is necessary to draw attention to an effect likewise involving dielectric capacity which may have a completely swamping effect as compared with any alterations in D produced by the salt. I refer to the effect of the protein itself upon the dielectric capacity of the solution. According to the measurements of Marinesco (60) upon methemoglobin (source, method of purification and pH not stated) an astonishingly great fall in the dielectric capacity of the "solution" in water occurs. Thus with 0.75 per cent methemoglobin $D = 58.3$, while with 2 per cent protein $D = 31.8$. Provided these dielectric capacity measurements are reliable, it would be inferred that for saturated solutions of methemoglobin (and therefore very probably for oxyhemoglobin) the numerical value of the group of terms collected in the numeral 0.5 in the numerator of the Debye equation, as well as one of the terms in the denominator would be so much altered as to give rise to too small values for z, the effective valency of the protein. The matter is raised at this point although it cannot be carried further owing to the complete lack of the necessary dielectric constant data.

Apart from the particular point just raised, it is evident that the Debye equation might be regarded as affording a basis for the increase in solubility exhibited by a protein by the addition of neutral salt, provided each unit of the protein carries effective electrical charges even though equal in number but of opposite sign. Such an effect is hardly to be distinguished from a peptization effect, which from this point of view would be ascribed to an electrical environmental effect. Any actual combination of either or both of the salt ions with the protein would on this basis be regarded not as a cause of peptization but merely as a concomitant. That the theory underlying the Debye equation cannot be regarded as covering the whole of peptization becomes

[17] We are here anticipating to a certain extent the topic discussed in a later section, *viz.* flocculation.

evident when we recall that peptization of material, whether electrically neutral or not, may be brought about by non-electrolytes. Further, in using the term peptization we have in mind not only an increased solubility effect, but likewise a preliminary (and essential) disaggregation of gross units of colloidal material. This implies that the cohesive forces operative between the colloidal units of a large aggregate can be sufficiently reduced by the peptizing agent. The existence of this phenomenon suggests the possibility of the applicability of another expression, namely the "distribution" equation of Bjerrum and Larsson (62). This equation is intended strictly to apply to the solubility of an electrolyte in ion-free media of different dielectric capacities. At first sight, therefore, it would appear to be wholly inapplicable to the present case. It is just possible, however, that a complex "tree-like" structure such as a protein molecule or aggregate may be so bulky that in comparison therewith even a salt solution may be regarded as a medium essentially homogeneous and of uniform dielectric capacity. The act of dissolution must certainly involve the protein molecule as a whole, though it may be dragged into solution, so to speak, by weakening of the localized forces operative between certain points on the contiguous protein molecules, these forces being associated with a limited number of groups (possibly the amino or carboxyl groups in the zwitterion form) likely to possess marked mutual affinity. From this point of view it seems not unreasonable to regard the effective valency as comparable with the number of amino or carboxyl groups in the zwitterion form. This would mean a number varying with the pH of the solution, being of the order 2 to 5 presumably in the neighborhood of the iso-electric region. It is evident that these electrically charged regions are widely separated from one another in the protein molecule as a whole. At pH values sufficiently far removed from the iso-electric region, the effective charge on any protein unit becomes predominately of one sign, with consequent mutual repulsions between contiguous units and therefore additional solubility—in agreement with experiment—on this ground alone quite apart from dielectric capacity considerations. We are concerned for

the moment more particularly however with the behavior in the region of the iso-electric point.

The Bjerrum-Larsson equation deals primarily with the distribution coefficient or ratio of solubilities of an electrolyte—in the case considered, the protein—in two different environments. Regarding the change in solubility as due solely to the alteration in the electrostatic forces owing to alteration in dielectric capacity, the equation is

$$\log_{10}\frac{c_1}{c_2} = \log_{10}\frac{S_1}{S_2} = \frac{z^2 \times 1.2 \times 10^2}{r}\left(\frac{1}{D_2} - \frac{1}{D_1}\right)$$

where S_1 and S_2 denote the solubilities of the protein in two media of dielectric capacity D_1 and D_2 respectively. z is the mean valency or charge (of either sign) upon the protein unit, and r the radius of the protein unit expressed in Ångstrom units.

Taking 65,000 as an average molecular weight for a protein, and treating the molecule or unit as though it were a sphere of unit density, it follows that $r = 30$Å. The value of z we shall consider as lying between 5 and 2. Whence the value of $\frac{z^2 \times 1.2 \times 10^2}{r}$ lies between 100 and 16. Taking a solution of 0.02 N NaCl as the first solvent, it follows from Schmidt's data that $D_1 = 93.6$. With 0.016 N NaCl, $D_2 = 80.8$. Whence $\left(\frac{1}{D_2} - \frac{1}{D_1}\right)$ is approximately 1.7×10^{-3}. Hence $\log_{10}\frac{S_1}{S_2}$ lies between 0.17 and 0.027. That is, $\frac{S_1}{S_2}$ lies between 1.48 and 1.06. From the graphs given in Mellanby's paper the increase in solubility of serum globulin in so far as it can be estimated at all for the NaCl range 0.016 — 0.02 N appears to lie between 20 per cent and 40 per cent, i.e. $\frac{S_1}{S_2}$ lies between 1.2 and 1.4. It is impossible to say whether there is substantial agreement or clear disagreement between the observed and calculated values, owing to the uncertainty of the dielectric constant data on the one hand and of the solubility data on the other. In the case of edestin, much more extensive

solubility data are available, but the concentration of salt employed is far beyond that for which any dielectric constant data are available. It may be well to emphasize here an essential difference between the Debye equation and the Bjerrum-Larsson equation in respect to the dielectric constant term. On the Debye equation the solubility of a protein would be expected to *fall* as the dielectric constant increases, i.e., log $S \propto 1/D^{3/2}$ (omitting the κ term of the denominator). On the Bjerrum-Larsson equation the solubility would be expected to *rise* as the dielectric constant increases.

In both cases the "response" of the protein to the surroundings depends upon the protein having one or more electric charges. If the material be a non-electrolyte, neither equation is applicable. Furthermore, as already mentioned, the Debye equation is intended to take account of the effects due to the presence of ions upon a given ionic species, while the Bjerrum-Larsson equation is intended to apply to an ion-free medium.

If the latter equation is applicable at all to the solubility of a protein, it would follow that addition of substances such as glycine and urea which, according to Fürth (63) raise the dielectric capacity of water, should increase the solubility, while addition of sugar or alcohol, which lower the dielectric constant, should decrease the solubility. Comparing absolute alcohol with water as solvents, one would expect on the Bjerrum-Larsson basis that the solubility of a protein in alcohol should be very much less than in water. This is true for certain proteins, e.g., gelatin, albumin, hemoglobin, but there are many known, e.g., the prolamines and the vegetable proteins, which are much more soluble in alcohol than in water. The environmental change here contemplated is so large that the solvent action upon material in the truly undissociated forms, considered by Bjerrum and Larsson but not included in the equation employed and discussed above, may well predominate.

Reference has been made to the possible effect of addition of urea to aqueous systems containing proteins, the effect anticipated on the basis of the Bjerrum-Larsson equation being that

of an increase in solubility.[18] In this connection a number of striking observations have been made by Ramsden (64) Spiro (65) and others.

Ramsden states that "globulins, caseinogen, acid and alkali albumin, copper albuminate, fibrin, and even heat-coagulated proteins swell up and dissolve in a saturated aqueous solution of pure urea." Also "coagulable proteins are not heat coagulable in its presence, but reacquire their coagulability when the urea is removed by dialysis." Ramsden's observations have been confirmed by Osborne and Harris for edestin (undenatured and crystalline). In water the edestin is practically insoluble. With addition of urea edestin gives "a clear and bright" solution.

The fact that swelling precedes the act of dissolution points to an effect of a physical character (primarily) upon the forces holding the constituent parts of gels together, i.e., the overcoming of the cohesion. Since the forces here involved may well be electrical in origin, it is possibly not too speculative to suggest that alteration in dielectric capacity due to the presence of the urea is an important factor in its effect. If this is so, it is of interest to see what kind of results may be obtained by applying the Bjerrum-Larsson equation.

To this end Booth, in the writer's laboratory, determined the solubility of hemoglobin—a British Drug Houses preparation which was found to be salt-free and on solution in water gave a solution of pH = 6.6 (very close to the iso-electric point)—in water and in 5 per cent urea at room temperature. By addition of a little alkali the pH of the water plus protein was brought exactly to that of the urea, the absolute value of the pH of which was determined to be in the neighborhood of 9.9. That it is essential particularly in the case of urea effects to make comparison at the same pH values was pointed out to the writer by Professor Ramsden. It was found that the solubility of the protein in 5 per cent urea solution was 1.52 times that in "water," that is the urea has

[18] That urea may react and combine with a protein in a purely chemical (stoichiometric) sense is of course highly probable. We are concerned at present however with only one possible aspect of its behavior, namely, the "environmental" effect.

increased the solubility by 52 per cent. According to the data of Schmidt (66), the dielectric constant of a 5 per cent urea solution is 1.04 times that of water. Taking D_{H_2O} as 80, it follows that $D_{urea} = 83.2$. Furthermore, taking z, the valency of hemoglobin as lying between 2 and 5, and setting $r = 30$ Å, it follows from the Bjerrum-Larsson equation that the electrical environmental effect produced by 5 per cent urea should cause the solubility of the hemoglobin to increase between 2 per cent and 12 per cent. Apparently the environmental effect to be expected on the Bjerrum-Larsson basis is small compared with the total effect actually produced by urea. However, no attempt has been made—nor can be made at present—to take into account the possible influence which may be exerted upon the magnitude of the Bjerrum-Larsson effect by the change in dielectric capacity of the system induced by the protein itself, since there are no data available for the dielectric capacity of solutions containing protein and urea together. Thus if 5 per cent urea in this case increased the dielectric capacity by 4 per cent (as before), then on taking the dielectric capacity of a saturated hemoglobin solution in water to be not greater than 30—a maximum value in the light of Marinesco's results already referred to—the Bjerrum-Larsson equation would lead to an increase in solubility between 5 per cent and 34 per cent, the lower value being the more probable of the two. It would appear that the environmental influence taken account of by the Bjerrum-Larsson equation is only likely to account for a part and possibly not a very large part of the effect exerted by urea. It would be necessary, however, to take it into account in any attempt to deal quantitatively with the total effect.

In concluding this section, it may be well to point out that no attempt is made to come to a definite conclusion as regards the relative merits of the Debye equation and the Bjerrum-Larsson equation in respect to their applicability to protein behavior. It is probable that both may be of assistance in interpreting different aspects of this behavior under different environmental conditions. At the present stage an exact test of either equation is impossible owing to lack of precise information upon protein

solubility in various media, the number of electric charges on the protein unit, and the dielectric capacities of aqueous solutions of salts and non-electrolytes.

III. FLOCCULATION

In this section we shall restrict ourselves almost entirely to the proteins of the albumin and hemoglobin type. As already mentioned, in the absence of salt the protein even when denatured may be maintained at the iso-electric pH without flocculation taking place. The coagulum when it is formed can be washed free from the neutral salt employed in its production. In other words, the ions of the salt do not form an essential feature of the structure of the amorphous colloid mass, the coagulum existing in virtue of cohesion operative between acidic and basic (probably carboxyl and amino) groups,[19] although in the denatured but dissolved state these groups do not *spontaneously* unite with any measurable speed at ordinary temperatures.

There appears to be no doubt as to the effect exerted by small quantities of neutral salt in bringing about rapid and complete flocculation on heating. The position is less clear in the absence of neutral salt. The early work such as that of Aronstein (67) and of Heynsius (68) is contradictory and confusing. The most reliable information would seem to be that obtained by Chick and Martin (*loc. cit.*) who worked with crystalline egg albumin. Solutions of this material were dialyzed, and it is to be presumed that the dialysis was continued until the material was really electrolyte-free, although no actual analytical data are given. With this material a milky solution was obtained on heating for 15 minutes in boiling water. The filtrate however contained protein, i.e., coagulation was incomplete. This behavior refers to the solution as prepared in distilled water. The pH is not recorded, but presumably it lay between 5 and 6. On adding hydrochloric acid and heating, Chick and Martin find complete flocculation possible, "provided the pH be carefully adjusted" to

[19] That the coagulum formed at the iso-electric point can be redissolved by a sufficient addition of acid or alkali may be taken as evidence that the links between the molecules composing the coagulum are ionizable acidic-basic in character.

the immediate neighborhood of the iso-electric point. On addition of a small amount of salt, the pH range over which flocculation is complete is greatly increased. Thus Chick and Martin find that a 1 per cent solution of undialyzed (albumin) crystals containing 0.3 to 0.4 per cent ammonium sulfate "will coagulate perfectly on boiling over a wide range of acidity (hydrogen ion concentration $10^{-3.1}$ N to $10^{-7.4}$ N)."

The usual explanation given for the favorable effect of heating is not only that the essential denaturation is brought about, but also that the protein salt of the acid, when such exists, is hydrolyzed to a greater extent. It may be pointed out that the decrease in dielectric capacity of the solution consequent upon the rise in temperature may be an important factor. Whether denatured protein in the absence of salt could be flocculated even at room temperature with sensible velocity by a suitable alteration in dielectric capacity of the system does not appear to have been investigated experimentally.

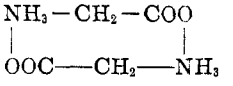
In addition to possible alteration in physical environment or in the chemical nature of the molecular individuals present, the main effect of increasing temperature will be of course the well-recognized one of increasing the intrinsic energy of the molecular species present. This aspect is taken up later in the present section in connection with the temperature coefficient of the flocculation process.

Apart from actual flocculation, however, as Chick and Martin have recorded, it is possible to get turbidity or opalescence even in the absence of salt on heating over a wide pH range from about 5 or 6 to about 2, so far as I can estimate it. From the point of view of stability of the protein solution, turbidity is of greater importance than complete flocculation since it indicates the initiation of aggregation. Further systematic work is required in this field.

It is a very remarkable fact at first sight that denatured protein molecules which contain amino and carboxyl groups, some of them probably in the zwitterion form, do not flocculate spontaneously and readily at ordinary temperature. It has to be remembered, however, as pointed out earlier, that to maintain a structure of the

type $^{+}NH_3 \cdot R \cdot COO^{-}$ it is necessary to assume that a considerable number of the lines of force associated with the two charged regions mutually form a closed field. If this were not the case one would naturally expect addition of H^{+} and OH^{-} (from the solvent) with formation of the unionized form $NH_2 \cdot R \cdot COOH$. Consequently, although the zwitterion looks at first sight to be a highly reactive form capable of forming chains of molecules through the operation of simple electrostatic forces, the fact that this is not the case can only mean that the two electrified regions have to a large extent neutralized each other's field. The function of increase in temperature would therefore seem to be that of opening up the electrical fields around both the basic and acidic regions. On the other hand, the function of the neutral salt would seem to be essentially that of destroying the symmetry of the zwitterion formation, thereby opening up one portion of the molecular field, thus enabling it to react with the opposite field of the molecules which have not interacted with the salt.

As pointed out in the Appendix the simplest amino acid, glycine, which according to Bjerrum (69) exists almost entirely in the zwitterion form, is capable of exhibiting even in water a small but appreciable degree of polymerization. If the polymer has the structure $^{+}NH_3 \cdot CH_2 \cdot COO \cdot NH_3 \cdot CH_2 \cdot COO^{-}$ it is evident that such polymerization would be the first step towards still further union. The fact that the polymerization only goes as far as the formation of the double molecule in the case of this very simple type of amino acid rather suggests that here the most stable form is the "inert"



structure. Whatever the structure in the case of glycine, the polymerization is detectable only with solutions which are at least 0.2 *M*. In the case of solutions of protein, the concentrations normally employed lie between 1 per cent and 10 per cent, that is, for a protein of molecular weight 43,000, the concentration is only of the order 10^{-3} to 10^{-4} *M*. It is evident, therefore, that

appreciable polymerization even to double molecules is unlikely to occur, provided of course, that the process be regarded as reversible, as it is in the simple case of the amino acid. It is evident that the ions of the neutral salt play a definite rôle and, since small amounts suffice, the effect is not to be attributed to a general alteration in the properties of the solvent as a whole, but must be essentially a localized effect. Parenthetically, we may contrast this effect with the function of the salt (e.g., ammonium sulfate) in large concentration when acting as an agent for bringing about crystallization of undenatured protein. In the latter case the effect is almost certainly a "general" one in the main—namely, competition with the protein for solvent molecules. In Hopkins's words "the protein crystals are formed under the influence of the electrolyte but not in association with it."

Returning to the phenomenon of flocculation near the isoelectric point in the presence of inorganic salts, it has been observed in electrophoresis experiments that, in certain cases, part of the protein travels towards the anode and part towards the cathode, the remainder (usually a considerable portion of the whole) remaining motionless. This behavior indicates the existence of individuals such as $^{+}NH_3 \cdot R \cdot COONa$ and $Cl \cdot NH_3 \cdot R \cdot COO^{-}$, small quantities of which may likewise be inferred from the e.m.f. experiments of Pauli and the membrane measurements of Northrop and Kunitz already discussed in an earlier section. Such units, having lost their zwitterion character, and having the field of force which emanates from the group carrying the free charge thereby greatly enhanced, may now be capable of uniting with one of the original zwitterions themselves, thereby giving rise to a double molecule of the form

$$Cl—NH_3—R—COO—NH_3—R—COO^{-} \quad \text{or} \quad {}^{+}NH_3—R—COO—NH_3—R—COONa$$

in which the group carrying the free charge has a similarly enhanced field, thereby rendering further addition possible. This probably represents the function of the neutral salt at the isoelectric point. Incidentally, if a very large amount of neutral salt be added, it is conceivable that, instead of getting the chain effect which is essential for flocculation, we might get simple fully

saturated complexes of the form $Cl \cdot NH_3 \cdot R \cdot COONa$, which would be useless for flocculation and would possibly lead to increased stability of the protein.[20]

If the above view of the function of the neutral salt as the accelerator of flocculation at the iso-electric point be correct—that is, if the flocculation or chain formation is caused not so much by neutralizing a free charge as by liberating the field of force around the charge which has *not* reacted with the neutral salt ion—it follows that the mechanism of flocculation of a protein is very different from that envisaged in, let us say, Smoluchowski's theory of coagulation of a hydrophobe colloid such as gold sol. The distinction is clearly not simply one dependent on great or small affinity for water, although this may play a secondary part. In the case of the proteins, flocculation is apparently brought about by the removal of the amphoteric character on the part of a small proportion of the protein units, these modified units being now characterized by the possession of an electric field of a single sign of enhanced intensity, which renders them capable of initiating chain formation. In the case of gold sol, the individuals to begin with are all of the one sign, and the effect of the electrolyte is to reduce the charge below a certain magnitude. For this reason a strong acid acts towards gold sol exactly as would other strong electrolytes. The behavior is very different in the case of proteins. We have now to consider the flocculation of protein in acid solution as distinct from flocculation at the iso-electric point.

Addition of strong acid or base alters the pH of the system and causes the zwitterion formation to disappear, the protein being now in the form of protein salt, e.g., protein chloride or sodium proteinate. H^+ ion and OH^- ion, in respect to their effect upon

[20] Actually it does not appear to be essential to postulate the production of definite chemical individuals of the type $^+NH_3 \cdot R \cdot COONa$ or $Cl \cdot NH_3 \cdot R \cdot COO^-$. Either of the ions of the neutral salt, if brought sufficiently close to the oppositely charged grouping of the zwitterion structure, would be expected to give rise to the effect without forming a constituent of the resulting coagulum. We might regard the function of the ion lying between two oppositely charged dipoles or zwitterion structures as that of locally lowering the dielectric capacity and thereby increasing the forces of attraction between the protein units.

protein, stand wholly apart from other ions in that their combination with protein is much more extensive and complete. For this reason the effect of adding acid is to give rise to protein units *all* similarly charged (cations) together with a greater or less proportion of unionized salt, e.g., protein chloride, the amount and composition of which is a function of the pH. The similarly charged protein ions repel one another and, so far as this portion of the protein material is concerned, the conditions for flocculation are even less favorable than they were prior to addition of the acid. On addition of neutral salt to the acid solution there is no doubt that union to some extent takes place between the anions of the salt and the protein cations in solution. Obviously this process could not conceivably lead to chain formation; in fact, addition of a very small amount of neutral salt would not be expected to have any appreciable influence. The only kind of effect to be anticipated would seem to be a mass action effect with alteration in solubility of the protein in the ordinary sense of the term. The idea that it is a mass action effect is not novel. It is substantially that put forward by Hardy in 1905 (70) in connection with the globulins.

Now it is evident that appreciable mass action effects cannot be brought about unless the neutral salt added increases appreciably the concentration of the anion already present by virtue of the acid added in the first place. That the effect of the addition of neutral salt under such conditions, i.e., at a pH well removed from the iso-electric point, is due to mass action, and that the process is one of precipitation rather than of flocculation, such as occurs on addition of salt *at* the iso-electric point, appears to be borne out by the experimental evidence available. This evidence may be illustrated by two quotations, one from the paper by Chick and Martin (71), the other from the paper of Hardy already referred to.

Chick and Martin state: "We found, however, that a solution of purified crystals of egg albumin which has been dialyzed until it is free of Am_2SO_4 does not form a precipitate on heating in the presence of HCl. Precipitation occurs, however, if neutral salts are added, but the amount of a chloride which is necessary to

effect agglutination makes the total chlorides to be estimated in the filtrate so great that the determination of any loss of chlorine (chloride ion) by fixation of HCl is rendered uncertain. A very small addition of a sulfate of sodium, potassium, or ammonium is able to bring about complete separation of the heated protein in a particulate form. In the presence of 0.014 *N* HCl and 0.36 per cent Na_2SO_4 we found that, although acid disappeared on coagulation, the whole or nearly the whole of the chlorine remained in the filtrate. On subsequent experimentation we ascertained, however, that a corresponding quantity of SO_4^{--} had combined with the coagulum. We therefore had recourse to H_2SO_4 for acidifying the solution of egg albumin crystals, and a small quantity of Na_2SO_4 was added to facilitate separation of the coagulum. Only a small amount of this salt is required because SO_4^{--} assists the agglutination of denatured egg albumin much more powerfully than Cl^-." The slightly misleading phrase in the above is "very small" addition of a neutral salt, as this would naturally be taken to mean an addition that would not materially affect the concentration of the significant ion, in this case the anion. That the concentration is in fact very materially affected is shown by the analytical data given by Chick and Martin for the case just referred to. On analyzing the precipitate it is found that "the protein acid salt formed on addition of H_2SO_4 to a solution of pure crystalline egg albumin is precipitated as such on coagulation by hot water." The data show clearly that practically equivalent amounts of hydrogen ion and sulfate ion are removed. Furthermore, the precipitate contains in round numbers 1×10^{-3} equivalents of either H^+ ion or SO_4^{--} ion per gram of protein, indicating that the precipitate is in fact undissociated protein salt with practically all the available amino and carboxyl groups in combination with H^+ ion and SO_4^{--} ion. In the actual case considered the solution in equilibrium with the precipitate was distinctly acid, namely, 9.6×10^{-3} *N*. Had no sodium sulfate been added "to facilitate separation of the coagulum," this figure would likewise represent the concentration of SO_4^{--} in equivalents. Actually, owing to the addition of Na_2SO_4, apparently in very small quantity (namely, to about 0.1 per cent) the concentration

of SO_4^{--} is raised to 25×10^{-3} N, a very appreciable increase and one not unlikely to throw the protein sulfate out of solution, owing to the operation of the solubility product principle.[21]

A rather sharp distinction has been drawn between the kind of structure to be expected in the coagulum formed at the iso-electric point and the structure of the protein salt precipitated at a pH removed from the iso-electric point. In the latter case removal of the excess neutral inorganic salt by washing and addition of water should cause the precipitate to redissolve. In the former case such treatment should be ineffective, re-solution being brought about only by addition of acid or alkali. This is in general agreement with experiment. It is evident that much valuable information regarding the structure of the precipitate at different pH values might be obtained by systematic x-ray measurements. So far as the writer is aware no such measurements have as yet been carried out.

Supplementing the findings of Chick and Martin quoted above in connection with egg albumin, we now give a short quotation from Hardy in connection with (serum) globulin.

"Globulin itself is insoluble in water—. Acid globulin and alkali globulin can be separated in the solid state by dissolving globulin with minimal amount of acid or alkali and evaporating to dryness *in vacuo* over sulfuric acid and caustic potash. When hydrochloric acid is used, the dried HCl-globulin is found on analysis by Carius's method to contain all the chlorine used to dissolve the globulin. HCl-globulin therefore is stable *in vacuo* in the presence of solid KOH, and the acid may be regarded as being in true combination. Acid globulin dissolves in water."

"Alkaline globulin can be separated in the solid state from, for example, solution in ammonia by drying *in vacuo* over sulfuric acid. When redissolved the solid shows the same order of molecular conductivity as the solution from which it was dried out. Both acid and alkali globulins ionize in solution."

"Solutions of acid or alkali globulin are precipitated by the addition of neutral salts; further addition brings about re-solution always in the

[21] It follows that the precipitate formed under these conditions is not flocculation in the sense of molecular chain formation, such as appears to be the case at the iso-electric point.

case of alkali globulin, sometimes in the case of acid globulin. Still more salt brings about reprecipitation. In the first solution the globulin is ionic, in the second solution it is not ionic."

"Now if the action between salt and colloid is chemical and identical, for instance, with the selective reaction in dyeing which discriminates between acid and basic dyes, it would be represented by a generalized equation of the form:

$$C'H' + B'S' = CB + HS.$$

That is to say, the colloid functions as an acid and by the law of mass action the compound CB is formed because of its insolubility."

As is generally realized, the behavior of globulins with respect to neutral salts is of a more complex character than the behavior of proteins of the albumin type. Until further information is available regarding the mechanism of flocculation and precipitation in the case of the latter, it will be readily understood that many features of the former are at the present time so obscure that speculation is valueless.

We may at this point draw a comparison between the mechanism of the precipitating action of a weak acid, such as acetic acid, to be anticipated on the basis of the considerations (neither novel nor original) which we have attempted in the foregoing to emphasize as at least offering a certain amount of guidance through a subject complex in itself and rendered more so by incomplete data. The two cases to be considered are (*a*) the precipitation of material of the albumin type and (*b*) the precipitation of a colloidal metal. In the case of the hydrophile colloid (albumin) the hydrogen ion and acetate ion would be expected to be equally involved in the precipitation, the hydrogen ion combining with the protein (initially iso-electric) to give rise to protein cations, which in the presence of a sufficiency of acetic acid unite with acetate ions to form protein acetate until, the solubility of the salt being exceeded, the protein would be expected to precipitate in the form of the salt.

In the case of the hydrophobe colloid (say gold sol) the hydrogen ion is primarily concerned, its rôle being that of diminishing or annulling the initial negative charge on the colloid particles,

which now aggregate as such (not as the acetate) in the manner envisaged in the theory of Smoluchowski. In the first case one would expect the precipitate to be crystalline; in the second case amorphous—or, more precisely, to have the same degree of lattice structure as the original sol particles themselves.

Critical increment of flocculation

This section may be concluded with a brief consideration of the influence of temperature upon the flocculation process. This has been investigated by Chick and Martin for diluted horse serum, dialyzed, and containing acetic acid, together with either sodium chloride or ammonium sulfate. As the behavior observed is similar in both cases we shall consider one of them only, namely, a diluted serum (3.3 per cent) containing 3.7 cc. of 0.1 *N* acetic acid in 100 cc. and NaCl at a concentration of 1 per cent. Apparently the solutions are milky to begin with. The flocculation rate is determined by noting the times taken for "visible particles" to appear. From the temperature coefficient data given I have calculated the apparent energy of activation or critical increment, *E*, of the process. The results are given in table 5. Chick and Martin remark upon the fact of a very high temperature coefficient at the lower temperature range which falls to a low value of normal magnitude at higher temperatures. This is shown more clearly by the variation in the *E* values, a "normal" limiting value of about 23,000 calories being eventually attained. This value presumably represents the energy required to activate or open up the valency field around at least one amino or carboxyl group to enable it to unite with a polar group of opposite sign. (Although the connection is a little remote, it may be mentioned that the energy required to break the peptide linkage —CO—NH— in the hydrolysis of this group has been found in the writer's laboratory to be 22,000 calories per gram-molar group (Escolme and Lewis (28)). That a critical increment of even "normal" dimensions is obtained anywhere for the flocculation process is a striking observation. It indicates clearly that the act of aggregation in the case of denatured protein is very different in nature from the aggregation of a colloid such as gold

hydrosol. In the latter case it has been shown in the writer's laboratory with some degree of precision (cf. Butler (72)), that when sufficient electrolyte has been added (and the quantity is small) to completely neutralize the charge on the colloid particles, the effect of temperature on the rate of flocculation is quantitatively accounted for by the alteration in viscosity of the medium (water). In such a case there is no true critical increment,[22] all collisions being effective. In other words, while the aggregation of colloidal gold under the conditions named is satisfactorily taken account of by the Smoluchowski theory, in the case of protein the process is much more analogous to a bimolecular chemical reaction.

It will have been noticed that the material employed by Chick

TABLE 5

Critical increment, E, of flocculation

TEMPERATURE °C.	*E* IN CALORIES
44	110,000
46	67,000
49	83,000
56	37,300
66	29,100
76	22,400
86	24,400

and Martin in connection with the influence of temperature is not a pure protein, nor were the initial solutions transparent. Furthermore the pH was evidently not determined. We do not know therefore whether the limiting critical increment is a function of pH or not. It would be desirable to know with some degree of precision the critical increment of pure crystallized egg albumin with respect to flocculation after preliminary denaturation. The values for the rate of flocculation of egg albumin at different temperatures recorded by Lepeschkin (18) are too inaccurate to

[22] Expressed as a fictitious critical increment, the viscosity effect amounts to 1000 calories (in round numbers) for a process occurring in an aqueous system in the neighborhood of 25°.

allow of any conclusion being drawn as to the magnitude of the increment or as to whether it varies with temperature or not.

The very great influence of temperature on the flocculation of horse serum at the lower temperature region is considered from a statistical standpoint by Chick and Martin. It would seem, however, that such statistical considerations applied to intrinsic energy relations for an individual process taken to be essentially the same in nature at different temperatures would be consistent with a constant value for the critical increment[23] and not with one rapidly altering as temperature rises.

The large value of E at the lower temperature range at first sight suggests that denaturation is occurring at that region and that in fact we are dealing not with a single process but with two consecutive processes. Chick and Martin, however, state that the material was denatured to start with. That such an explanation based on two processes could not explain the fall in E with rise in temperature may also be shown on theoretical grounds. Let us suppose that there are two processes $A \rightarrow B \rightarrow C$, in which $A \rightarrow B$ with a velocity constant k_1 is the slow process, and $B \rightarrow C$ with velocity constant k_2 is the faster process. On this basis the observed critical increment over any feasible temperature range should be the critical increment of the slower process and should not vary with temperature. It is equally useless to assume that two simultaneous side processes $A \begin{matrix} \nearrow B \\ \searrow C \end{matrix}$ occur, as it may be shown in this case that the critical increment observed should be sensibly that of the *faster* process and again would be expected to be independent of temperature. If we are to explain the variation in E on the basis of two processes it would seem to be necessary to assume the existence of *independent* processes of which the one with the higher critical increment peters out at a fairly low temperature. This would be a purely *ad hoc* assumption for which there is no evidence at all in the system examined,

[23] With $E > 20{,}000$ calories, statistical theory shows that over any temperature range at which a liquid system could exist as such, the fraction of molecules having the necessary critical energy is exceedingly small compared with the total. Consequently Chick and Martin's argument does not apply.

although the assumption of successive processes giving rise to breaks in the log k–temperature curve has been made in the much more complicated case investigated by Crozier and his co-workers (73) in connection with the chemical processes underlying certain vital activities in organisms. It would seem necessary therefore in the protein flocculation case, to revert to the concept of a single process and to attempt to account for the marked decrease in E as temperature rises by assuming that each protein unit itself undergoes some internal change—differing completely from denaturation, however—with increase in temperature, the change being of such a nature that the chemical groups—almost certainly the amino and carboxyl groups[24]—at which flocculation may reasonably be regarded as initiating itself do not become more numerous but are possessed of a higher average energy content than is allowed for in ordinary statistical treatment. The suggestion amounts to the assumption that with increase in temperature there is not simply an all round increase in energy content distributed amongst a fixed number of degrees of freedom, but that in the protein unit a rise in temperature brings new degrees of freedom into operation. That is, a degree of freedom associated with some chemical bond may be in a quantized state at the lower temperature region with energy content small compared with that demanded on an equipartition basis, but on raising the temperature the energy content may increase towards the equipartition value. If now we regard the energy required to cause a given bimolecular chemical process to occur as being contributed partly by certain degrees of freedom within the chemical unit and partly by the kinetic energy of collision, as in the well-known theory of Hinshelwood and of Fowler (cf. Hinshelwood (74)), it follows that the assumption of a variable, as distinct from a fixed, number of degrees of freedom, whose energy is available for transfer in this sense, will

[24] The fact that the precipitate can be dissolved by sufficient acid or alkali points to this. The attempt was made by Cubin (*loc. cit.*) in this laboratory to confirm this by using formaldehyde in the expectation that the flocculation would consequently be hindered. A slight hindering effect was observed but the results were not as conclusive as had been hoped.

modify the effect of temperature upon the chemical process and thereby conceivably give rise to an observed critical increment, which shall itself be a function of temperature and not independent thereof, as would be expected on the basis of a fixed number of degrees of freedom. I am indebted to Dr. A. McKeown of this Department for working out the consequences of this assumption. In brief, the assumption of an increase in effective[25] degrees of freedom with rise in temperature does in fact lead to a decrease in the observed or apparent critical increment with rise in temperature, and by inserting certain numerical values for the theoretical or true critical increment,[26] the number of effective degrees of freedom, and the rate of increase in degrees of freedom with temperature, an "apparent" critical increment altering with temperature in much the same way as that observed by Chick and Martin may be obtained. The real drawback to the treatment, possibly a fatal drawback, is the high value (>0.1) which has to be ascribed to df/dT (the increase in the number of effective degrees of freedom per degree) in order to account for an apparent (or observed) critical increment, which can vary, as found by Chick and Martin, by as much as 50,000 calories for a rise of 40° in temperature.

No conclusion can therefore be drawn at the present time regarding the magnitude of the critical increment of flocculation and its apparently high variation with temperature. The problem, however, seemed to be of such importance as to justify a certain amount of space being given to it in the present connection.

[25] The effective degrees of freedom (f) are to be distinguished from the real internal degrees of freedom (g), the energy of which is quantized. The number, f, is in fact defined by the relation

$$f\,RT = g\,E(\nu_1 T)$$

where on the simplest basis

$$E(\nu_1 T) = \frac{h\nu}{e^{h\nu/kt} - 1}$$

In general f will be small while g would necessarily be considerably greater.

[26] This may with some confidence be taken as being in the neighborhood of 20,000 calories. It is in fact not far from the limiting value obtained by Chick and Martin at the high temperature region which they regard as the true value for the flocculation process.

APPENDIX

Note on the physicochemical behavior of glycine in solution

On the zwitterion theory, glycine exists in solution at its isoelectric point (pH = 6.08) mainly in the form $^{+}NH_3 \cdot CH_2 \cdot COO^{-}$. In aqueous solution it is conceivable that electrically charged individuals of this kind, although not "free" ions in the ordinary sense, could exert a mutual influence analogous to that taken account of by the Debye-Hückel theory of interionic attraction. To test whether any such effect is operative a number of determinations of the lowering of the freezing point were carried out by Mr. W. J. Loughlin in the writer's laboratory. If Δ is the observed lowering of freezing point, and Δ_0 the theoretical lowering on the basis of a molecular weight of 75, then $\frac{75\Delta_0}{\Delta}$ represents the apparent molecular weight. Furthermore the ratio Δ/Δ_0 is the osmotic coefficient f_0 as defined by Bjerrum. The values obtained are given in table 6.

TABLE 6

Glycine in water

GRAM-MOLES OF GLYCINE (M = 75) IN 1000 GRAMS OF WATER	APPARENT MOLECULAR WEIGHT	$f_0 = \frac{\Delta}{\Delta_0}$	$x = 1 - \frac{\Delta}{\Delta_0}$	$K = \frac{(\text{GLYCINE})^2}{(\text{DIGLYCINE})}$
0.210	75.35	0.995	0.005	[41.2]
0.385	78.05	0.961	0.039	8.4
0.572	79.09	0.948	0.052	8.8
0.765	80.37	0.933	0.067	8.6
0.986	81.92	0.916	0.084	8.1
1.674	85.28	0.880	0.120	8.1
2.176	86.58	0.866	0.134	8.7
			Mean value =	8.4

On plotting the values of $(1 - f_0)$ against the concentration, a curve was obtained which differed significantly from that given by KCl. Thus with KCl the values of $(1 - f_0)$ rise rapidly at first and then much more slowly with increase in concentration of the salt. This change is taken account of in a reasonably satisfactory way by the Debye-Hückel interionic attraction theory, in which

allowance is made for the mean effective diameter of the ions in solution and for the thickness of the interionic attraction sphere. With glycine, on the other hand, the values of $(1 - f_0)$ at first remain low, then rise, passing through a point of inflexion with increase in content of glycine. It seems reasonable to conclude, therefore, that these osmotic abnormalities are not to be ascribed to forces of the "interionic attraction" type. It would seem justifiable to ascribe the increase in apparent molecular weight to actual polymerization, for which a zwitterion structure would be expected, on general grounds, to be eminently suited. On this basis, if x denotes the fraction of each mole of glycine polymerized to the diglycine form, then

$$\frac{\Delta_0}{\Delta} = \frac{1}{1 - x}$$

and at a concentration c the mass action expression becomes

$$K = \frac{c\,(1 - 2x)^2}{x}$$

This quantity is given in the final column of table 6. In view of the constancy of K there seems little doubt but that polymerization does in fact occur. Thus a 1 M solution is polymerized to the extent of 10 per cent in round numbers. The existence of this polymerization has of course to be allowed for in any measurements upon the behavior of glycine in solutions of salts before one can assert with certainty that union of glycine with the salt occurs in *solution*.

Although the glycine zwitterions do not exert a detectable interionic attraction upon one another—in the sense of the Debye-Hückel theory—it was of interest to investigate the possible influence of a simple electrolyte, sodium chloride, upon the behavior of the glycine individuals. To this end a number of determinations of the lowering of the freezing point of solutions of glycine in 0.1, 0.2, and 1.0 N NaCl has been carried out in the writer's laboratory by Mr. A. M. Maiden. Selecting two concentrations of glycine—namely, 0.38 and 0.76 M (M being taken as 75 for the purpose of expressing molarity), the following results

were obtained. The values obtained with pure water as solvent are taken from table 6. It will be observed that there is an increased osmotic abnormality caused by the presence of the NaCl and expressed in the form of an apparent molecular weight. The osmotic coefficient of Bjerrum is given in the final column, Δ_0 being replaced by Δ to allow for the fact that even apart from NaCl there is an osmotic abnormality (due to polymerization). Before attempting even a partial analysis of the above figures it is necessary to consider the evidence existing for chemical union between one or both of the ions of NaCl with glycine.

TABLE 7

Glycine in NaCl solutions

CONCENTRATION OF NaCl	APPARENT MOLECULAR WEIGHT	$\Delta'/\Delta = f_0$
Concentration of glycine = 0.38 M (M = 75)		
0	78.05	1
0.1	79.5	0.98
0.2	87.2	0.90
1.0	94.5	0.83
Concentration of glycine = 0.76 M (M = 75)		
0	80.4	1
0.1	82	0.98
0.2	88.7	0.91
1.0	95	0.85

Oryng and Pauli (75) have measured by the e.m.f. method—using the calomel electrode in the presence of glycine—the influence of glycine upon the activity of the Cl^- ion in KCl. Naturally the experiment gives no indication as to whether any union occurs between K^+ ion and glycine. Employing the data of Oryng and Pauli, and introducing the data for the activity coefficient of Cl^- ion, ($\alpha_{Cl^-} = \alpha_{K^+}$) in KCl, as given in Lewis and Randall's "Thermodynamics," we may calculate the concentration of Cl^- in equilibrium with the glycine. The difference between this "free" Cl^- ion and that obtaining initially in the KCl solution employed (assuming complete ionization) will be taken as representing the amount of Cl^- combined with the

glycine. It is convenient to denote glycine by the symbol Gl. It is evident from table 8 that the e.m.f. data, particularly at the higher range of concentrations of KCl, are not sufficiently reliable to permit of any precise calculation. Nevertheless an attempt will be made to get at least an approximate idea of the magnitude of K_1 the equilibrium constant of the process

$$Gl + Cl^- \rightleftarrows GCl^-,$$

in order to estimate the order of the combination effect upon the

TABLE 8

Influence of glycine upon the activity of the Cl^- ion in KCl

1.6 per cent glycine = 0.2133 *M*

INITIAL CONCENTRATION OF KCl	ACTIVITY OF Cl^- ION IN (KCl + Gl) AS OBSERVED BY ORYNG AND PAULI	CONCENTRATION OF FREE Cl^- ION	CONCENTRATION OF BOUND Cl^- = CONCENTRATION OF $GlCl^-$
N			
0.05	0.04346	0.051	(negative)
0.02	0.01760	0.0194	0.0006
0.01	0.00882	0.0095	0.0005
0.005	0.00413	0.0044	0.0006
0.002	0.00151	0.00155	0.00045
0.001	0.00071	0.000724	0.00028

freezing point data obtained by Maiden for (greater) concentrations of NaCl. Strictly, K_1 is defined by

$$K_1 = \frac{\alpha_{Gl\,Cl^-}}{\alpha_{Gl} \times \alpha_{Cl^-}}$$

We shall assume that the activity coefficient of glycine itself is identical with that of the glycine-Cl^- complex. That is,

$$K_1 = \frac{c_{Gl\,Cl^-}}{c_{Gl} \times \alpha_{Cl^-}}$$

The values thus obtained are given in table 9. It may be pointed out that the values of the equilibrium constant of the process calculated on the basis of allowing for union with metallic cation simultaneously with the Cl^- ion, would necessarily show still greater increase with dilution of KCl.

With higher concentrations of KCl, *viz.* 0.1, 0.2 and 1.0 N, it is evident that the value of K_1 would probably be considerably lower than 0.1. Taking this as a maximum value, however, we may make an attempt to calculate a maximum value for the degree of union of glycine under these conditions. With glycine at an initial concentration of 0.38 M, we thus find that in presence of 0.1, 0.2, and 1.0 N KCl the concentration of the resulting $GlCl^-$ is 0.003, 0.0056, and 0.023 M. That is, the percentage of glycine in the form of the complex is 0.8 per cent, 1.5 per cent and 6 per cent respectively. In so far as glycine unites with one of the ions of KCl in a solution of this salt, the depression of freezing point caused by the glycine is thus less than it should be, and a correction factor has to be introduced to allow for that portion of the glycine which has been rendered osmotically in-

TABLE 9

Values of the equilibrium constant at different concentrations of KCl

	INITIAL CONCENTRATION OF KCl					
	0.05N	0.02N	0.01N	0.005N	0.002N	0.001N
K_1	—	0.16	0.266	0.68	1.4	1.9

active. This correction corresponds to the percentages of the total glycine concentration (0.38 M) already calculated as combined with chloride ion in 0.1, 0.2, and 1.0 N KCl solution, respectively. This would lead us to expect the values 0.99, 0.98 and 0.94, respectively, for Δ'/Δ in table 7 in place of the observed values 0.98, 0.91 and 0.84. Evidently the osmotic abnormality shown in table 7 (i.e., the increase in apparent polymerization due to the presence of sodium chloride) can be ascribed only to a limited extent to union of glycine with Cl^- ion as calculated from the somewhat unsatisfactory data of Oryng and Pauli. In fact, if the total observed "excess" abnormality as expressed by the apparent molecular weight 94.5 for glycine in 1.0 N NaCl, were to be ascribed entirely to combination of a certain fraction of the glycine with Cl^- ion, it would be necessary to conclude that 0.066 moles of glycine were thus

combined out of a total of 0.38 moles; in other words, that the degree of union was 17 per cent. While this is, of course, not impossible, it is far greater than anything suggested by the results of Oryng and Pauli.

The excess osmotic abnormality due to the presence of NaCl could conceivably be ascribed, not to true polymerization nor to chemical union with Cl^- ion, but to an interionic attraction effect in the sense of Debye and Hückel, exerted by the ions of the salt upon the zwitterions of glycine. This possibility has now to be considered.

Treating the excess osmotic abnormality in terms of the Debye-Hückel theory, it is necessary in the first place to convert the osmotic coefficients f_0 for glycine recorded in table 7 into the corresponding activity coefficients (α). Since we have shown that the osmotic coefficients are sensibly the same for 0.38 M and 0.76 M glycine, in the presence of sodium chloride, we shall take the mean values of these as a starting point and assume that the α values in this case are related to f_0 values in the same way in which they are related in the case of ionized KCl.[27] This is naturally an assumption based solely upon the univalency of the charges in the two cases compared. In this way one obtains the values of α and of $-\log \alpha$ for glycine, recorded in table 10 as "obtained from the freezing point data." To compare this result with that of the Debye-Hückel theory we shall employ the expression

$$-\log_{10} \alpha = \frac{z^2 \times 0.5 \sqrt{\Sigma\, c\, \nu^2}}{1 + 1.5 \sqrt{\Sigma\, c\, \nu^2}}$$

See Cohn and Prentiss (53).

In this expression α stands for the activity coefficient of the glycine zwitterion of valency z in the presence of NaCl, the concentration of either Na^+ or Cl^- being c, and ν, the valency thereof, being unity. Setting $z = 1$ it follows that

$$-\log_{10} \alpha = \frac{0.5 \sqrt{2\, c}}{1 + 1.5 \sqrt{2\, c}}$$

[27] For this case at the fairly high concentration range here considered the purely empirical relation $f_0 = \sqrt[3]{\alpha}$ was used for purposes of computation. The same relation holds very approximately for cane sugar in solution.

The values of $-\log_{10}\alpha$ are likewise included in table 10. From the degree of agreement exhibited between "calculated" and "observed" values of $-\log_{10}\alpha$, it is practically impossible to decide whether interionic action plays any appreciable part or not.[28] If the value of $-\log_{10}\alpha$ obtained for glycine at the lower concentration of salt be regarded as definitely disproving the applicability of interionic attraction, we would be forced to regard the apparently high molecular weights of glycine in the presence of salt as indicating, in fact, increased polymerization. No decision on this important question is possible until further experimental data are available, especially in the region of low salt content.

TABLE 10

Activity coefficients of glycine in the presence of NaCl

CONCENTRATION OF NaCl	MEAN OSMOTIC COEFFICIENT, f_0, OF GLYCINE	MEAN VALUE OF α "FROM FREEZING POINT DATA"	$-\log_{10}\alpha$ (FROM PREVIOUS COLUMN)	$-\log_{10}\alpha$ (DEBYE AND HÜCKEL THEORY)
0	1	1	0	0
0.1	0.98	0.94	0.024	0.134
0.2	0.91	0.75	0.123	0.162
1.0	0.84	0.59	0.227	0.230

Similar inconclusiveness exists in connection with the attempt to calculate the influence of salts upon the solubility of glycine. Taking Pfeiffer's data (42), which refer to salt concentrations of the order of 0.5 to 1.0 M, we find that $\log_{10}\frac{S}{S_0} = 0.0342$, where S_0 stands for the solubility of glycine in water and S for its solubility in 0.97 M LiCl. On the theory of interionic action we would expect this quantity to be very much greater—namely, 0.225. Similarly for 0.57 M $CaCl_2$, the observed value of

[28] If allowance were made in column 2 of table 10 for the fraction of glycine combined with Cl^-, the mean osmotic coefficients for the free glycine would not have fallen so rapidly as the values of column 2 indicate. The corresponding α values obtained therefrom would likewise not fall so rapidly and hence the value $-\log_{10}\alpha$ obtained from them in turn would rise less rapidly than the values given in column 4. This would mean that the discrepancies between columns 4 and 5 would be slightly greater than they appear to be in the table.

$\log_{10}\frac{S}{S_0}$ is 0.092, the calculated value being 0.245. In neither case has any allowance been made for union of Cl^- ion with the glycine. Also no allowance has been made for the change in dielectric capacity of the saturated solution which, owing to the enormous solubility of glycine in water, may well be considerable, in view of Fürth's observation (76) that glycine solutions possess a greater dielectric capacity than water itself. Thus, with a glycine content of 16.6 weight per cent, the dielectric capacity D attains an apparently asymptotic value of 93.0 instead of 80.5, the value taken by Fürth for pure water. The factor 0.5, which appears in the numerator of the Debye-Hückel expression already employed, contains $1/D^{\frac{3}{2}}$. The denominator term 1.5 contains $1/D^{\frac{1}{2}}$. Neglecting the effect of the latter, it follows that the calculated value of $\log_{10}\frac{S}{S_0}$ would become 0.180, instead of 0.225, for a solution of glycine containing 0.97 M LiCl. This is still vastly greater than the observed value 0.0342.

Finally, it is of interest to see what effect is produced upon the osmotic coefficient of glycine when sucrose is added to the solution, especially in view of the fact that sucrose diminishes the dielectric capacity. Thus Fürth (in agreement with others) finds that the dielectric capacity of molar sucrose solution is 63 (water = 80.5). Using a 1 M solution of sucrose as the solvent, Maiden, in the writer's laboratory, has measured the depression of freezing point produced by the addition of different amounts of glycine. The volume concentrations of glycine varied between 0.23 and 0.96 M. In computing the actual mass concentrations, it is necessary to refer the mass of glycine to a certain mass of water. An ambiguity arises as to whether or not allowance should be made for the water of hydration of sucrose (6 moles of water per mole of sucrose). On general grounds one would expect that the water bound by the sucrose would be unavailable as solvent for the glycine. The results of both methods of computation are given in table 11. A further point arises as to the correct value to assume for the theoretical molecular depression Δ_0. For water this is taken as 1.86°. For molar sucrose the

value obtained by plotting the molecular depression of sucrose against concentration in solutions of sucrose alone in water is 2.103°. Results are recorded on the basis of both.

Table 11 contains sets of values for the apparent molecular weight. The second column gives the value of M calculated on the basis $\Delta_0 = 1.86$, the total water in the solution being regarded as solvent; similarly for the fourth column. The third column gives the value of M on the basis $\Delta_0 = 1.86$, the effective solvent being total water less the amount bound by the sugar; similarly for the fifth column. The point is whether these relatively high values for M in the presence of sucrose represent real polymerization in excess of that in water alone (shown in the last column of

TABLE 11

Apparent molecular weight of glycine in 1.0 M sucrose solution

GLYCINE, VOLUME CONCENTRATION, MOLES/LITER OF SOLUTION	M CALCULATED ON THE BASIS OF $\Delta_0 = 1.86$ W_{Total}	M CALCULATED ON THE BASIS OF $\Delta_0 = 1.86$ W_{Free}	M CALCULATED ON THE BASIS OF $\Delta_0 = 2.103$ W_{Total}	M CALCULATED ON THE BASIS OF $\Delta_0 = 2.103$ W_{Free}	M OF GLYCINE IN WATER
0.233	82.4	95.6	93.4	108	75.8
0.470	82.5	95.7	93.5	108	78.5
0.483	82.9	96.2	94.0	109	78.7
0.70	84.9	98.6	96.2	111	80.1
0.72	84.3	97.3	95.5	111	80.2
0.96	84.6	98.2	95.9	111	81.9

table 11), an effect which would, on general grounds, be favored by the decreased dielectric constant, or whether, quite apart from polymerization, the activity of the glycine has been diminished by combination with sucrose or otherwise to the extent represented by the figures. We shall consider first the question of increased polymerization of glycine due to the presence of sucrose. The most direct way of testing this would be by calculating the equilibrium constant for the assumed polymerization process. Denoting the (new) equilibrium constant by K' (to distinguish it from K, the value of the constant obtained for glycine in water alone) we may write

$$K' = \frac{c\,(1 - 2x)^2}{x}$$

The quantity x is given by $(M - M_0)/M$, where M is the apparent molecular weight. c is taken as the number of moles ($M_0 = 75$) of glycine per liter of solution. Naturally the values of K' differ according to the basis of calculation employed for M, as shown in table 11. On calculating the values of K' it is found *in all cases* that K' apparently increases approximately three-fold as the volume concentration of glycine is increased from 0.233 to 0.96 moles per liter. For this reason it is not thought worth while to record the actual values obtained for K'. It follows that the concentration of diglycine does not increase sufficiently rapidly with increase in total glycine. In other words, to obtain a constant value for K' the apparent molecular weights should have increased with concentration more rapidly than they have been observed to do. Incidentally, the assumption of the union of an appreciable amount of the glycine with sucrose (considered below), could not account for the observed molecular weight being too low, since obviously any such union would tend to give fictitiously large molecular weights. It would seem, therefore, that the conclusion to be drawn is that the osmotic abnormality exhibited by glycine in the presence of sucrose is not to be attributed to further polymerization. We are left therefore with the alternative—namely, that the activity coefficient of glycine may be diminished owing to the presence of the sucrose. It would be natural to ascribe this in the first place to union of sucrose with glycine.

On this basis the extent of union has been calculated from the data of table 11, postulating the simplest type of complex—namely, one molecule of glycine with one molecule of sucrose. The corresponding dissociation constants of the complex have likewise been calculated. Naturally different extents of union are obtained according to the column of table 11 chosen. In all four series, however, roughly constant values for the dissociation constant are obtained, the values obtained on the basis of $\Delta_0 = 1.86$, W_{Total} being the least satisfactory. No distinction can be drawn as between the columns 3, 4, or 5 of table 11. A more extensive series of measurements would be required. It may be of interest to record the values thus obtained for the extent of

association of glycine with sucrose when 0.5 mole of glycine is present in a 1.0 *M* sucrose solution. The degrees of association amount to 5 per cent, 18 per cent, 16 per cent and 27 per cent according as the data of columns 2, 3, 4, or 5 of table 11 are employed for the calculation. None of these values is inherently improbable.

Mr. W. J. Shutt of this Department has determined the solubility of glycine in water and in 1.0 *M* sucrose, respectively, at 0°C. The analytical method involved formol titration of the weighed sample, using 0.1727 *N* NaOH, and phenolphthalein. The solubility in water was found to be 14.20 grams of glycine per 100 grams of water. In the presence of 1.0 *M* sucrose the solubility[29] was found to be 9.90 grams per 100 grams of sugar + water. Since the density of the sugar solution is 1.131, the solubility may be expressed as 14.19 grams per 100 grams of total water. If all the water present were regarded as available solvent, one would conclude that the sucrose has no effect at all upon the activity coefficient of glycine. On the more probable assumption that only part of the water is available, it follows that there is a real increase in solubility, that is, a decrease in the activity coefficient of glycine, due to the sucrose either as an environmental effect or due to complex formation. This may be expressed quantitatively on the basis that the sucrose holds 6 molecules of water per molecule of sugar, this quantity of water being unavailable as solvent. We thus calculate that the solubility of glycine, in the presence of 1.0 *M* sucrose, is 16.4 grams per 100 grams of "free" water. Hence the activity coefficient, α, of glycine in the presence of the sucrose, relative to its value in water, is 14.2/16.4 or 0.866. Assuming that this value would hold for any concentration of glycine in the presence of sucrose, and employing the empirical relation $f_0 = \sqrt[3]{\alpha}$ we obtain for f_0, the osmotic coefficient of glycine in the presence of molar sucrose, the value 0.954. The apparent molecular weight in the presence of sucrose would therefore be expected to be the observed value in water (corresponding to the concentration of glycine employed) divided by f_0. Hence for concentrations 0.470 and 0.72 *M*,

[29] The solid phase was pure glycine.

respectively, the apparent molecular weights should be 82.3 and 84.1. (The corresponding values in water are 78.5 and 80.2.) Although these are close to the results given in the first column of table 11, it would be unsafe and indeed illogical, to assume that this column rather than the remaining ones was to be preferred, in view of the approximate nature of the empirical relation used to connect α with f_0, and also in view of the assumption regarding the applicability of the α value, obtained from solubility data, to the region of more dilute glycine solutions. Owing to the absence of data it is impossible at the present time to carry out a more satisfactory analysis of the osmotic behavior of glycine in the presence of sucrose.

The behavior of glycine in solution outlined in this Appendix, and the general inconclusiveness which exists at the present time regarding many aspects of its behavior may at least serve to indicate how much still remains to be done before a quantitative treatment of the behavior of protein solutions in terms of activity and interionic attraction can be effected.

Addendum

Since the above account was written a paper by M. Frankel (77) has appeared upon molecular weight determinations (boiling point as well as freezing point) for a number of amino acids in water,—in the absence of salts. Frankel finds that association is exhibited by glycine, *l*-asparagin, *d*-arginine, and *d*-dioxyphenylalanine. On the other hand, *d*-, *l*-, and *dl*-alanine have a normal molecular weight. In the case of glycine, the most concentrated solution employed contained 1.809 moles per 1000 grams of water. At this concentration the molecular weight was found by Frankel to be 88.3, which is somewhat higher than the value obtained in the writer's laboratory—namely, 85.3 at a concentration of 1.674 moles per 1000 grams of water, and 86.6 at a concentration of 2.176 moles. More recently still a paper by Borsook and MacFadyen (78) has appeared dealing with "The effect of isoelectric amino acids on the pH of a phosphate buffer solution," in which the relative merits of the "classical" and zwitterion conceptions of amino acids are discussed. The importance of

dielectric capacity measurements is pointed out, as has likewise been done independently in the present paper.

Summary of appendix

1. From freezing point data it is shown that glycine in water is polymerized, to the extent of 10 per cent (in round numbers) in a 1.0 *M* solution. Apparently the glycine zwitterions do not exert an appreciable "interionic attraction" upon one another.

2. In the presence of sodium chloride, there is an increased osmotic abnormality. It is shown that the extent of union of glycine with chloride ion is probably too small to account for more than a part of this abnormality. Evidence is also afforded that the effect is not due to increased polymerization. On ascribing the effect to interionic attraction the results obtained are inconclusive, especially in the region of low sodium chloride content.

3. In the presence of sucrose the freezing point data indicate osmotic abnormality in the sense of an increase in the apparent molecular weight. From an analysis of the results it is concluded that further polymerization does not occur, but that the effect is to be referred to a decrease in the activity coefficient of glycine due to the presence of the sucrose and probably due to complex formation. This is confirmed by solubility determinations in so far as this procedure is justifiable.

In conclusion the writer wishes to express his very great indebtedness to his colleagues Dr. R. O. Griffith, Mr. W. J. Shutt, and Dr. A. McKeown for their helpful criticisms and suggestions in connection with a number of the points considered and discussed in the above article.

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