# Protein Hydration and Behavior

Many aspects of protein behavior can be interpreted in terms of frozen water of hydration.

Irving M. Klotz

It is the purpose of this article to reexamine some of the distinctive properties of protein molecules in the light of certain ideas which have appeared sporadically in the literature and which, if applied on a broad scale, provide a more unified and plausible interpretation of the singular behavior of these substances. This reexamination was stimulated by certain problems which we encountered in studies of what is perhaps the simplest reaction of protein molecules, combination with a proton in aqueous solution:

$$P + H^+ = PH^+$$

Consequently, a description will be given also of some of the unusual features of this reaction. However, we shall not discuss this reaction per se but rather analyze it in the context of what it might reveal about the structure and behavior of protein molecules.

## Some Problems in Protein Behavior

As a prefatory background, therefore, let us consider some of the chemical and physical properties which mark proteins as unique. That is, let us examine some reactions or aspects of physicochemical behavior of proteins for which there are counterparts in simple nonprotein systems but in which the introduction of a protein environment produces some marked differences. The particular examples which one might choose to illustrate the unique character of proteins are largely a matter of taste. A reasonable selection from among many alternatives might be the following four.

*Masked groups.* From an analysis of the amino acid composition of proteins, one knows or expects certain functional groups to be present—for example, phenolic or mercaptan groups. Nevertheless, these may not be detectable in the native protein although they do become evident in the denatured material (*1*). Mercaptan groups have been the most extensively examined in this connection. As a typical example, an amperometric silver titration of mercaptan in hemerythrin is shown in Fig. 1. When the protein is dissolved in aqueous salt solution (curve A), added silver ion produces an immediate increase in diffusion current, indicating that the metal ion is not bound to protein mercaptan. On the other hand, in the presence of urea, even an immediate titration (curve *B*) shows a much slower rise of free silver than in water alone, and a titration after 1 hour in urea (curve *C*) shows no free silver until the total metal exceeds a value (about 0.5 $cm^3$) corresponding to the mercaptan content of this protein. Thus, the mercaptan groups are evidently present in the protein, but in the native state they are inaccessible to a particular reagent—silver in the experiments of Fig. 1. The molecular basis of this masking has been the subject of much speculation, suggestions having ranged from that of a chemically modified state (thiazolines) (*2*) to that of steric blocking by hydrogen-bonding or by side-chain groups (*2, 3*).

*Denaturation.* An examination of the meaning of this term in general would involve a prolonged discussion which would distract from the principal objective of this article. Let us consider, therefore, merely one typical example of denaturation, that of the enzyme aldolase. The activity of this enzyme (*4*) in aqueous solutions of increasing urea concentrations is fairly constant up to 1*M* urea and then falls rapidly, reaching zero at about 3.5*M* (Fig. 2). In this particular example, activity is regained if the enzyme in urea solution is diluted to reduce the urea concentration markedly—that is, the denaturation can be "reversed." In molecular terms, denaturation is usually ascribed to a disorientation of the structure of the protein molecule (see Fig. 3), renaturation to a reorganization of the structure to its original form.

*Volume changes.* Proteins undergo striking volume changes when they are denatured chemically or when they are denatured and hydrolyzed enzymatically. The latter phenomena have been studied extensively in the Carlsberg laboratories (*5*), and some of the observations made there are summarzied in Fig. 4. For orientation purposes, a hatched region is shown for the small volume change, −15 to −20 $cm^3$/mole, normally found for the hydrolysis of simple peptides. In contrast, the hydrolysis of β-lactoglobulin by trypsin at, for example, 0°C is accompanied by a marked decrease in volume, particularly for the splitting of the first few bonds. It is evident that a pronounced collapse in effective volume of β-lactoglobulin occurs in the early stages of tryptic hydrolysis, the contraction being more than five times as great as that found with simple peptides.

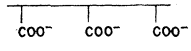
*Cooperative effects.* There are many instances in protein behavior where combination of the macromolecule with one small ion or molecule makes it much easier for succeeding molecules to become attached. Perhaps the best examples of such cooperative effects are the oxygen-carrying proteins, notably hemoglobin. The familiar S-shaped curve for oxygen uptake (Fig. 5) (*6*) indicates clearly that oxygen is bound more readily after some has already been taken up by the hemoglobin molecule. These effects can be expressed quantitatively if the observed equilibrium constants ($k_i$) for the uptake of oxygen by each of the four sites of the protein molecule, are compared with the values to be expected if each site acted completely independently (Fig. 6) (*7*). With independent sites, $k_1 > k_2 > k_3 > k_4$, simply for statistical reasons (*8*). The observed values of $k_i$ for hemoglobin are much higher than the statistical values (Fig. 6). Such behavior is not a property of the heme groups as such, however, for cooperative effects are also present in the heme-lacking oxygen pigments hemocyanin and hemerythrin (*9*).

## Uptake of Protons by Protein Molecules

With these unusual properties of protein molecules in mind, let us turn to an examination of some aspects of their

The author is on the staff of the department of chemistry of the College of Liberal Arts, Northwestern University, Evanston, Ill.

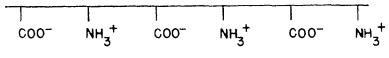
titration behavior. As an introduction let us consider the behavior of a very simple system, a mole of formate ion, $HCOO^-$, in solution, as $H^+$ ions are added in progressively increasing amount. As Fig. 7*A* illustrates, below *p*H 6, the amount of $H^+$ which combines with formate, to form HCOOH, increases in the form of a typical (reversed) S-shaped titration curve as the *p*H drops. A plateau of 1 mole of $H^+$ per mole of total formate originally present is reached at about *p*H 2. As is well known, the *p*H of the midpoint of this titration curve equals the *pK* of formic acid. Essentially the same curve would be obtained if 100 moles of formate were originally present, and if the ordinate were moles of $H^+$ combined with 100 moles of formate. Likewise, Fig. 7*A* would represent correctly the acid titration of 100 moles of carboxyl groups joined in one large polymer molecule I




(I)

if these groups were spaced at sufficiently large intervals so that they had no electrostatic influence on each other.

Let us examine now the actual behavior found in the titration of carboxyl groups in protein molecules. Illustrated in Fig. 7*B* are the ideal curve for the behavior of 100 identical groups without electrostatic interactions and the actual curve observed in the acid titration of the approximately 100 carboxyl groups in serum albumin (*10*). With albumin the uptake of $H^+$ is spread out over a wider *p*H range. A possible reason for this flattening is readily evident, however, as soon as one recognizes that a protein contains charged groups in addition to its carboxyls, and that the distance between substituents is limited by the over-all size of the molecule. Schematically we may represent the situation by II.




(II)

From this diagram it becomes apparent that when, for example, one proton has combined with one —COO- group, the protein will carry a net positive charge due to the cationic groups, and this charge may make it more difficult for succeeding protons to combine with the protein. In essence, then, we shall need a lower *p*H—that is, a stronger proton pressure—to force a proton onto a —$COO^-$ group; therefore, the titration curve is stretched toward lower *p*H's.

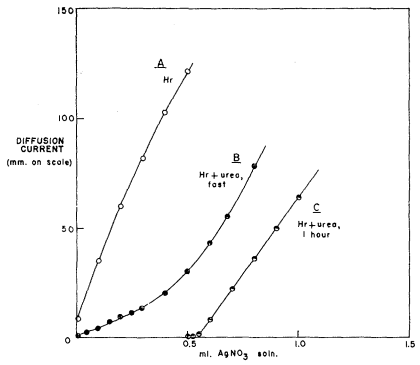



Fig. 1. Amperometric titrations of hemerythrin with silver ion: *A*, hemerythrin in aqueous solution; *B*, hemerythrin in 8*M* urea solution upon immediate titration; *C*, hemerythrin in 8*M* urea, silver ion added and solution allowed to stand for 1 hour before further titration. [I. M. Klotz, T. A. Klotz, H. A. Fiess (*50*)]

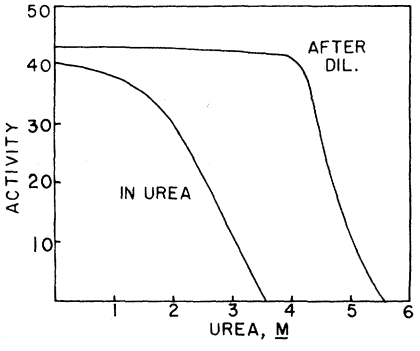



Fig. 2. (Lower curve) Activity of aldolase as a function of concentration of urea in solution. (Upper curve) Activity of aldolase after solution of enzyme in urea of given concentration is diluted with water to a urea concentration of 0.08*M*. [A. D. Swenson and P. D. Boyer (*4*)]

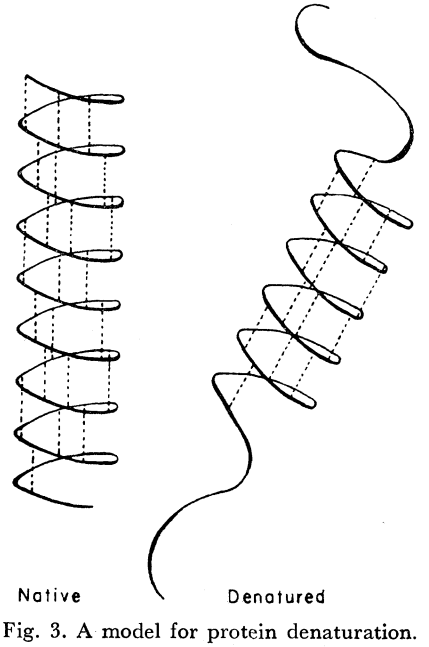



Fig. 3. A model for protein denaturation.

These electrostatic considerations can be formulated in quantitative terms and have been so expressed (*11*). The titration curves of some proteins can then be accounted for quantitatively reasonably well. More often, however, there are large deviations from predictions based on electrostatic theory. Discrepancies can still be explained by various additional assumptions for specific cases (*10*). Alternatively, one could also account for an observed acid titration curve by assuming, quite reasonably, that each or most of the carboxyl groups are not really identical, for their environments, in terms of which amino acid residues are their neighbors, are very likely quite different. Consequently, the observed behavior of carboxyl groups in a protein may actually be the composite result of, for example, nearly one hundred different carboxyl groups with different *pK*'s, the distribution of *pK*'s being such as to produce the observed flattened titration curve.

It seemed to us that a promising approach to the assessment of the contribution of electrostatic and various specific factors to the behavior of protein molecules might be a study of the proton equilibria of a *single* group in a protein. If electrostatic effects are influential, they should still modify the ionic behavior of even a single residue, for as the *p*H of the solution is lowered, the net positive charge of the protein molecules will be increased. Consequently, those molecules still containing the singled-out residue in its basic state should not take up hydrogen ions as easily; the titration curve should be stretched out and should look like the actual curve of Fig. 7*B*, even though only a single residue is titrated. On the other hand, if the residue is not subject to electrostatic influences from other side-chains, the titration curve should be in a form approximating the ideal curve of Fig. 7*B*.

From these considerations it follows that carboxyl groups of proteins are not suited for our purposes, since so many of these are normally present in a protein. There are usually fewer imidazole or tyrosine groups, but even these are commonly present in appreciable number, and their proton equilibria overlap those of amino groups so that a sharp separation cannot be made by electrometric methods. The proton-donor group usually present in smallest number in proteins is the sulfhydryl. Here again, however, a direct study of its dissociation by electrometric methods is not feasible, since the equilibria of amino and tyrosine groups would obscure the contribu-

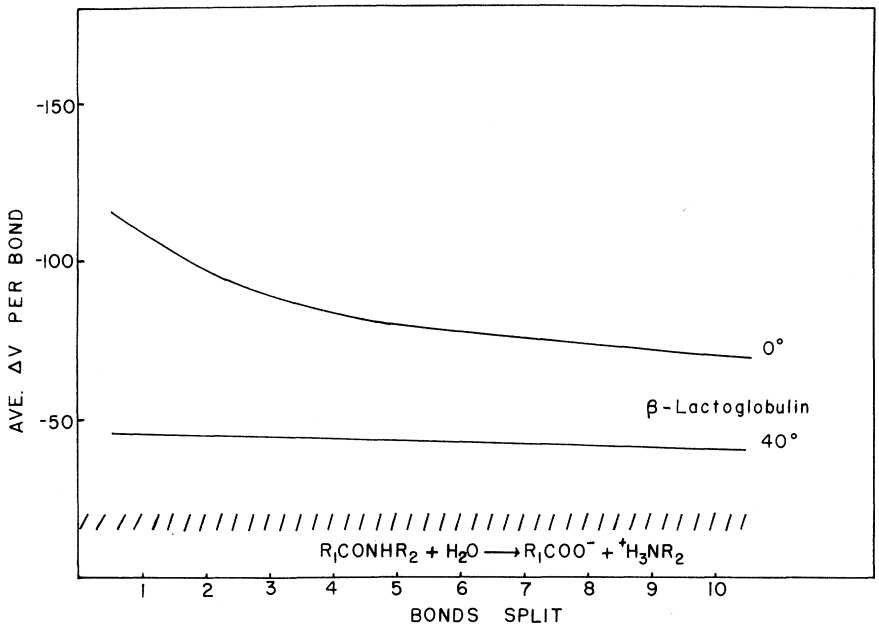
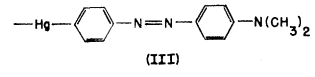



Fig. 4. Volume changes accompanying hydrolysis. [K. Linderstrom-Lang (*5*)]

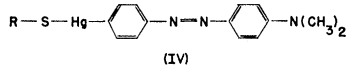
tion of the thiol. An alternative approach, which could still take advantage of the small number of —SH groups, would be to introduce a molecule which reacts specifically with the mercaptan side chain, and which contains a substituent whose proton uptake, or release, is accompanied by a marked change in visible spectrum (*12*). In this way one could follow the ionic equilibrium of the marked residue without any obscurant effects from the many simultaneous proton equilibria of other side chains, since the latter are all uncolored. An example of a suitable labeling molecule is the azomercurial III




(III)

which combines specifically with the mercaptan groups of proteins and undergoes marked alterations in absorption of light over a characteristic *p*H range.

The *pK* of the $-N(CH_3)_2$ group in III is generally unaffected by changes in substituents at the opposite end of the azobenzene nucleus (*13*). Nevertheless, it seemed prudent to examine the proton equilibrium of III when it is attached to a small-molecule mercaptan IV




(IV)

so that any effect of the sulfur-mercury linkage to be use in attaching this molecule to the protein could be evaluated in a non-protein environment. As anticipated, the *pK* of the cationic form of IV, 3.4, was not significantly different from the *pK*'s of other para-substituted azobenzenes, about 3.4.

When the azomercurial (III) is attached to a protein, however, some surprising behavior is observed (Fig. 8). The *shape* of the titration curve of the dye in the protein, from *p*H 3.5 down to almost 0, is not greatly different from that of the dye attached to a small molecule (for example, cysteine) in aqueous solution. In the cysteine complex a rise in absorption becomes detectable with increasing acidity immediately below *p*H 5. When the dye is attached to serum albumin, with an isoelectric point near *p*H 5, one would expect, if electrostatic effects were important, that the optical density would also rise as soon as the *p*H passes below 5 and would continue to rise with decreasing *p*H, but at a somewhat slower rate due to the increasingly positive charge on the proton. Actually, the rise in absorption due to uptake of $H^+$ by the dye attached to protein really begins only as the *p*H drops below 3, and then goes up sharply. The titration curve of $-N(CH_3)_2$ attached to the protein is not stretched out over a wider *p*H range than in the cysteine complex.

It would seem, therefore, that the ionic behavior of the $-N(CH_3)_2$ group is not affected by the presence of other charged groups in the protein molecule. Confirmation of this conclusion is also obtained from another titration, in the presence of 30 dodecyl sulfate anions per protein molecule. Since these negatively charged ions are all bound to the positively charged protein, one would expect an appreciable decrease in the electrostatic effect of groups on the protein molecule; no such change is observed (*12*).

In view of the absence of electrostatic effects from neighboring side chains on the ionization behavior of the $-N(CH_3)_2$ group on the protein, the relative position of the titration curve for dye-albumin in Fig. 8 is most peculiar. The mid-point of the titration, 1.7, which we might call the apparent *pK*, is very low compared to the value of 3.4 observed for the dye attached to a small-molecule mercaptan. Such behavior, furthermore, is not limited to this single protein. Experiments have been carried out also with ovalbumin and β-lactoglobulin. Although there are individual differences between these proteins, they both show essential similarities to serum albumin in that their *pK*'s are quite low —about 1.2 and 2.3, respectively.

Thus, with all three proteins, it is harder for a hydrogen ion to get on to the $-N(CH_3)_2$ substituent when the latter is attached to the macromolecule than when it is in simple aqueous solution attached to cysteine. Such behavior is reminiscent of the difficulty $Ag^+$ ions have in combining with mercaptan groups on native proteins, as was described earlier. We might say, therefore, that the $-N(CH_3)_2$ group attached to the protein behaves as if it were "masked." Evidently a "masked" group can be introduced synthetically as well as in natural biosynthesis.

Because of the analogy with the characteristics of naturally occurring masked groups, we might be curious to know whether unmasking of the $-N(CH_3)_2$ can be produced by denaturing agents. One of the most common denaturants is urea. Strikingly enough, the *pK* of the azomercurial $-N(CH_3)_2$ group attached to proteins but in concentrated urea solu-

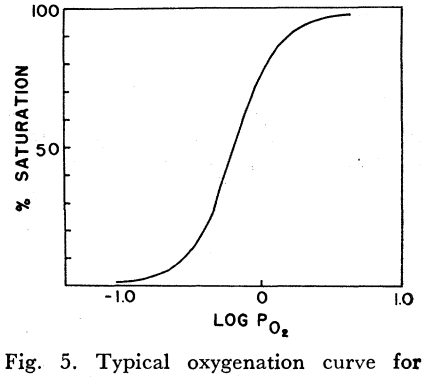



Fig. 5. Typical oxygenation curve for hemoglobin, percentage saturation being plotted as a function of the logarithm of the oxygen pressure. [From A. F. Riggs (*6*)]

tions is 3.3, experimentally indistinguishable from the $pK$ of this group when the azomercurial is attached to cysteine. Furthermore, in urea all three proteins—serum albumin, ovalbumin, and β-lactoglobulin—show the same $pK_a$ for the azomercurial, even though they are readily differentiable in the native state.

### An Interpretation of the Behavior of Proteins in Solution

We are thus faced with the question of what is the molecular basis of this masking. Since it also occurs with the artificial group which we have introduced into proteins, an explanation based on steric interferences from side chains of conveniently adjacent residues in all three proteins seems unlikely, for it is difficult to see why the protein configuration should be just so arranged in every case as to be able to mask the *artificially introduced* side chain. Furthermore, the degree of "maskedness" of the original —SH groups in serum albumin, ovalbumin, and β-lactoglobulin is markedly different. For example, in serum albumin, there seems to be little or no interference with the sulfhydryl group, as judged from titrations with $Ag^+$ or an organic mercurial, whereas in β-lactoglobulin there is much interference. Nevertheless, in both proteins, the artificially introduced group is masked—in fact, more masked in serum albumin than in β-lactoglobulin, as judged from the shifts in $pK_a$. Such behavior is difficult to understand in terms of steric interference because of protein configuration. An alternative approach based on the following considerations seems more fruitful.

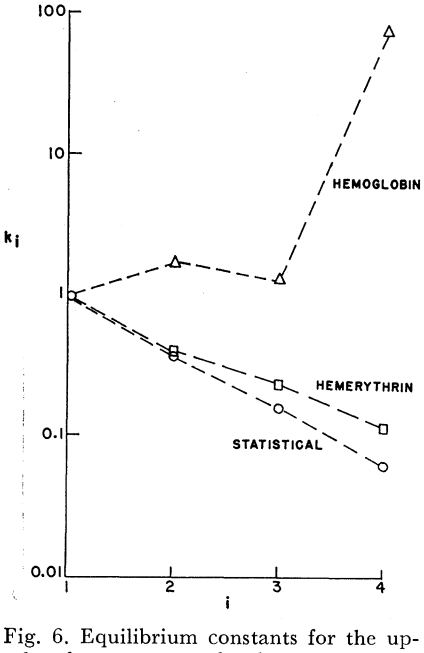



Fig. 6. Equilibrium constants for the uptake of an oxygen molecule at each of the four sites of hemoglobin (*7*) and of hemerythrin (*9*) compared with the relative values to be expected purely from probability considerations.

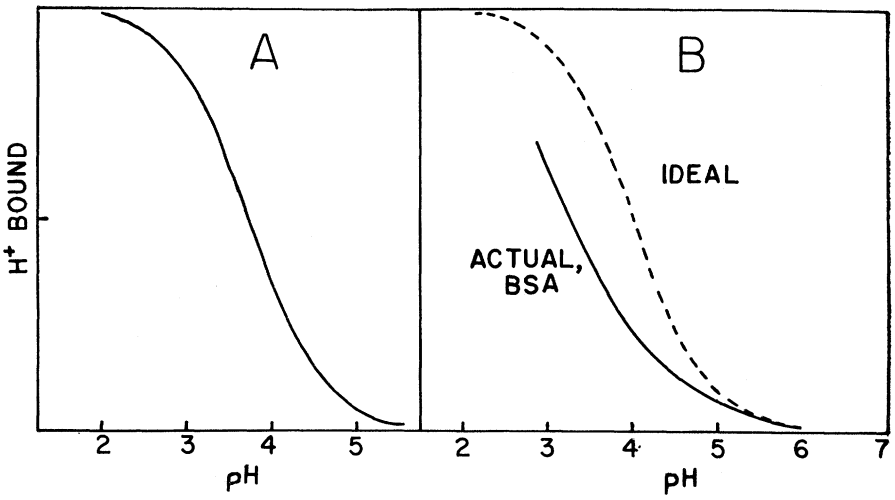



Fig. 7. (A) Uptake of hydrogen ions by a carboxylate ion such as $HCOO^-$; (B) uptake of hydrogen ions by 100 carboxylate ions under ideal conditions, compared with the actual curve observed for bovine serum albumin. [C. Tanford, S. A. Swanson, W. S. Shore (*10*)]

A $H^+$ ion can clearly reach the $-N(CH_3)_2$ group of the protein, although, as judged from the lower $pK_a$, the environment is not as hospitable to it. It would seem, therefore, that the azomercurial on the protein is in an aqueous environment but not a normal one. One is led to ask, therefore, whether the water in the vicinity of the protein is not different in structure from that in the bulk of the solution.

It has long been recognized, of course, that in aqueous solution macromolecules as well as small molecules are hydrated. Nevertheless, the first strong emphasis on the importance of "icebergs" around solute molecules in water was probably that made in the papers of Frank and Evans (*14*; see also *15–18*). Since that time this concept of "frozen" hydration shells has been utilized in interpretations of various phenomena involving macromolecules. For example, in connection with studies of entropy changes in the formation of protein complexes (*19*) we have suggested that disorganization of "frozen" water of hydration plays a dominant role. Reasoning from analogous thermodynamic grounds, Eisenberg and Schwert (*20*) have interpreted their observations on the reversible denaturation of chymotrypsinogen in terms of the partial dehydration (*21*) of icelike water on the protein molecule. Similarly, Jacobson has shown from nuclear magnetic resonance (*22*) and other studies (*23*) that nucleic acids in solution are surrounded by a hydration shell of lattice-ordered water. An analogous concept has been used by Szent-Gyorgyi (*24*) in the interpretation of fluorescence experiments and their relationship to problems of energy transfer in biological systems.

An examination of the titration behavior, and of some of the other unique characteristics of proteins described earlier in this article, from the viewpoint of the "iceberg" nature of the hydration water reveals a pattern for the explanation of many seemingly unrelated phenomena. It is necessary first to keep in mind the various ways in which a solute can affect the structure of water in its neighborhood. An ion imposes a structure on the first hydration shell in which all the water molecules are oriented in a centrosymmetric fashion. Somewhat farther from the ion, the electric field is usually not strong enough to effect the same orientation but generally is sufficient to disrupt any lattice structure organized by cooperative effects in the water itself (*16*). With a nonpolar solute, however, any disorientation effect due to the electric field disappears, and a lattice-ordering effect predominates, as seems evident from many physicochemical properties of nonpolar solutes in water (*14–18*). Nonpolar side chains in proteins might be expected, likewise, to induce a crystalline, cagelike, arrangement of hydration water, with the added possibilities of long-range cooperative

effects due to the presence of many such side chains bound to the frame of the protein molecule. It is in terms of the icelike nature of this hydration water (*14–18*) that we can account for much of the puzzling behavior of proteins described above.

*Titration behavior.* Within the framework of this concept we would view the drop in $pK_a$ of the dimethylamino group in the environment of the protein as a consequence of the rigid iceberg structure in the neighborhood of the macromolecule (Fig. 9). If the proton attached itself to the $-N(CH_3)_2$, some disruption of the stable cagelike hydration lattice of the protein would be required, and this rearrangement would be more difficult to carry out in the protein than in an ordinary water environment.

With serum albumin (in contrast to ovalbumin), a detailed examination (*12*) of the shape of the titration curve of the bound $-N(CH_3)_2$ group indicates that this cagelike freezing becomes particularly extensive below *p*H 4.2. Evidently the extent and rigidity of the hydration envelope increases substantially and may contribute to changes in hydrodynamic properties and optical characteristics described in the literature (*25*).

The effect of urea, bringing the $pK_a$ back up to 3.3, is then easy to understand. In essence, in the presence of urea, the dye attached to protein behaves in its ionization just like the dye in water, free from protein. An appropriate inference might be, therefore, that urea, because of its strong hydrogen-bonding characteristics, breaks down the "frozen" structure of the water envelope of the protein and transforms it into one more nearly like that of the bulk aqueous environment.

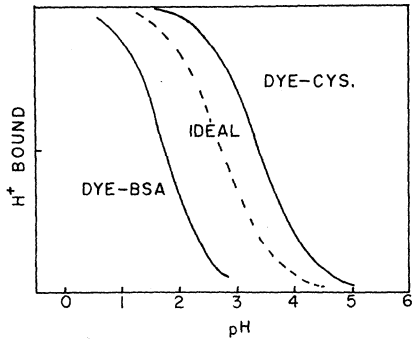



Fig. 8. Titration curves for III when attached to bovine serum albumin or to cysteine, as compared to its behavior in ideal circumstances. The dye-cysteine curve could be superimposed on the ideal. The dye-protein curve almost fits the ideal, but is probably slightly steeper, certainly not flatter.

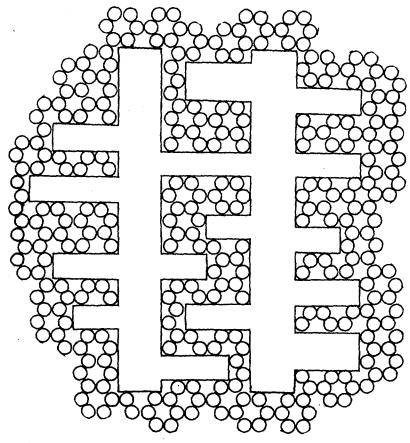


Fig. 9. Schematic diagram of icelike character of hydration sheath of protein molecule.

*Volume measurements.* Let us examine first in this connection the molal volumes of proteins in aqueous solution. McMeekin and Marshall (*26*) have shown that the value of this volume computed from the sum of the specific volumes of the constituent amino acid residues agrees very well with observed numbers. As Edsall (*27*) and Waugh (*28*) have pointed out, such agreement is very surprising since no correction is made for the electrostriction effects of charged residues. For human serum albumin, for example, electrostriction ought to reduce the volume by about 1800 cm³ per mole of protein molecules; for ovalbumin, by 730 cm³ and for β-lactoglobulin, by 830 cm³. Since these decreases in volume are not observed, it seems reasonable to infer that some other phenomenon produces a compensating increase in volume when the amino acid residues are joined in a protein. We would suggest that this increase in volume comes from the fixation of the hydration water of the protein into an icelike lattice (Fig. 9). There is good reason to believe that the hydration ice around nonpolar solutes is similar in density to ordinary ice (*15, 17, 18*). By analogy with the freezing of ordinary water one would expect, therefore, an increase in volume upon formation of the hydration iceberg of the protein. Some interesting calculations can be made in this direction. The freezing of water is associated with a volume increase of about 2 cm³ per mole. If one attributes the 1800 cm³ volume increase of serum albumin, which compensates for electrostriction decreases, to the freezing of water, one can compute the number of water molecules fixed and, thereby, the percentage hydration of the protein. Similar computations can be made for ovalbumin or β-lactoglobulin. The results give hydrations of close to 20 percent by weight, in very good agreement with the best measured values (*29*).

The volume changes accompanying proteolysis (*5*) (Fig. 4) also follow as a natural consequence of the iceberg picture. In particular, the large values of $\Delta V$ at the onset of proteolysis would seem to be a reflection of extensive disarrangements in the water lattice at the surface of the substrate protein molecule at the beginning of the enzymatic attack. Thus, the common observation that proteolytic enzymes seem to denature the substrate protein before hydrolyzing it would be a natural expectation. Likewise, one could readily interpret the general experience that these enzymes act more readily on previously denatured substrates.

It is also of interest to note in Fig. 4 that the volume decrements during hydrolysis at 0°C are substantially larger than those observed at 40°C. Such behavior would be eminently reasonable from the iceberg viewpoint, for one would expect more extensive freezing at the lower temperature.

Some quantitative estimates of hydration can also be made from the observed volume changes during hydrolysis. For complete hydrolysis of β-lactoglobulin, Linderstrom-Lang (*5*) has estimated a $\Delta V$ of 700 cm³ (at 40°C), after correction for electrostriction effects. This volume change corresponds to about 17-percent hydration, again in good agreement with generally accepted values.

*Masking and denaturation.* Masking of naturally occurring functional groups of proteins, as of the artificially introduced $-N(CH_3)_2$ group, can be attributed to a "freezing" of the water structure in the neighborhood of that site (Fig. 9). Lack of reactivity of such hindered groups (for example, mercaptan) is usually only relative and dependent on the reagent. The effectiveness of a reagent in combining with a masked group might very well be related to its ability to dissolve in, or to disorient, the lattice structure of the iceberg covering the group. Similarly, the effects of denaturing agents, such as urea, in "unmasking" blocked functional groups may be due to the rearrangements which these reagents produce in the structure of the water envelope characteristic of the native protein.

In terms of the melting of the water of the "metastructure" of the protein, it

is to be expected that denaturation would be accompanied by an over-all volume contraction. It is striking that such indeed is the case, not only for the enzymatic proteolyses described above but also for denaturation by urea, or alkali (*5, 28, 30*). Volume contractions of about 250 $cm^3$ per mole of protein have been observed in alkaline denaturation, and as high as 300 $cm^3$ in urea denaturation.

The larger volume changes accompanying enzymatic hydrolysis are probably due to a much greater disruption of the macromolecular structure in the hydrolytic process.

The reversibility of some denaturation processes—for example, after removal of urea or after cooling of a heated solution—is also more readily understandable in terms of the iceberg picture. If the effect of urea or heat is to disrupt the hydration lattice but not to disturb the polypeptide configuration, then it is easy to understand that the removal of urea or heat should lead to a reformation of the specific hydration lattice originally present. Since the shape and extent of the iceberg would depend on the nature and disposition of protein side chains, if the latter have not been separated from each other by unfolding of the polypeptide backbone, these should continue to provide the framework for the reconstruction of the original hydration lattice once the disturbing denaturing influence has been removed. On the other hand, irreversible denaturation would follow after an unfolding of the fundamental polypeptide configuration, since a refolding into the highly specific original orientation is an improbable process.

It is perhaps of interest to note in connection with reversible denaturation that the $pK_a$ of the $-N(CH_3)_2$ group attached to serum albumin showed changes in the expected direction. As was pointed out above, the $pK_a$ changed from 1.7 to 3.3 upon addition of urea; when the urea was then dialyzed out for 24 hours, the $pK_a$ returned to 1.85.

It has been known for some time that anions, in small amounts, can protect serum albumin against denaturation by urea or heat (*31*). If this protection arose from a stabilization or further extension of the hydration lattice due to the bound anion on the protein, one might expect the $pK_a$ of the $-N(CH_3)_2$ group to be lowered even further in the presence of suitable anions. Some experiments were carried out, therefore, with small amounts of dodecyl sulfate anions and serum albumin; the observed $pK_a$ was below 1.3, as compared to 1.7 when the protein was in water alone. Furthermore, if low concentrations of dodecyl sulfate were added to urea solutions, the $pK_a$, instead of being 3.3, as for urea alone, was returned downward to 2.4. All of these observations fit readily the view that polarizable organic molecules increase the order of water structures. It is reasonable, therefore, that, when bound to a protein surface, long-chain aliphatic anions might extend even further the lattice-ordered hydration layer. Other surprising effects of anions or neutral small organic molecules on serum albumin, such as the large changes in optical rotation produced by a small number of bound anions (*32*) or the marked effects of decanol and oleate on the dielectric increment of serum albumin (*33*), are also more readily visualized in terms of a reorientation of the hydration layer than on the basis of a rearrangement of the configuration of the peptide chains.

The literature also indicates that concentrated solutions of sugars and sugar alcohols inhibit the denaturation of some proteins (*34*). We have made, therefore, a few measurements of $pK_a$'s of the $-N(CH_3)_2$ group attached to serum albumin in solutions of 40- to 60-percent sorbitol. It is certainly pertinent to note that the $pK_a$'s dropped to 1.4 to 1.3.

In contrast to these stabilizers against denaturation, high hydrostatic pressures inactivate enzymes and cause coagulation of proteins (*35*). This effect is of course what one would expect in view of the indications cited above that denaturation involves a decrease in volume as the hydration icebergs are converted into liquid water. In recent years, the masking of hydrogens on proteins has been approached through studies of rates of exchange with deuterium (*36*). Many of the effects reported, such as increased rates of exchange in the presence of urea, can be readily interpreted in terms of changes of hydration structure, as well as of modifications in configuration of the protein chains. Of particular interest in this connection are the observations of Hvidt (*37*) on the marked decrease in rate of exchange of deuterium in ribonuclease as the temperature is lowered. Such behavior would seem very plausible on the basis of the iceberg viewpoint, since the extent of the rigid lattice structure should surely increase as the solution is cooled.

A similar effect of temperature on masking has been recently reported by Murayama (*38*) in experiments on the uptake of silver ion by crystallized dialyzed hemoglobin. Substantially more sulfhydryl groups became available as the temperature was raised from 0° to 38°C—that is, in terms of our viewpoint, as the iceberg melted (*39*).

Thus it is apparent that many of the specific phenomena that are included in the broad terms *denaturation* and *masking* can be interpreted very plausibly in terms of the behavior of the hydration sheath of protein molecules.

*Cooperative effects.* Since many aspects of the oxygenation reaction have been investigated, it is of interest to examine the oxygen-carrying proteins in some detail. From the "iceberg" point of view, the binding site of the deoxygenated protein may be visualized as having an aqueous covering sheath whose lattice structure is determined by the nature of the residues (and metal) at the active site and by the amino acid side chains in the vicinity of the site. Under appropriate conditions these crystalline water islands may be so extensive that they merge into an interwoven lattice. The subsequent attachment of an $O_2$ molecule to the binding site would then produce an incongruity in this lattice because of the larger size of $O_2$ as compared to $H_2O$ and because of the differences in hydrogen-bonding properties. As a result, the hydration lattice might be disturbed for an appreciable distance around the active site. This disturbance could affect the hydration structure of neighboring sites and hence change their affinity toward oxygen molecules. In this way an S-shaped oxygenation curve may arise (Fig. 5).

There are several features of this model which seem more plausible than those recently proposed by others (*40, 41*). For one, cooperative interactions, which have been studied most extensively in hemoglobin, exist also in hemocyanin and hemerythrin, which do not contain heme groups and in which, therefore, the bulkiness of the heme can play no direct steric role. Furthermore, with hemocyanin, for example, the extent of interaction between sites depends very much on *p*H (*42*); one would anticipate changes in hydration as the charges on groups of the protein are varied. Likewise, one can interpret readily the effects on the affinity of hemoglobin for oxygen of salts which presumably change only the ionic strength (*43*), as well as the influence of specific ions (*44*) on the interactions between sites in hemocyanin. All of these substances could modify the

extent and nature of the lattice structure of the hydration sheath of the protein. In a similar vein, changes in solubility of hemoglobin upon oxygenation seem more plausible. The structure of the hydration lattice would depend on the state of oxygenation as well as, presumably, on the number, type, and position of amino acid side chains. When the interaction of the protein with solvent molecules in the hydration sheath is altered, one might reasonably expect differences in the interaction with solvent molecules in the liquid phase—that is, changes in solubility. Likewise, relatively small changes in composition of a hemoglobin —for example, the single different amino acid (*45*) in sickle-cell hemoglobin (half molecules)—could have the profound effect on solubility that they have because the nature of the hydration layer around one specific side chain could affect the structure of the lattice for some distance in its neighborhood.

It is also of interest to examine further certain interrelationships among properties of hemoglobin pointed out by Wyman and Allen (*40*). As these investigators have shown, there exists a parallelism of interactions between hemes when the process is oxidation-reduction and when it is oxygenation; when one heme is oxidized, the second follows along immediately, as in oxygen uptake. Likewise, there is a parallelism in interactions when the equilibrium involves carbon monoxide and when oxygen is the gas. On the other hand, in examining correlations between the Bohr effect and other properties of heme proteins, Wyman and Allen (*40*) point out that these changes in oxygen affinity with *p*H do not seem to be related to the change in bond type of the iron. Thus, myoglobin shows no Bohr effect, although oxygenation produces the same change in unpaired electrons of iron as it does in hemoglobin, which shows a large Bohr effect. Thus, if the Bohr effect is attributed to changes in the nature of the bonding of an imidazole side chain to the iron, as has been described by Coryell and Pauling (*46*), one must assume that a histidine-iron linkage does not occur in myoglobin. As an alternative explanation of these phenomena, Wyman and Allen (*40*) propose that the protein molecule changes its configuration on oxygenation, or oxidation, and that it is these changes which transmit the interactions between hemes or affect the acidity of certain amino acid residues.

It seems apparent that if we consider the "iceberg" viewpoint, characteristics of heme proteins are more readily interpretable. While the uptake of an oxygen molecule or the removal of an electron from the heme iron could conceivably alter the configuration or folding of peptide chains, it seems more plausible that these changes would influence the interaction of the iron and heme with the aqueous hydration sheath. (Certainly, $Fe^{++}$ and $Fe^{+++}$ in simple aqueous solution differ very greatly in their interaction with $H_2O$ molecules). As indicated above, a disturbance at one point in the water lattice could, reasonably, affect the hydration structure for some distance in the vicinity. One might anticipate, therefore, changes in the oxidation or oxygenation characteristics of neighboring hemes, as well as of the acidity of acid-base groups of amino acid residues in the vicinity.

In these terms one can also understand more readily why nonheme oxygen carriers also show Bohr effects. Hemocyanins of the lobster and crab (*47*), for example, become more acidic on oxygenation; hemocyanin of the horseshoe crab (*Limulus*) does not. It could be, of course, that the copper is bound to imidazole in the first two cases and not in the latter. However, a more plausible explanation seems available in terms of differences in the structure of the hydration lattice, particularly when one notices that the extent of interaction between binding sites for oxygen parallels the magnitude of the Bohr effect, being large for lobster and crab and small for *Limulus*. Along the same lines it is further of interest to note that interactions between sites in hemerythrin (*9*), the other class of nonheme oxygen-carrying pigment, are small (Fig. 6) and that, furthermore, this protein gives no evidence of a Bohr effect (*48*).

Thus it seems feasible to use a more unified interpretation of many aspects of the behavior of all classes of oxygen-carrying pigment in terms of the character of the "frozen" water in these proteins.

## Conclusion

We have thus reexamined a variety of unique features of protein behavior and have seen that a thread of likeness can be drawn through them when the hydration sheath is viewed as an icelike lattice. These correlations in interpretation of behavior serve to emphasize the important role played by the solvent in fixing the structure and properties of the solute molecule, as well as the influence of the solute in imposing a structure on the solvent. It is this mutual interaction which has perhaps not been fully appreciated in the interpretation of the behavior of proteins in solution. The topics reviewed in this light in this article were representative but hardly exhaustive. They will undoubtedly suggest other phenomena which might be profitably reinterpreted in terms of the concepts discussed here (*49*).

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# The Conservation of Intellectual Talent

## The reasons why some able students do not attend college and the uses of scholarships are assessed.

Donald L. Thistlethwaite

Estimates of the number of highly talented students who fail to go to college are alarming. Even if we take the most conservative of the available estimates (*1*), about 34,000, or 28 percent, of the highest-ranking tenth of the nation's high school graduates do not enter college as full-time students. For students in the highest 30 percent in respect to ability, the estimated number not going to college increases fourfold. In 1955 one study (*2*) reported that some 150,000 students in the top 30 percent of ability failed to go to college, but would have gone if scholarships had been available.

These figures suggest the need for more financial aid. Certainly existing funds are small compared with available estimates of the number of drop-outs among students of high ability, and who could be induced to go to college by financial aid. Fortunately, a national scholarship competition has features which tend to reduce the number of students who drop out after high school (*3*). Public recognition of the excellent performance of high-scoring students seems to motivate both the students and scholarship donors. Holland and Stalnaker (*4*) report that 98.7 percent of a group of near winners in the first National Merit Scholarship program went to college, and that approximately 65 percent of these students obtained scholarships from other sources. Students who rank high in any nationwide testing program become highly visible; as a consequence, they are stimulated to go to college and they frequently attract offers of scholarships from colleges and other sources. Thus loss is reduced.

Previous appraisals of talent loss were made before the initiation of the large-scale Merit Scholarship program. Under present conditions, the number of drop-outs among students of high ability is only half as large as the lowest figure estimated in earlier studies. The second annual Merit Scholarship program included a greatly enlarged sample of very able students. Over 166,500 high-school seniors in some 12,500 secondary schools took the initial qualifying test, and the top-scoring 15,000 students from this group were surveyed in respect to their college-going behavior. This population provides the best available means for appraising the current talent loss and for identifying the social and psychological factors which contribute to it (*5*).

In the present study, the amount of talent loss which occurs under existing conditions was estimated from the number of near winners in the 1957 program who failed to enroll in college. The study also sought to estimate the number of drop-outs who might attend college if additional scholarships were available.

Students ranking in the top 5 or 10 percent of the nation's youth cannot be expected to make full use of their talents without further specialized training. The loss of these students to higher education is an undeniable waste of the nation's intellectual talent. To throw light

The author is associate director of research, National Merit Scholarship Corporation, Evanston, Ill.