SOME FACTORS IN THE INTERPRETATION OF PROTEIN DENATURATION[1]

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I. Introduction

The changes that take place in protein molecules during denaturation constitute one of the most interesting and complex classes of reactions that can be found either in nature or in the laboratory. These reactions are important because of the information they can provide about the more intimate details of protein structure and function. They are also significant because they challenge the chemist with a difficult area for the application of chemical principles.

The chemist, wishing to apply the concepts and methods with which he is familiar to the study of denaturation, is faced with many problems. The first and most obvious one is that of being able to recognize the phenomenon when he sees it. Many writers on the subject have wrestled with this problem—which ultimately requires finding an acceptable definition of the word, denaturation. At the present time there is no general agreement on a precise meaning of this word, and the feeling of vagueness that this has generated leads some protein chemists to avoid using the term alto-

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gether. Nevertheless, the phenomena that have in the past been given the name denaturation do exist and demand our attention. It therefore seems desirable to have a word which identifies them; either the word denaturation will have to continue in use or else a new word will have to be agreed upon. (The new word will, of course, also have to be defined.) The writer prefers to continue to use the old word, fully realizing the difficulty that "denaturation" has meant different things to different people and that there is much to be said for coining a new term, such as "transconformation reactions" (Lumry and Eyring, 1954).

There is little doubt that when one is confronted with specific examples of what protein chemists would agree to call denaturation, one almost invariably finds on close inspection that there have been changes in the way the polypeptide chains are arranged within the protein molecule. One can be reasonably sure, therefore, that one is talking about the same phenomenon as is the protein chemist if denaturation is used to denote *a process (or sequence of processes) in which the spatial arrangement of the polypeptide chains within the molecule is changed from that typical of the native protein to a more disordered arrangement.* (The terms *configuration*, *conformation*, and *state of folding* can be substituted for *spatial arrangement* in this definition).

The chief objection to a definition of this type is that it is not operational, since it says nothing about the experimental criteria to be used in detecting changes in chain configuration or in deciding whether these changes increase the degree of disorder in the protein molecule. The objection is most pertinent, and Section II of this article has been written in order to deal with it.

The definition given here is similar to those that have been given in the extensive reviews of denaturation by Neurath *et al.* (1944) and by Putnam (1953), among others, except that it permits the term to be used in conjunction with the changes in configuration that may accompany the rupture of primary chemical bonds in the protein. For instance, the reduction and oxidation of disulfide cross-linkages in various proteins are known to be accompanied by considerable changes in the configuration of the polypeptide chain. Similar changes accompany the hydrolysis of certain peptide linkages in some proteins. The writer (see also Tanford, 1958) feels that it is unreasonable not to consider these changes in chain configuration as examples of denaturation merely because they accompany the rupture of primary linkages. (Of course, if a large fraction of peptide links in a protain is broken it becomes meaningless to speak of a polypeptide chain, so one would not refer to an extensively hydrolyzed protein as denatured.)

Anson (1953) suggests that we do not yet know enough about denaturation to be able to define it rigorously, though he gives a molecular interpretation of the phenomenon which is very close to what is contained in the

definition set forth above. Anson then goes on to make an important point, which is "presented more for discussion and as a notion for holding my review together than as something proven." This point is "that the change from the native to the denatured state involves usually the breaking of a considerable number of the looser protein linkages and that these linkages are usually broken in an 'all or none' way. By the expression 'all or none' I do not mean to imply that the linkages are not broken one at a time, but rather that the intermediate forms are relatively unstable." Dr. Anson's reason for hesitating to offer a definition is therefore his belief that the denatured state of proteins will some day be found to be more clearly definable than it is now, and that an essential characteristic of denaturation will be omitted if a definition of the phenomenon fails to account for the absence of states intermediate between the native state and the denatured protein. The writer does not share this hesitation because he feels that, according to all that we know now about protein structure, we have good reason to believe that disorder might be introduced into a protein in small increments. Whether or not disorder actually is introduced in small increments in proteins is an important point which is unfortunately very difficult to establish experimentally. It therefore seems more profitable to focus attention on the *process* of denaturation rather than on an attempt to characterize a hypothetical and possibly undefinable denatured *state* of a protein. The problem here may be compared with that which faces us in describing the changes in the acidic and basic groups of a protein when the pH of the medium is varied. Here we habitually speak of the *process* of ionization whereas the realization that proteins are amphoteric ions has made it meaningless to attempt to distinguish between the un-ionized and the ionized *states* of proteins.

The objection that the proposed definition (and others similar to it) oversimplifies the problem thus meets with this reply: first, that the definition emphasizes what protein chemists have agreed is the essential characteristic of the *process*, and second that the existence of a clearly defined denatured *state* is a separate issue, not involved in the definition of the *process*.

Some workers prefer to take a purely operational point of view and therefore define denaturation in terms of a change in a particular experimental property, such as the solubility in water at the isoelectric point, or the loss of some typical biochemical characteristic possessed by the protein (enzymatic activity, ability to combine with antibodies, etc.). It is easy to understand a preference for this approach to the problem, but it faces a number of serious disadvantages. The first of these disadvantages is that definitions in terms of solubility or activity draw attention away from the most significant aspect of the phenomenon, namely its intimate relation-

ship to protein structure. Even more serious, however, is the fact that strict adherence to such a definition can in some cases lead one to overlook important structural changes in the protein. As was pointed out by Anson and Mirsky (1933) this is especially true where the experimental criterion selected for denaturation cannot be applied in the presence of the denaturing agent. For instance, serum albumin is unfolded in salt-free acid and in strong urea solutions, but when the acid or urea is removed the protein returns to a form that is highly soluble at the isoelectric point. If denaturation were studied merely in terms of solubility at the isoelectric point, the changes undergone by serum albumin in urea and in acid would be overlooked.

Let us suppose, then, that the chemist has been furnished with reasonably definite specifications for the phenomenon of denaturation. The next question that naturally arises is how he is going to investigate the phenomenon. In particular, he will have to decide which properties of the protein he will use in order to study the configuration changes that take place. This question will be considered in Section II, where the properties of proteins that are likely to be useful in such a study are listed. The relationship of the properties to protein structure is discussed briefly, and the limitations and advantages inherent in the use of each of them are considered.

When the chemist begins to interpret the results of his measurements he finds it necessary to consider numerous further questions. He will find himself in a position to give positive answers to some of these questions. For the answers to other questions he will have to look to studies other than denaturation. Some of these other questions can as yet be given only indefinite answers. For instance, one would like to know the types of structures actually present in native and denatured proteins. It is believed that the α-helix of Pauling *et al.* (1951) may be present in globular proteins, but not much evidence is yet available and there are undoubtedly large regions of the molecule in which other, perhaps less regular structures are developed. The denatured protein in a good solvent such as urea is probably somewhat like a randomly coiled polymer, though the large optical rotation of denatured proteins in urea indicates that much local rigidity must be present in the chain. The structure of the denatured protein in poor solvents, where the intrinsic viscosity is much lower than in urea, must be far from a completely random coil, but next to nothing is known about it. These structural questions will not be considered in this article because what is known about protein structure has been fully and frequently reviewed elsewhere, and because so little can be said as yet, even in speculative respects, about the additional structural elements that might be present in native and denatured globular proteins.

Another important question that arises in trying to interpret experimental observations on protein denaturation concerns the nature and characteristics of the intramolecular bonds which stabilize the native form of the protein. It is, of course, the rupture of these bonds that leads to the disordering of the native protein structure, which has been specified as the characteristic feature of denaturation. Section III will consider this question. The special aim of this section is to examine the thermodynamic properties of the intramolecular bonds that might be expected to exist in proteins. In order to obtain such thermodynamic information it is necessary to turn to simple model systems which would be expected to illustrate the various possible types of bonds and permit us to estimate their properties. Although relatively little will be said about proteins in this section, the pertinence to proteins should be self-evident.

It is convenient in the next two sections to refer at times to a classification of protein structures that has been proposed by Linderstrøm-Lang (1952). Lang speaks of primary, secondary and tertiary structures within a protein molecule. The primary structure is that expressed by the structural chemical formula and depends entirely on the chemical valence bonds that the classical organic chemist would write down for the protein molecule. (The sequence of the amino acids along the polypeptide chain, and the locations of disulfide bonds in relation to this sequence, relate to the primary structure.) The secondary structure is the configuration of the polypeptide chain that results from the satisfaction of the hydrogen bonding potential between the peptide N—H and C=O groups. (The α-helix and β-sheet structures of Pauling *et al.* (1951) are illustrations of possible secondary structures for polypeptides.) The tertiary structure is the pattern according to which the secondary structures are packed together within the native protein molecule. The term denaturation as used in this article is intended to include changes in both the secondary and tertiary structures.

As applied to globular proteins, the distinction between the secondary and tertiary structures must be regarded as tentative because it is still by no means certain that the structural patterns resulting from hydrogen bonding between peptide links are inherently stable and can exist apart from the stabilization brought about by other types of intramolecular bonds.

II. Experimental Methods Available for Observing Changes in the Configurations of Polypeptide Chains in Proteins

It is possible to make a useful and relatively clear-cut distinction between two classes of properties which depend in very different ways on the polypeptide chain configuration of a protein. One class of property (typified by the solution viscosity and the friction ratio) depends on the over-all shape or envelope of the molecule without being directly affected by the

exact spatial relationships between any particular atom in the molecule and its immediate neighbors. The other class of property (typified by the optical rotation and the infrared and ultraviolet spectra) is influenced by changes in the immediate vicinity of individual groups in the molecule, whether such changes alter the general external shape of the molecule or not. We shall call the two respective classes of properties "shape properties" and "short range properties." Most of the properties that have been used in studying protein denaturation are classified into these two categories in Tables I and II. The nature of the expected relationships between the individual properties and the state of folding of the polypep-

TABLE I

Shape Properties of Proteins

Hydrodynamic properties
A. Friction ratio (from sedimentation-diffusion)
B. Viscosity increment
C. Rotatory diffusion constant (flow birefringence, dielectric relaxation, fluorescence depolarization)
Radiation scattering (angular dependence)
A. Light scattering
B. Small angle X-ray scattering
Long range electrostatic effects on titration curves (Linderstrøm-Lang's "w")
Electron microscopy
Second virial coefficient at moderate salt concentrations
Surface properties
A. Force-area curves at moderate pressures
B. Surface dipole moment (?)
C. Area of solid film
Dipole moment and/or Kirkwood-Shumaker effect
Diffusion through membranes with controlled pore sizes (Craig)

tide chain is outlined below, but first certain more general remarks will be made about the two classes of properties.

(1) Both classes of properties must be studied in order to obtain a complete understanding of the phenomenon, but short range properties can potentially give a much more detailed picture of what happens during denaturation. We must therefore expect that the most important contributions to our understanding of denaturation will ultimately come from the study of short range properties.

(2) In order to obtain structural information from an observed property of a protein it is, of course, necessary to know something about the general relationships between the property and molecular structure. There are two ways of discovering these relationships: (a) by deducing them from theoretical principles, and (b) by studying the behavior of the property in

model compounds of known structure, i.e., by establishing empirical correlations. At the present time the basic theory of shape properties is much better understood than is the basic theory of short range properties. (Reliable theoretical predictions can be made for the light scattering and diffusion constant of an elliptical molecule dissolved in water, but at most only qualitative and not very confident theoretical predictions can be made

TABLE II

Short Range Properties of Proteins

Thermodynamic properties
A. Energy and heat capacity
B. Entropy
C. (Free energy)
D. Volume, compressibility, and coefficient of expansion
E. Solubility, activity, distribution between solvents
Optical properties
A. Optical rotation and dispersion
B. Infrared absorption
C. Visible and ultraviolet absorption
D. Wide angle X-ray diffraction
E. Index of refraction (polarizability, anisotropy)
F. Depolarization of fluorescence (in some cases)
Chemical properties
A. Reactivity of groups
B. Intrinsic pK's of acidic and basic groups
C. H-D or H-T exchange
D. Binding of small molecules, dyes, ions, etc.
E. Immunochemical properties
F. Digestibility by proteolytic enzymes
G. Biological activity
H. Electrophoresis (isoelectric point, zeta potential)
Nuclear and electronic magnetic resonance
Surface phenomena
A. Spreadability
B. Surface viscosity
C. (Surface dipole moment)

about the effect of hydrogen bonding on the absorption spectrum of tyrosine or the contribution to the optical rotation arising from the interaction of a pair of amino acid residues.) Consequently the molecular interpretation of a short range property depends almost entirely on the study of model systems, and the usefulness of short range properties to protein chemists depends on the availability of suitable model compounds. The importance of synthetic polypeptides arises from just this consideration.

(3) It is important to utilize properties that are sensitive to different kinds of structural changes in the protein. For instance it has been sug-

gested that the optical rotation is particularly sensitive to changes in the amount of α-helix present in a protein (Yang and Doty, 1957) and not so sensitive to the tertiary structure. The partial specific volume, on the other hand, ought to be sensitive to changes in both the secondary and tertiary structures. The infrared absorption by the peptide N—H and C=O groups should depend on the secondary structure. The ultraviolet absorption spectrum in the vicinity of 280 mμ will reflect local changes in the vicinity of chromophoric groups such as tryptophan. A really detailed understanding of denaturation can come only when many such properties have been studied concurrently on a great many proteins.

(4) Most of the properties that are observed for proteins in the solid state are short range properties. (The principal exceptions are the crystalline form and the X-ray diffraction pattern of crystalline proteins, which depend in part on the external molecular shape.) Measurements on protein crystals and fibers have the advantage that the molecules may be oriented in space, so that the variations of certain properties in different directions within the molecules can be studied. In general, however, it is easier to make measurements when the molecules are dissolved, and more different types of measurements are possible.

(5) Proteins are generally rather highly hydrated. One can distinguish two types of water of hydration. One type merely fills voids between the chains in the molecule (for instance, the water which penetrates the serum albumin molecule when it is swollen by bringing the pH below 4 in the absence of salt). The other type of water of hydration is relatively tightly bound, for instance, to the polar groups in the molecule. As a rule the first type of hydration is best detected by studying shape properties, whereas the short range properties are affected only by the second type of hydration.

(6) Aggregation generally has a much greater effect on shape properties than on short range properties.

A. Shape Properties of Proteins

1. Hydrodynamic Properties

Hydrodynamic properties have to do with the factors that influence the motion of the protein molecule through a solvent—translational motion in the case of sedimentation and diffusion, rotational motion in the cause of rotatory diffusion, and more complex motions in the case of the modification of viscous flow by suspended particles. When proteins are denatured there are marked changes in these properties. In most cases when there is no change in molecular weight the diffusion constant is decreased, and the friction ratio, viscosity increment and rotational diffusion time are in-

creased. These changes are consistent with a change from a compact, dense structure to a more elongated or a more open structure. In either case it is necessary to infer that there has been a considerable change in the arrangement of the polypeptide chains within the protein molecule in order to account for the observations.

The hydrodynamic properties of macromolecules have been interpreted in terms of two different types of models—the rigid, solvated ellipsoid and the random coil. The rigid ellipsoid is probably the more suitable for native proteins and for denatured proteins when they are dissolved in poor solvents (which tend to make the polypeptide chain coil up compactly, though less regularly than in the native protein). The random coil model is probably better suited for denatured proteins dissolved in urea and guanidinium salts.

Scheraga and Mandelkern (1953) have presented a procedure for applying the rigid ellipsoid model by combining data from the friction constant, viscosity and rotatory diffusion constant. They are able to derive an axial ratio and an effective volume for a hypothetical equivalent ellipsoid which would have the same hydrodynamic properties as the protein, provided that the shape of the protein molecule is itself not too far from a relatively impenetrable ellipsoid. If the shape is not ellipsoidal, consistent dimensions cannot be obtained for the equivalent ellipsoid from the three hydrodynamic properties. If the molecule is not compact, and the solvent can flow through it, the equivalent ellipsoid may have rather different dimensions and solvation from those of the actual molecule.

No detailed systematic application of the random coil model has yet been made to the hydrodynamic properties of denatured proteins, although the model may be a reasonably good representation of such systems as ovalbumin and serum albumin in concentrated urea (Kauzmann, 1954).

Recent detailed discussions of hydrodynamic properties from the rigid ellipsoid point of view have been given by Edsall (1953), Sadron (1953), Gosting (1956), Weber (1953), and Frisch and Simha (1956). The random coil model is discussed by Flory (1953) and by Riseman and Kirkwood (1956).

An important recent development is the method of transient electrical birefringence to obtain rotational diffusion constants (Benoit, 1951 a,b; O'Konski and Haltner, 1957).

2. Angular Dependence of Radiation Scattering

The variation in different directions of the intensity of the radiation scattered by a molecule depends on the spatial distribution of the atoms inside the molecule. In going from the native form of a protein to the denatured form this distribution becomes less compact for most proteins and the

scattering pattern is changed. [For a discussion of the theory, see Edsall (1953).]

If λ is the wave length of the radiation and θ is the angle between the direction of the incident radiation and the direction of the scattered radiation, then the intensity of the scattered radiation at angle θ will be appreciably less than the intensity at smaller angles if the largest dimension of the particle is of the order of magnitude $\lambda/\sin\theta$. As the ratio of the particle dimensions to the wave length increases the scattering pattern becomes more complex and more and more information can be deduced from it concerning the arrangement of scattering matter within the molecule. This means that if the particle is too small compared with the wave length, the angular dependence of the scattering intensity may be too slight to be observed. It is found in practice that a readily observable variation of the scattering intensity with direction is obtained when the longest dimension of the particle is greater than about one-tenth of the wave length of the radiation. With visible and ultraviolet light the method is therefore not very useful for studying shape if the proteins have low molecular weight (under about 100,000) and at best only a rather general impression of the shape of the molecule can be ascertained. In principle, however, one ought to be able to obtain a great deal of information about the arrangement of atoms inside the molecule by studying the small angle scattering of X-rays, whose wave length is approximately 1000 times shorter than that of ultraviolet light. The technical difficulties involved in small angle X-ray scattering studies are rather formidable, and the method has not been used systematically in the study of protein denaturation. It has, however, been applied to native proteins both in the solid state and in solution (see, for example, Arndt and Riley, 1955; Kratky, 1956 a,b,c; Tomlin and Worthington, 1956).

3. Long Range Electrostatic Effects on Titration Curves

If a protein contains titratable groups such as NH_2 and COOH, the fraction α of the groups of a given type that are uncombined with a proton at a given pH is given to a first approximation by

$$\log \frac{1-\alpha}{\alpha} = pK_0 - pH - 0.868wZ \qquad (1)$$

where pK_0 is the intrinsic pK for the dissociation of the group in the absence of other electrically charged groups in its vicinity, Z is the net electrostatic charge on the molecule (expressed in units of the charge on a proton) and w is a constant typical of the protein, and depending on the dimensions and shape of the molecule, as well as on the ionic strength of the solvent. The term $0.868wZ$ [which is due to Linderstrøm-Lang, (1924)] takes ac-

count of the electrostatic interactions between the charges already on the molecule and the proton that is added to or removed from the average dissociating group. The factor w is large if the molecule is compact and spherical and it is small if it is swollen or elongated. It is found, as expected, that w decreases considerably when a native protein unfolds (Tanford and Roberts, 1952; Tanford *et al.*, 1955).

The relationship between molecular shape and the titration curve is discussed by Tanford (1955b, 1958) and by Steinhardt and Zaiser (1955), where further references will be found. Calculations of w for various shapes are given by Hill (1955, 1956a,c), and by Tanford and Kirkwood (1957) and Tanford (1955a, 1957b,c). It should be noted that it is difficult experimentally to distinguish between the effects of a configurational change in the molecule on w and the effects on pK_0 which will be discussed later.

4. Electron Microscopy

The thermal denaturation of tobacco mosaic virus has been followed using the electron microscope by Hart (1956), who observed that under conditions that did not lead to the formation of a precipitate the rodlike native molecules changed into spheres, the change taking place exclusively at the ends of the rods. Because of the difficulty in observing the shapes of molecules having low molecular weights, this approach has not yet been useful with the more common globular proteins. The method also suffers the disadvantage that the molecules can be observed only in the dry state.

5. Second Virial Coefficient

The dependence of the osmotic pressure, π, of a protein solution on the protein concentration, c, may be expressed as a power series in c,

$$\pi/RTc = (1/M) + Bc + Cc^2 + \cdots \tag{2}$$

where M is the molecular weight, R is the gas constant, T is the absolute temperature, and B and C are constants. If there are no intermolecular attractions (that is, in a good solvent), the constant B is directly related to the "excluded volume" of the molecule (see Edsall, 1953, and Flory, 1953). The excluded volume depends quite strongly on the shape of the molecule as well as on its true volume, being much larger for ellipsoids and random coils than for spheres. Thus it would be expected that B would undergo a considerable change on denaturation. The relationship between shape and the magnitude of B is discussed by Zimm (1946), Schulz (1947) and Onsager (1949) for rigid molecules, and by Flory and Krigbaum (1951) for flexible chains.

The constant B can also be determined from a study of the concentration dependence of light scattering.

6. Surface Properties

The force-area curves for mono-layers at moderate surface pressures deviate from the ideal gas type of law because of interatomic attractions and repulsions. In principle these deviations should be interpretable in terms of the same concepts of excluded volume and solvent-solute affinity as those that are employed in understanding the second virial coefficient for the osmotic pressure. The effects of intermolecular attractions on surfaces may, however, be more difficult to avoid because the protein may not spread if the solvent below the interface is a good one. Furthermore, the surface forces exert a powerful denaturation action on proteins, so it is difficult to compare the excluded surface area for the native and denatured forms.

Numerous attempts have been made to interpret the behavior of randomly coiled polymer molecules on surfaces from the same point of view as that employed rather successfully for polymers in solution. [See Cheesman and Davies (1954) and Frisch and Simha (1957), and earlier papers referred to therein.] It is difficult to evaluate the success of these attempts at the present time.

Hamaguchi and co-workers (Hamaguchi, 1955, 1956; Isemara *et al.*, 1955) have reported that the addition of urea to the substrate liquid increases the area occupied by each molecule for lysozyme and synthetic polypeptides, indicative of an unfolding action by urea. Further information about the shapes of protein molecules can be obtained from the solid film area, from which a molecular thickness can be deduced. This thickness generally turns out to be considerably less than the smallest dimension of the native molecule—which indicates that unfolding or some other type of disintegration must have occurred on the surface. Bull (1956) has found that the thickness obtained for bovine serum albumin adsorbed from water onto glass is consistent with the adsorption of native molecules provided the pH is greater than 4. Ovalbumin adsorbed onto glass also gives a more compact film than that observed at an air-water interface (Bull, 1957).

7. Dipole Moment

The dipole moment of a protein depends on a distribution of charged groups throughout the molecule and on the dimensions of the molecule, so it is best classified as a shape property. An important contribution to the apparent dipole moment is probably made by fluctuations in the charge distribution under the influence of the electric field applied to measure the dipole moment (Kirkwood and Shumaker, 1952). The polarization of the ionic atmosphere surrounding the molecule also probably makes a contribution that might be confused with a permanent dipole moment (O'Konski and Haltner, 1957; Dintzis *et al.*, 1954). Because the relative magnitudes

of these effects are difficult to evaluate, the structural significance of the apparent dipole moment deduced from the dielectric increment is not at this time clear, and this property is not able to tell us much about the molecular structure.

Jacobson (1955) has suggested that the large observed dielectric increments of proteins, deoxyribonucleic acid (DNA) and other macromolecules are largely determined by the dielectric properties of a relatively thick blanket of frozen water surrounding the molecule. This layer is supposed to have a considerably higher dielectric constant than ordinary water. Efforts to detect this layer of water in dilute DNA solutions by X-ray diffraction have, however, failed (Rich, 1958).

8. Rate of Diffusion through Membranes

The rate at which proteins diffuse through membranes having controlled pore sizes should be a function of the size and shape of the protein molecule. (If the membrane is charged, then the charge on the protein may also have an important effect on the rate of diffusion through it.) This method is under study by Craig and King (1955). It is found that the denaturation of insulin and ribonuclease in 6 *M* urea greatly decreases the rate of passage through membranes (Craig *et al.*, 1957).

B. Short Range Properties of Proteins

1. Thermodynamic Properties: The Enthalpy (Heat Content)

The change in enthalpy on denaturation may be measured directly in a calorimeter (see Sturtevant, 1954, for a review). If the reaction

$$\text{native protein} \rightleftharpoons \text{denatured protein}$$

is reversible and an equilibrium constant can be measured at more than one temperature, the enthalpy difference between the two forms, ΔH, may be determined from the temperature coefficient of the equilibrium constant by well known thermodynamic procedures. Most measurements that have been made so far by either method indicate that the denatured protein has a higher enthalpy than the native protein, typically by as much as 100 kcal per mole or more. [An exception is the reversible denaturation of methemoglobin by salicylate, where ΔH is close to zero (Anson and Mirsky, 1934).] The values of ΔH for denaturation show large changes with pH, which have not yet been explained. The kinetics of pepsin denaturation as followed in the calorimeter appear to be distinctly different from the kinetics obtained by following the loss of enzymatic activity (Buzzell and Sturtevant, 1951, 1952).

It is most unfortunate that for technical reasons the calorimetric measurement of ΔH during denaturation is rather difficult. The energy is one

of the most important and best understood molecular properties and ΔH is directly related to the stability of the native protein, so that much insight into detailed structural questions could probably be obtained if the enthalpy changes could be measured at different stages of the unfolding process. The interpretation of the structural significance of ΔH for denaturation in water is, however, subject to some rather serious uncertainties. As will be discussed in a later section, the contribution to ΔH due to the rupture of hydrophobic bonds in water may very well be negative, whereas the contribution due to the rupture of hydrogen bonds is positive. It is therefore not meaningful to estimate the number of bonds broken during denaturation by dividing the observed ΔH by an estimated bond strength for one type of bond, as is frequently done.

The temperature dependence of the enthalpy, $C_p = (\partial H/\partial T)_p$ (i.e., the specific heat) has not been extensively studied for native and denatured proteins and would undoubtedly show interesting differences, though it is not easy to measure. Buzzell and Sturtevant (1952) found that ΔH for pepsin denaturation near pH 7.0 may be strongly dependent on the temperature, indicating a possible large difference in the specific heats for the native and denatured forms.

2. Thermodynamic Properties: The Entropy

Values of the entropy change, ΔS, accompanying denaturation have been obtained from the study of equilibrium constants for reversible denaturations at several temperatures, using the relationship

$$\Delta S/R = \ln K + (\Delta H/RT) \tag{3}$$

Available values are tabulated by Lumry and Eyring (1954) and by Kauzmann (1954). The denatured protein invariably has a larger entropy than the native protein by several hundred entropy units. This is presumably a reflection of the greater randomness in the configuration of the polypeptide chain in the denatured protein.

In principle it should be possible to determine ΔS for irreversible denaturation by calorimetric measurements utilizing the third law of thermodynamics. It is doubtful, however, if this will ever prove feasible because of the difficulty of getting the denatured protein into a state of sufficiently low disorder at 0°K in a calorimeter.

Schwert (1956) reports that in spite of a large increase in entropy in the reversible thermal denaturation of chymotrypsinogen, there is no appreciable change in either the sedimentation constant or in the optical rotation (see also J. A. Schellman, 1958f; Rasper, 1957; and Stauff and Rasper, 1958). This probably means that in this protein reversible heat denaturation does not result in any change in the secondary structure, and it is

doubtful if the observed entropy change can arise from an increased randomness in coiling, as has been suggested by Kauzmann (1954). Furthermore, as will be discussed in a later section, the rupture of hydrophobic bonds brings about a large decrease in entropy; unless the magnitude of this contribution is known there is little point in interpreting the entire entropy change on denaturation in terms of the number of configurations available to the polypeptide chain. Changes in the state of ionization and solvation of acidic and basic groups may also make large contributions to the entropy change.

3. Thermodynamic Properties: The Volume

The change in volume accompanying denaturation can be followed most readily by allowing the process to occur in a dilatometer (Linderstrøm-Lang, 1940; Linderstrøm-Lang and Lanz, 1938). Charlwood (1957) has compared the densities of solutions of native and denatured proteins using the very sensitive magnetic float method. This method is not so convenient as the dilatometer method, but it can give absolute values of the volume, and not merely changes. Rosenberg and Klotz (1955) studied volume changes in serum albumin when it binds organic ions using a pycnometer to measure solution densities. Although it has not yet been applied to the study of proteins, the extremely sensitive method of Frivold (1920) should be mentioned. Using this method Masterton (1954) has been able to detect density differences of one part in ten million. The volume change can also be determined from the effect of pressure on the equilibrium constant for reversible denaturation, using the relation

$$\Delta V = -RT(d \ln K/dp)$$

This approach has been used especially by F. H. Johnson and co-workers (review in Chapter 9 of F. H. Johnson *et al.*, 1954).

The partial molar volume of a protein in solution depends on several factors: (1) The constitutive volume, as calculated by the procedure of Traube, or its equivalent, from tables of atomic or groups volumes; (2) the presence of voids in the molecule because of imperfect atomic packing; (3) the presence of charged groups which are strongly solvated by the solvent water—this results in a very considerable contraction (for instance, the reaction R—COOH + $H_2O \rightarrow$ R—COO^- + H_3O^+ is accompanied by a volume decrease of 11 ml per mole of carboxyl groups, which is a considerable fraction of the constitutive volume of the groups concerned in the reaction (see Section III,C); (4) structural changes in the water in the vicinity of short hydrocarbon chains and other non-polar groups (see Section III,B). It is interesting that the effects of (2), (3), and (4) for native proteins in water apparently tend to cancel almost completely, because

the partial specific volume can be calculated quite accurately from the constitution, not taking account of the electrostriction or other effects (see Chapter 16 of Cohn and Edsall, 1943; McMeekin and Marshall, 1952; and p. 565 of Edsall, 1953).

There is evidence from studies of the volume changes of several proteins that denaturation by acid, by urea, or by enzymatic attack preliminary to proteolysis results in a volume decrease amounting to several hundred milliliters per 100,000 grams of protein (Linderstrøm-Lang and Jacobsen, 1941; Christensen, 1952; Simpson and Kauzmann, 1953; Charlwood, 1957; and Kauzmann, 1958). This presumably results from the disappearance of voids and the rupture of hydrophobic and ion pair bonds in the native protein. Johnson and co-workers have, however, been able to interpret the effects of pressure at higher temperature for various biological systems by assuming that the reversible thermal inactivation of enzymes results in an *increase* in volume [see p. 307 ff. of F. H. Johnson *et al.*, (1954) for a review]. It is possible that ΔV for denaturation changes its sign at higher temperatures.

There is a contraction of 5–8% of the protein volume when anhydrous proteins are dissolved in water (Chick and Martin, 1913; Hipp *et al.*, 1952). According to McMeekin *et al.* (1954), who studied the volume changes that occur on the hydration of β-lactoglobulin crystals, only about half of this contraction is a consequence of the electrostriction of the water by the charged groups of the protein. Cohen (1935) had also concluded that electrostriction cannot account for all of the observed contraction when water is added to other anhydrous proteins. The remainder of the volume change is presumably due to the filling of some of the voids in the anhydrous protein by water, and by improved space-filling by the protein when water is present.

Waugh (1954) has presented some interesting speculations relating the volume occupied by a protein to its structure and composition.

The temperature coefficient of the volume (coefficient of thermal expansion) and the pressure coefficient of volume (compressibility) have not been studied for native and denatured proteins in water, but might one day reveal interesting information. If it is indeed true that at high temperatures there is a volume increase on denaturation, whereas at low temperatures there is a volume decrease, then the denatured protein must have a much larger coefficient of thermal expansion than the native protein.

4. Thermodynamic Properties: Solubility and the Distribution of Protein between Phases

The solubility depends on the relative affinity of the protein molecule toward the solvent and toward itself. The distribution coefficient between

a pair of solvents depends on the relative affinities toward the two solvents. Denaturation invariably results in a marked decrease in solubility in aqueous solvents, and the loss of solubility at the isoelectric point is one of the classical criteria of denaturation. Presumably denaturation results in the rupture of intramolecular bonds, so that the denatured protein contains many unsatisfied potential bonding points. Therefore, if denatured molecules come into contact, some or all of the ruptured bonds reform intermolecularly. If the denatured molecules are not greatly swollen (as is the case at the isoelectric point and in the presence of salts) a precipitate is formed. If the molecule is swollen (as in the presence of a good solvent such as strong aqueous urea, or at pH's far from the isoelectric point) a gel may be formed.

If a protein can denature reversibly and if the rate of reforming the native molecule from the denatured one is rapid, it may be rather difficult to interpret solubility changes in terms of configuration changes. For instance, bovine serum albumin changes its optical rotation immediately on exposure either to acid or to urea, and on neutralization of the acid or dilution of the urea the rotation returns immediately to the value characteristic of the native protein. This indicates that there is a rapid reversible configuration change in this molecule. The rapid addition of an excess of isoelectric sodium sulfate to bovine serum albumin in urea will result in a partial precipitation. Dilution of the urea before adding the sodium sulfate, or slow addition of the sodium sulfate, causes no precipitate to form (Kauzmann and Douglas, 1956). The formation of a precipitate depends on the relative rates of formation of intermolecular and intramolecular bonds, and evidently sodium sulfate inhibits the formation of those intramolecular bonds required for the return to the native state. Similarly, heating bovine serum albumin in acid at 60–70°C causes an immediate and reversible, though small, increase in levorotation (J. A. Schellman, 1958d). Levy and Warner (1954) have heated bovine serum albumin at low pH and observed a slow production of protein insoluble in isoelectric ammonium sulfate solutions at room temperatures. The kinetics of this change were carefully investigated. The process cannot, however, be the same as the unfolding that is responsible for the instantaneous change in rotation observed by J. A. Schellman. In systems of this kind it is clear that unless solubility changes are studied along with other properties that are more closely related to protein structure and which can be measured in the actual presence of the denaturing condition, it will be very difficult to gain a meaningful interpretation in molecular terms of what is taking place.

Solubility is also strongly affected by chemical cross-linking reactions such as those involved in the interchange reaction between thiols and disulfides (C. Huggins *et al.*, 1951; Hospelkorn *et al.*, 1954; Halwer, 1954;

Frensdorff *et al.*, 1953a,b; Kauzmann and Douglas, 1956). These reactions are secondary changes which depend only indirectly on the state of folding of the protein.

The interpretation of distribution coefficients for proteins between different solvents may also be rather complex if rapid, reversible unfolding takes place. If suitable auxiliary studies of the state of folding are made, however (e.g., by measuring the optical rotation, intrinsic viscosity or friction factor in the solvents employed), important quantitative information and insight into the intramolecular forces acting in proteins ought to be obtainable.

An interesting survey of the solubility of 23 proteins in 37 non-aqueous solvents has been made by Rees and Singer (1956), who found that most proteins are soluble in an unfolded state in hydrazine and ethylene diamine. The solubility behavior suggested an unexpected similarity between zein and insulin. Yang and Doty (1957) found that proteins could be dissolved in the unfolded state in dichloroacetic acid and that when ethylene dichloride was added the polypeptide chain refolded into a helical structure.

5. Optical Properties: Optical Rotation and Its Variation with Wave Length

With few exceptions, native proteins are levorotatory and on denaturation their levorotation increases. The amount of the change in rotation varies depending on the protein and on the denaturing conditions, but it is usually so large that this property is among the most convenient available for following the denaturation process.

The physical principles that underly the phenomenon of optical rotatory power are rather complex and cannot be described here [see J. A. Schellman (1958a) and Kauzmann (1957a) for elementary accounts]. It will be sufficient for our purposes to state that optical rotation arises through the interactions of electrons ("vicinal actions") in different groups in asymmetric molecules. The physical nature of these interactions is understood in a general way, and it is known that the contribution to the optical rotation by a given pair of interacting groups depends very much on their relative positions and orientations in space. Any structural change which alters the relative positions of the groups in an asymmetric molecule therefore produces a marked change in the optical rotation, even though the chemical nature of the molecule may not have been altered. It is unquestionably these changes in spatial relationships between different parts of the protein molecule that give rise to the observed change in optical rotation on denaturation. Optical rotatory power is thus unusually well suited to the detection of configuration changes in general and to the study of the denaturation reaction in particular.

Unfortunately it is not possible to be much more specific than this in

using theory alone to deduce the detailed nature of the structural changes in the protein from the observed changes in the optical rotation. From the fact that native proteins are all levorotatory we may conclude that there is probably a basic structure more or less common to all proteins, although there is a considerable variation in rotation (and, therefore, also probably in structure) from one protein to another. From the fact that denaturation invariably brings about an increased levorotation we may conclude that the changes that occur in all proteins on denaturation are similar.

Fortunately the inadequacies of our theoretical understanding of optical rotatory power are to a considerable degree circumvented by the availability of excellent model compounds (the synthetic polypeptides) whose optical rotatory characteristics are now being actively studied by Doty and co-workers. Consequently, it is beginning to be possible to say something about the nature of the common basic structure responsible for the levorotation of proteins and its increase on denaturation. A word of caution is, however, called for at this point. It has been found that when synthetic L-polypeptides in their helical form are transformed into the random coil form they become more levorotatory (Yang and Doty, 1957). This observation leads one to the tempting hypothesis that whenever a change in the environment makes a protein more levorotatory, there has been a disordering of the polypeptide chain, and whenever the protein becomes less levorotatory the fraction of the polypeptide chain that is folded in a helical structure must have increased. Without independent evidence for ordering and disordering, however, this hypothesis may lead to erroneous conclusions. The optical rotation is, after all, but a single number, and it might change for many reasons other than the ordering and disordering of the polypeptide chain. For instance, any molecule adsorbed onto a protein might become subject to vicinal actions from the protein which could change the observed optical rotation without any structural change whatsoever having taken place in the protein molecule. A further example of the possible dangers in this hypothesis might be found in the observation (Markus and Karush, 1957; Turner *et al.*, 1958) that the splitting of the cystine disulfide groups of serum albumin either by reduction or by oxidation results in a decrease in the levorotation of the protein. Markus and Karush have interpreted this to mean that the disulfide groups in serum albumin introduce constraints which reduce the amount of helical structure possible for the polypeptide chain. On the other hand, Turner and coworkers have noted that cystine and cystine-like compounds have unusually high levorotations, which are markedly reduced when the disulfide link is ruptured; they have shown that the order of magnitude of the decrease of the levorotation in serum albumin and other proteins is just what one would

expect on the basis of their cystine content if the rotation change were caused entirely by the change in the contribution of the cystine residues to the optical rotation. It is, of course, not possible to say which of these two interpretations is correct without some independent means of determining the amount of helical structure present in the protein before and after the disulfide groups are split; no such independent evidence appears to be available at the moment. A third example of the easily conceivable inadequacies of the hypothesis is illustrated by considering what one would find if a polypeptide chain in a protein could exist in several different ordered structures in addition to the helix. If an environmental change altered the relative amounts of these structures, one would expect the optical rotation to be affected. Such a change could hardly be referred to as denaturation if it happened to increase the levorotation, or as reversal of denaturation if it happened to decrease the levorotation. Until we know more about the relationship between protein structure and optical rotation, it is therefore not safe to interpret changes in rotation solely in terms of ordering and disordering the configuration of the polypeptide chain, without at least acknowledging the possibility of other interpretations.

Recently the theoretical considerations of Moffitt (1956) and the empirical observations of Yang and Doty (1957) and Linderstrøm-Lang and J. A. Schellman (1954) have drawn attention to an interesting relationship between denaturation and the variation of the optical rotation with the wave length of the light used in its measurement. The classical and quantum mechanical theories of the phenomenon both predict that the optical rotation at wave length λ should be given by

$$[\alpha]_\lambda = K\sum R_i\lambda_i^2/(\lambda^2 - \lambda_i^2) \tag{4}$$

where K is a positive universal constant, λ_i is the wave length of the ith absorption band, and R_i is a constant (known as the *rotatory strength* of the ith absorption band) whose numerical value is determined by the molecular structure. The sum in (4) is taken over all electronic absorption bands of the molecule. For proteins all bands that have large values of R_i (and hence make significant contributions to the optical rotation of visible light) lie in the ultraviolet below about 2300 A. (The absorption band of proteins at 2800 A makes a negligible contribution to the rotation at visible wave lengths.) It is found that, provided one does not use light having wave lengths too close to those of the active absorption bands of a compound, it is often possible to express the optical rotation empirically by means of the expression

$$[\alpha]_\lambda = A/(\lambda^2 - \lambda_0^2) \tag{5}$$

where A and λ_0 are empirical constants. When this is the case $[\alpha]_\lambda$ in-

creases monotonically with decreasing wave length as one approaches the absorption bands in the ultraviolet from the visible region. Such behavior is called *simple dispersion*, and equation (5) is called the *simple one term Drude dispersion equation*. J. A. Schellman (1958a) has shown that at long wave lengths equation (4) can be approximated by equation (5), with

$$\lambda_0^2 = \sum R_i \lambda_i^4 / \sum R_i \lambda_i^2 \tag{6}$$

Thus λ_0 is a weighted average of the λ_i's and does not necessarily correspond to any particular absorption band of the molecule, though it is related to the absorption properties. It sometimes happens that an electronic transition with a large rotatory strength, R_a, absorbs at a wave length, λ_a, which is considerably longer than the wave lengths of the remaining transition of the molecule. Under these conditions the terms $R_a\lambda_a^4$ and $R_a\lambda_a^2$ dominate the sums in the numerator and denominator of (6) and it turns out that

$$\lambda_0 \cong \lambda_a \tag{7}$$

This is the case with many aldehydes and ketones, where λ_0 usually corresponds to the carbonyl absorption band at 2900 A. It is not the case with proteins, however.

It is sometimes found that the observed dispersion cannot be expressed by a simple one term Drude equation of the form (4), but that the observations can be expressed by a Drude expression containing two terms,

$$[\alpha]_\lambda = \frac{K_1}{\lambda^2 - \lambda_1^2} + \frac{K_2}{\lambda^2 - \lambda_2^2} \tag{8}$$

If K_1 and K_2 happen to have opposite signs and if $|K_1| > |K_2|$ and $\lambda_1 < \lambda_2$, then $[\alpha]_\lambda$ will have the same sign as K_1 at long wave lengths, but as one approaches λ_2 from the long wave length end of the spectrum, the second term in (8) will become larger than the first term and $[\alpha]_\lambda$ will change sign. This is called *anamolous dispersion.*

If an optically active molecule contains a pair of identical groups, it can be shown that an absorption band of the isolated group whose wave length is at λ_1 will split into two bands at λ_1' and λ_1'', having rotatory strengths, R_1 and $-R_1$, which are equal and opposite in sign (see Kuhn, 1930). If the contributions to the optical rotation due to the remaining interactions in the molecule can be expressed as a simple one term Drude equation, then it is found (Kauzmann, 1957b) that the dispersion can be described to a close approximation by the expression

$$[\alpha]_\lambda = \frac{K_0}{\lambda^2 - \lambda_0^2} + \frac{K_1}{[\lambda^2 - \lambda_1^2]^2} \tag{9}$$

where λ_0 is a weighted average similar to that defined by equation (6). An equation similar to this has been derived by Moffitt (1956) from a spe-

cific quantum mechanical model of a helical polypeptide chain, but the general form does not require the existence of a helix in the molecule.

The optical rotation of all proteins in both the native and denatured forms obeys the simple one term Drude equation. Linderstrøm-Lang and J. A. Schellman (1954) have observed, however, that on denaturation the dispersion constant, λ_0, of many proteins decreases from values above about 2400 A to values below 2400 A. [More recently, native proteins have been found by Jirgensons having λ_0 well below 2400 and well within the range of values typical of denatured proteins, e.g., γ-globulin, with λ_0 = 2000 to 2200 A; chymotrypsin, with λ_0 = 2230 A; Bence-Jones protein, with λ_0 = 2030 A; pancreatic amylase, with λ_0 = 2190 A; pepsin, with λ_0 = 2130 A; and trypsin inhibitor, with λ_0 = 2100 A (see Jirgensons and Straumanis, 1957; and Jirgensons, 1958a,b). The significance of the observation of J. A. Schellman and Linderstrøm-Lang is therefore now open to some question.]

Yang and Doty (1957) have made the important discovery that synthetic polypeptides in the helical form show an optical dispersion obeying an equation of the form of equation (9) (with the added simplification that $\lambda_0 = \lambda_1$), whereas the dispersion of the random coil form obeys the simple one term Drude equation. They have also shown that the conversion of the helical form of synthetic polypeptides to the random coil form results in an increased levorotation. The change in levorotation is somewhat larger than the increase observed when proteins denature. Yang and Doty have shown that if a protein consisted of a mixture of helical and randomly coiled polypeptide chains with more than about 60–70% of random coil, the dispersion in the visible and near ultraviolet would be simple. Furthermore, as the fraction of the helical form decreases, the dispersion constant λ_0 in the simple Drude equation would decrease in the manner actually observed during denaturation. Yang and Doty conclude that the common native proteins contain a considerable fraction of their polypeptide chains in configurations resembling that of the random coil.

The results of an extensive study of the optical rotatory properties of proteins have recently been reported by J. A. Schellman (1958b,c,d,e,f) and their significance has been discussed by C. Schellman and J. A. Schellman (1958).

The optical rotation is commonly measured in solution, where the molecules are randomly oriented in space. The theory predicts, however, that the rotation should depend on the direction of the light as it passes through the molecule, so that if the molecules are oriented the optical rotation will vary with the direction in which the light passes through the specimen. The theory of this effect has been discussed by Tinoco and Hammerle (1956) for molecules oriented by an electric field and by Tinoco (1957a) for mole-

cules oriented by a hydrodynamic field. Tinoco (1957b, 1959) has observed a change in the rotation of poly-γ-benzyl-L-glutamate helices in an electric field brought about by orientation. More recently, Tinoco has shown that the rotations along the axis of the helix and perpendicular to it are opposite in sign, show different dispersion behavior, and are much larger than the average rotation for unoriented helices (Tinoco, 1958).

6. Optical Properties: Infrared Absorption and Dichroism

Certain frequencies in the infrared absorption spectrum of organic molecules can be associated with particular functional groups. It is found that small changes in the absorption frequencies of many groups can be brought about by changing the environment of the groups either by chemical changes within the molecule or by changing the solvent in which the group is immersed. As a result of extensive studies with model compounds a considerable body of empirical information is available for interpreting these small frequency shifts in structural terms. The results obtained with synthetic polypeptides have been summarized by Bamford *et al.* (1956). Especially useful are the carbonyl stretching band at about 1650 cm^{-1} and the N—H deformation band at about 1530 cm^{-1}, since the exact frequencies of these bands are characteristic of the folded structure in which the peptide groups are present. Furthermore, the intensity of the absorption depends on the orientation of the group relative to the electric field in the incident light, so that if the molecules can be oriented in a beam of polarized infrared radiation it is possible to gain information about the orientation of the groups inside the molecule. An extensive review of this field is also given by Bamford *et al.* (1956). (For a shorter account see Kauzmann, 1957b.)

The most serious limitation on the study of proteins by means of infrared is the absorption of water at the carbonyl and amide frequencies. This difficulty may be overcome by using solutions in heavy water or by making use of the much weaker overtone and combination bands, many of which occur at frequencies at which ordinary water is transparent. The interpretation of the overtone and combination bands is less simple than that of the fundamental bands, however.

7. Optical Properties: Electronic Spectra (Visible and Ultraviolet)

The electronic absorption spectra of functional groups in organic molecules are altered by changing the solvent and other environmental factors. These changes are manifested in both the shapes of the absorption bands and in their positions. For many proteins the band at 2800 A is most convenient for study. Absorption peaks in this region are due to tryptophan, tyrosine and phenylalanine, the first two of these being, however, by

far the more strongly absorbing. Beaven and Holiday (1952) have summarized what is known about environmental effects on the fine structure of the 2800 A band in proteins. The interpretation of these effects must, of course, depend on studies with suitable model systems.

Ribonuclease is particularly favorable for studies of this kind because it contains no tryptophan, so that practically all of its absorption at 2800 A is caused by tyrosine. Shugar (1952) has pointed out that the tyrosine absorption in ribonuclease is abnormally high as compared with free tyrosine, and the absorption maximum is shifted to longer wave lengths by about 25 A. He ascribed the shift to the peptide linkage, but Harrington and J. A Schellman (1956), who confirm the shift and the intensification, find that disruption of the secondary structure by urea or by oxidation of the disulfide groups shifts the peak back to the wave length observed for free tyrosine and eliminates the intensification. The shifts must therefore be caused by environmental changes in the vicinity of the tyrosine side chain, and not by the peptide bond. Harrington and J. A. Schellman attribute them to hydrogen bonding between the hydroxyl group of the tyrosine and an adjacent carboxylate ion. Wetlaufer (1956) finds that the addition of acetate ion or urea to aqueous solutions of phenol brings about spectral shifts that are qualitatively similar to the differences between free tyrosine and tyrosine in native ribonuclease.

Laskowski *et al.* (1956) have described a useful differential method for detecting changes in absorption due to denaturation or other changes in proteins. They have found by this method that the removal of a tyrosine-containing heptapeptide from insulin by treatment with trypsin is accompanied by a spectral shift to shorter wave lengths that they ascribe to the rupture of a carboxylate-tyrosine hydrogen bond in the native protein. Scheraga (1957) has applied the method to ribonuclease, where he finds a similar spectral shift on changing the pH from 7 to 2. The unfolding of bovine serum albumin in urea or in acid, and of ovalbumin in urea, is also accompanied by a movement of the absorption maximum toward shorter wave lengths (Glazer *et al.*, 1957).

Wetlaufer *et al.* (1958) have found that the ionization of a proton from the carboxyl or amino group of tyrosine or *O*-methyl tyrosine causes a small shift in the absorption peak toward longer wave lengths. Intramolecular hydrogen bonds involving the phenolic group are impossible in these amino acids, and intermolecular hydrogen bonding between two tyrosines was shown not to be present. Therefore it is evident that changes in the electric charges near a tyrosine residue of a protein, rather than the rupture of hydrogen bonds, might be responsible for the spectral shifts observed in protein denaturation.

If a protein contains a prosthetic group or a transition metal ion, changes

in the environment of this group or ion are especially suitable for spectrophotometric study (see Williams, 1953).

In favorable instances a colored derivative of a protein may be prepared by coupling a dye at a well-defined locus. The spectrum of this group can then be studied as the protein is denatured in order to detect changes in a localized region of the molecule. Klotz and Ayers (1957) have attached an azo dye radical to the single sulfhydryl group of bovine serum albumin making use of a mercury derivative of the dye. Treatment of the dye-protein compound with urea and with detergent was found to produce large changes in the dye spectrum which could be ascribed to changes in the environment of the dye.

8. Optical Properties: The Index of Refraction and the Polarizability

These two properties are related through the Lorenz-Lorentz equation,

$$\frac{n^2 - 1}{n^2 + 2} = \frac{4}{3}\pi \sum_{\substack{\text{all} \\ \text{species}}} N_i \alpha_i$$

where n is the refractive index, α_i is the polarizability of a molecule of species i and N_i is the number of molecules of species i present in unit volume. Since very small changes in the index of refraction can be measured by means of interferometers and differential refractometers, it should be possible to observe small changes in the polarizability in protein solutions as a result of denaturation. Such measurements are complicated, however, by the volume changes that accompany denaturation, as mentioned above in Section 3. Because of these volume changes, the numbers of molecules, N_i, of the various species present in unit volume changes during denaturation. Measurements by Stauff and Rasper (1958) and by the writer (unpublished measurements made at the Carlsberg Laboratory) indicate that the observed changes in the refractive index of chymotrypsinogen solutions on heating and of ovalbumin in urea can be accounted for by these volume changes. It therefore appears that the changes in protein polarizability accompanying denaturation may be too small to be readily detected.

Measurements of the flow birefringence and of the electrical birefringence of protein solutions make it possible to obtain information on the optical anisotropy of the protein molecule. In the case of tobacco mosaic virus, which is particularly convenient for this kind of measurenent, the two principal indices of refraction of the particle are nearly the same, and the flow and electric birefringence come primarily from form birefringence (Donnet, 1954; O'Konski and Haltner, 1957). Therefore, for this protein changes in birefringence on denaturation probably arise chiefly from a change in the molecular shape.

9. Optical Properties: Depolarization of Fluorescence

Weber (1953) has reviewed the method originally proposed by him, using the depolarization of fluorescence as an independent method of determining the rotational diffusion rates of rigid macromolecules. The method depends upon the fact that the mean lifetime of the excited states of molecules which have absorbed a photon and are capable of re-emitting the photon is of the same order of magnitude as the time required for protein molecules to rotate through 90° due to Brownian motion. If, therefore, a fluorescent dye is rigidly coupled to a protein so that it can change its spatial orientation only if the protein molecule rotates, and if the molecule is made to fluoresce by a beam of polarized light, the degree of depolarization of the light re-emitted by the coupled dye should depend in a predictable way on the relative rates of fluorescence and of rotatory Brownian motion.

When the method is applied to serum albumin (Harrington *et al.*, 1956a), reasonable rates of rotatory diffusion are obtained in neutral solution, but the measurements of depolarization indicate greatly increased rates of rotation in acid and in alkali. At first this was taken to mean that the serum albumin molecule dissociates into small fragments at high and at low pH, but it is now realized that the observation is a consequence of the greatly increased flexibility of the serum albumin molecule which results from partial unfolding. As a result of this flexibility the dye moiety is able to change its spatial orientation without the rotation of the entire serum albumin molecule. Similar behavior has been observed with fumarase (P. Johnson and Massey, 1957). Thus this method, if associated with a measurement of the molecular weight, makes it possible to study the flexibility of protein molecules in the vicinity of points to which fluorescent molecules can be attached.

Recent technical refinements and applications of the method have been described by Harrington *et al.* (1956b) and by Steiner and McAlister (1957).

10. Wide Angle X-ray Diffraction

If a protein can be obtained in crystalline form, the wide angle X-ray diffraction pattern is potentially the most promising method of obtaining detailed information on the structure. The field is undergoing active development and progress has been reviewed repeatedly (see, for instance. Kendrew and Perutz, 1957). An important example of the kind of structural information that may be expected to come from this activity over the coming years is to be found in the recent deduction by Kendrew *et al.* (1958) of a three-dimensional model for myoglobin. This model shows what is believed to be the general path through the molecule of the coiled polypeptide chain.

Since denatured molecules cannot be crystallized, these methods will never be able to determine the structure of denatured molecules in solution, nor will they be able to answer the awkward question of whether native protein molecules have the same structure in solution as they have in the crystalline state. Interesting information on denatured proteins has been obtained by precipitating them and drawing them into fibers, which are then used to diffract X-rays. (Astbury *et al.*, 1935; Senti *et al.*, 1945). These studies do not, of course, tell us very much about the structure of the dissolved denatured protein molecule. Arndt and Riley (1955) have shown how information on the internal structure of dissolved proteins can be obtained from the wide angle scattering pattern of protein solutions, making use of the Debye theory for the scattering of X-rays by randomly oriented molecules. The experimental technique is difficult and the interpretation appears not unambiguous at the present time. It is greatly to be hoped that this method can be further developed in the future.

11. Chemical Properties: The Reactivities of Groups on Amino Acid Side Chains

The marked increase in the reactivity of the functional groups of proteins on denaturation is among the most striking characteristics of the process. Most of these changes have been adequately reviewed elsewhere (Neurath *et al.*, 1944; Anson, 1945). The reasons for the changes in reactivity are still far from understood. One theory is that the reactive side chains in the native protein are inaccessible to the reagents with which they are supposed to react, presumably because the side chain is buried within the tightly folded native protein molecule. This theory encounters the difficulty that there often appears to be little relationship between the size of a reagent molecule and its ability to react with a functional group of a native protein. An alternative theory is that the reactivity of the functional group is markedly changed by its environment in the protein (e.g., by forming unusually strong hydrogen bonds with other groups in its vicinity). This theory has been developed in some detail by Laskowsky and Scheraga (1956), who point out that the ability to rupture a primary valence bond in a native protein will be markedly changed if the bond is a part of a folded fabric that is stabilized by a great many hydrogen bonds and other secondary bonds.

12. Chemical Properties: The Intrinsic Dissociation Constants of Acidic and Basic Groups

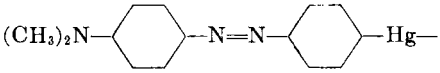
The titration curve of a denatured protein will differ from that of a native protein if, say, some of the carboxyl groups are able to participate in hydrogen bonds with other groups which are present in their vicinity only

in the native form, and if the carboxyl group in the ionized state forms a stronger hydrogen bond than when it is in the un-ionized form. Such an interaction will cause the intrinsic pK of the carboxyl group to be different in the native and denatured protein. Very considerable shifts of the dissociation constants of ionizable groups are also to be expected if the groups are located within the native molecule, where they are inaccessible to the aqueous phase, so that the charged form cannot be stabilized by an environment of high dielectric constant (Tanford and Kirkwood, 1957; Tanford, 1957b, c). In practice, however, it is difficult to distinguish between an anomaly in the titration curve that arises in this way and one that results from a change in the long range electrostatic effects that have been discussed previously (Section A, 3, above). Tanford (1957a) has shown, for instance, that the time-dependent uptake of protons by hemoglobin that has been observed by Steinhardt and Zaiser (1955) can be largely accounted for by a reduction in the long range electrostatic effect (w-factor in equation (1) of Section A, 3) due to the unfolding of the molecule that is known to accompany the uptake of acid. Steinhardt and Zaiser, on the other hand, had accounted for this uptake by a marked change in the intrinsic dissociation constants of the basic groups that were responsible for the uptake.[2] Similarly, Foster and Sterman (1956) believe that the anomalous titration curve of serum albumin in acid is caused by an increased basicity of the carboxylate ions resulting from the weakening or destruction of intramolecular structures when the molecule unfolds in acid; Tanford ascribes the marked uptake of protons in acid to a reduction in long range electrostatic repulsions resulting from the unfolding of the molecule to a more expanded state.

The marked intensification of the absorption spectrum of tyrosine on ionization of its hydroxyl group makes it possible to determine the dissociation constant of this group spectrophotometrically. Crammer and Neuberger (1943) were able to show by this method that the tyrosine groups of native ovalbumin must have considerably larger pK's than those of normal tyrosine. Shugar (1952) and Tanford *et al.* (1956) showed in a similar fashion that about half of the tyrosine residues of ribonuclease have their normal pK, whereas the other half can ionize only after the molecule has unfolded. The method has also been applied to bovine serum albumin (Tanford and Roberts, 1952), lysozyme (Tanford and Wagner, 1954) and β-lactoglobulin (Tanford and Swanson, 1957).

[2] More detailed studies by Nozaki and Tanford (1959) and by Beychok and Steinhardt (1959) seem to show conclusively that a large number of basic groups (probably histidine) do not react with hydrogen ions in the native protein even at pH 4, the time dependent uptake of protons by hemoglobin being only partially explained by the change in w.

Klotz and Ayers (1957) determined spectrophotometrically the ionization equilibria of the dimethylamino group of the azomercurial,



which had been coupled to bovine serum albumin through the single thiol group of the protein. By comparing the pK of this group when attached to the protein with its value when attached to cysteine they were able to make some interesting speculations on the environment of the group when it was coupled to the protein. Denaturation of the protein with urea or with detergent eliminated the difference between the pK's of the protein-coupled and the cysteine-coupled groups.

13. Chemical Properties: The Rate of Deuterium-Hydrogen Exchange

Lenormant and Blout (1953, 1954) showed by means of the infrared spectrum that some of the hydrogen atoms on the peptide groups of serum albumin, ovalbumin and γ-globulin exchange relatively slowly with water unless the molecules have been unfolded by heat or alkali. The effect has been observed by others on these and other proteins using infrared techniques [in keratin and other fibrous proteins by Parker (1955) and by Fraser and MacRae (1958a, b); in human hemoglobin by Larson and McLaughlin (1955); in insulin, serum albumin, myoglobin, β-lactoglobulin, ribonuclease, ovalbumin, γ-globulin, hemoglobin, tobacco mosaic virus, lysozyme and deoxyribonucleic acid by Haggis (1956, 1957)]. Lenormant (1956) finds that all of the peptide hydrogens of the soluble silk fibroin extracted from silk glands exchange rapidly at the isoelectric point and in acid.

Linderstrøm-Lang and co-workers have been able to follow both the kinetics and the extent of the exchange more quantitatively by measuring the density of the solvent in a gradient column after various times of exposure of the protein (reviewed by Linderstrøm-Lang, 1955). Simple peptides and the isolated A-chain of insulin are found to exchange all of their nitrogen-bound hydrogen atoms in less than one minute, but in native insulin 29 out of 89 potentially exchangeable hydrogens exchange only over a period of many hours at 0°C and pH 3. The exchange is catalysed by alkali (Hvidt and Linderstrøm-Lang, 1955) and by urea and guanidinium chloride. Leach and Scheraga (1958) observe a slow exchange in the isolated B-chain of insulin (though not so slow as in insulin under the same conditions). The B-chain appeared to be aggregated in these experiments. A slow exchange of some hydrogen has also been observed in ribonuclease (Hvidt, 1955) and in β-lactoglobulin (Hvidt and Linderstrøm-Lang, 1955). Berger and Linderstrøm-Lang (1957) have found that there is a slow ex-

change of some of the peptide hydrogens of poly-DL-alanine. The kinetics of the exchange indicates a wide range of exchange rates at different portions of the chain, and there is reason to believe that exchange near the ends of the chain occur by unravelling of the helix whereas exchange near the center of the chain occur at "breaks" resulting when several adjacent N—H···O=H hydrogen bonds rupture simultaneously. Urea had no effect on the exchange rate but there was a strong pH effect believed to be due to the direct attack of acid or base on the peptide hydrogen atoms. Elliott and Hanby (1958) have found that the exchange of deuterium with hydrogen in the helically folded form of poly-γ-benzyl-L-glutamate is very much less rapid than the exchange in the randomly coiled form.

As a result of this work we have strong independent evidence for the existence of stable structures within proteins in which the reactivity of the peptide group has been markedly reduced. We also have a means for obtaining information on how much of the molecule is tightly folded, how tightly folded it is, and some of the factors that bring about unfolding.

14. Chemical Properties: The Binding of Small Molecules

The ability of a protein to adsorb small molecules (dyes, ions, lipids, etc.) depends on the existence of suitable adsorption sites in the regions of the protein that are accessible to the small molecules. When the protein changes its configuration some or all of these sites may be destroyed, and new sites may also become available. Thus denaturation may result in great changes in the ability of a protein to bind other substances, and these changes reflect changes in the molecular architecture which a sufficient understanding of the binding process might enable us to decipher. At the present time we do not have enough information to exploit this phenomenon, however.

The effect of a denaturing agent on the binding properties of a protein may not operate solely through a change in the configuration. Competition between the denaturing agent and the bound substance for sites on the native protein would result in a decrease in binding in the presence of the denaturant even if no change in protein configuration had taken place.

If a substance is more strongly bound to the native protein than to the denaturated form, it should act as an inhibitor of denaturation. The inhibition of the denaturation of serum albumin by small amounts of detergent and other organic ions (Boyer, 1945; Boyer *et al.*, 1946; Luck, 1947; Duggan and Luck, 1948) undoubtedly arises in this way. The denaturing action of urea on proteins in general, on the other hand, presumably reflects the greater binding power of urea to denatured protein than to native protein—probably because the most important binding sites are the peptide bonds, and these are largely buried within the native protein molecule. Urea destroys the ability of serum albumin to bind dye molecules (Klotz

et al., 1948), possibly because urea competes for the same sites as the dye, but more probably because urea unfolds the molecule and thus destroys the binding sites. Irreversible denaturation by heat and by alkali also destroys the ability of serum albumin to adsorb dyes (Klotz *et al.*, 1948). It is interesting that, in contrast to serum albumin, the denatured forms of some proteins (e.g., chymotrypsin, tobacco mosaic virus, ovalbumin) bind dyes more strongly than do the native forms (Oster, 1951). Ermolenko and Ginzberg (1954) found that exposure of the proteins of white and yellow lupines to 2 *M* urea (which presumably causes some unfolding, although maximal unfolding occurs only at much higher urea concentrations) increases the adsorption of azobenzene; higher urea concentrations apparently did not bring about any further increase in the adsorption.

15. Electrophoretic Properties

The relationship between electrophoretic mobility and protein structure is rather complex, involving the binding of ions, the factors underlying the titration curve, the shape of the molecule, and the ionic strength. This tool therefore does not seem to be a particularly promising one at the present time for helping to unravel the structural changes that occur during denaturation. It is, of course, admirably suited for observing changes in the isoelectric point and for the detection of heterogeneity after denaturation.

16. Biological Interactions with Other Molecules

The ability of a protein antigen to combine with its homologous antibody is believed to result from a complementarity between the surfaces of the two molecules. It would therefore be expected that the immunological properties of a completely denatured protein (either the antigen or the antibody) would bear little resemblance to those of the native protein. MacPherson and Heidelberger (1945) found that denaturation of ovalbumin either by heat, acid or alkali markedly reduced its interaction with antibodies for native ovalbumin (see also Lobachevskaya, 1953). Wu *et al.* (1927) had earlier shown that native ovalbumin fails to cross-react with antisera produced from alkali-denatured ovalbumin. On the other hand, Heidelberger (1954) reports that denatured serum albumin is rather similar immunologically to native serum albumin, showing that for this protein the native and denatured forms are less dissimilar than for ovalbumin. Karush (1958a) reports that in 8 *M* urea an antibody loses the ability to bind the haptenic dye which had induced it, but the binding ability is recovered on removal of the urea by dialysis. If the disulfide groups in the antibody are reduced before removing the urea, the ability to bind the hapten is not recovered. Wright (1944, 1945) and Wright and Shomaker (1948a) have studied the irreversible loss of the ability of diphtheria antitoxin to combine with toxin

due to exposure to urea (see also Tsipis, 1949), and Wright and Shomaker (1948b) have found a similar irreversible inactivation of staphylococcus antitoxin in urea.

It is well known that proteins are generally more resistant to attack by proteolytic enzymes in the native form than in the denatured form. An interesting recent example of this is to be found in the study by Harris (1956) of the effect of urea on trypsin [see also Viswanatha and Liener (1955) and Neurath *et al.* (1956)]. Viscosity and activity studies show that urea causes a reversible reaction in which trypsin is converted into an unfolded, inactive form. At the equilibrium point of this reaction, trypsin exists entirely in the native form if the urea concentration is less than 2 *M*. At urea concentrations greater than 5 *M*, trypsin is present entirely in the inactive unfolded form. At urea concentrations between 2 *M* and 5 *M* trypsin undergoes a slow, irreversible loss of activity that is caused by the digestion of the unfolded form by the native form. The autolysis occurs at a maximum rate in 4 *M* urea, where equal amounts of the native and unfolded forms are present. If trypsin is allowed to stand for long periods in 4 *M* urea or in 8 *M* urea and its activity is then measured after greatly diluting the urea, it therefore appears to be more stable in 8 *M* urea than in 4 *M* urea. One thus gains the false impression that urea stabilizes the protein at high concentrations.

The effects of denaturing agents on the biological functions of proteins may be complicated by a competition between the denaturing agent and the substrate of the protein. An interesting recent example is found in the action of urea on ribonuclease (Anfinsen *et al.*, 1955). Harrington and J. A. Schellman (1956) and Anfinsen (1956) found from studies of the viscosity, optical rotation, sedimentation constant, hydrogen-deuterium exchange, and ultraviolet absorption that ribonuclease is extensively unfolded in 8 *M* urea. Nevertheless 8 *M* urea had no noticeable effect on the rate at which ribonuclease decreases the viscosity of RNA solutions or on the rate of production of dialysable or non-precipitable nucleotide fragments. The conclusion which was at first drawn from this was that the enzymatic activity of ribonuclease did not depend on the state of folding of the molecule. More recent work by Sela and Anfinsen (1957) and Sela *et al.* (1957), however, indicates that the unfolding of ribonuclease in urea is inhibited by polyvalent anions such as sulfate, phosphate and pyrophosphate. This observation suggests that ribonuclease in the presence of RNA (which is also a polyvalent anion) may not be unfolded in 8 *M* urea.

17. Nuclear Magnetic Resonance

The nuclear magnetic resonance spectrum of a substance is affected by the chemical environment of those nuclei that have a nuclear spin. With sufficient resolution and sensitivity one might expect to observe complex

and possibly interpretable changes in the NMR spectrum of a protein as a result of the configuration changes brought about by denaturation. Such changes do not yet seem to have been reported, though several studies of the proton resonance spectrum of proteins are described in the literature. Shaw and Palmer (1951) observed a sharp line superimposed on a broad band for solid proteins at 40% relative humidity. Stepwise removal of the water reduced the intensity of the sharp peak, which ultimately disappeared. At 100°C the lines were slightly less broad than at 25°C. In collagen the thermal contraction brought about a more marked narrowing, and on cooling to 25°C the line broadened again, becoming indistinguishable from the original collagen.

Jacobson *et al.* (1954) found that the proton resonances of ovalbumin and hemocyanin in water were almost identical with pure water, showing that no extensive structural changes in the water had occurred due to hydration. Indications for some lattice ordering in the hydration shell around DNA was obtained, however.

Saunders *et al.* (1957) have observed several separate peaks due to protons in ribonuclease dissolved in heavy water, but these appear to reflect aspects of the amino acid composition rather than of the secondary and tertiary structure of the molecule (O. Jardetzsky and C. D. Jardetszky, 1957). These studies have been extended to other proteins (Saunders and Wishnia, 1958).

In concluding this survey of the experimental means at our disposal for the study of protein denaturation, one point seems to be clear: no single one of the properties that have been discussed holds unusual promise as the magic key to the solution of the problems that await explanation. As has already been stressed repeatedly in discussions of protein denaturation, an understanding of the phenomenon will come only through a study of the changes of many properties, each sensitive to different aspects of protein structure. Since some properties are much easier to measure and to interpret than others, they are bound to be more frequently exploited. There is, however, a need to develop a better understanding of some of the more unusual properties and to study their changes during protein denaturation on a wider scale. The tools that are at hand for studying the phenomenon are not very potent, considering the magnitude of the problems that must be solved, and it is not easy to be optimistic about the progress that they will make possible. We cannot afford to overlook any conceivable potential aid to this progress.

III. Forces Responsible for Maintaining the Native Configurations of Proteins

An understanding of how and why protein molecules change their configuration requires an examination of the various kinds of internal bonds

that might be expected to form in a native protein, and the means by which denaturing agents break these bonds. Because of the many different kinds of functional groups present in proteins and because of the aqueous environment in which they normally exist, this is a complicated matter. Furthermore, several different types of internal bonds are undoubtedly important in stabilizing any given native protein molecule, and the predominant types may differ in different proteins. The problem is a difficult one, but its importance is so great that we must try to progress along all of the paths that offer any conceivable hope of helping to attain a solution.

In this section we shall attempt to estimate the strengths and, where possible, other properties of the different kinds of secondary bonds likely to be present in proteins. An understanding of these bonds is best gained by studying model systems. Information acquired in this way may be conveniently expressed in the following manner. Suppose that two functional groups, A and B, which are present in a protein, tend to adhere to one another when the protein is dissolved in water. Then in an aqueous solution of compounds containing the same two groups A and B there ought to be a tendency for an equilibrium

$$\text{R—A (aq)} + \text{R}'\text{—B (aq)} \rightleftharpoons \text{R—A} \cdots \text{B—R}' \text{ (aq)} \tag{1}$$

to take place. If the equilibrium constant of this reaction can be found, the relation

$$\Delta F = -RT \ln K$$

provides us with a quantitative measure of the strength of the bond, which should be useful in evaluating the importance of similar bonds in proteins. (Naturally it is important to choose model compounds in which the residues R and R′ do not interfere with the reaction). Furthermore, the heat of the reaction, ΔH, can be determined from the variation of ΔF with temperature or by calorimetric measurements. The entropy change of the reaction can be found from the relation

$$\Delta S = (\Delta H - \Delta F)/T$$

If the concentrations used in calculating the equilibrium constant are expressed in moles per liter, then ΔS is the entropy change when one mole of R—A(aq) and one mole of R′—B(aq), each at a concentration of one mole per liter, react to give one mole of the complex, R—A $\cdots$ B—R′(aq), also at a concentration of one mole per liter. If different concentration units are used, ΔS will have a different value. It is desirable to eliminate this rather arbitrary factor before trying to interpret the magnitude of ΔS in terms of the molecular structures present in the solution. This may be

accomplished in the following way. If the solution is sufficiently dilute to be ideal, then the partial molal entropy of, say, R—A is given by

$$S_A = S_A^0 - R \ln x_A$$

where x_A is the mole fraction of R—A. If R—A is not charged and if its molecular weight is not too high, this expression will be very nearly true for most aqueous solutions at concentrations up to one mole per liter and even higher. The quantity S_A^0 represents the contribution to the entropy due to the interaction of R—A with the solvent; it is the entropy that would be obtained if one mole of R—A were added to a large excess of solvent, all of the R—A molecules being completely surrounded by solvent, but with the R—A molecules forced to remain at fixed positions in the solution. (The last restriction eliminates the contribution $R \ln x_A$ in the expression for S_A.) S_A^0 is the inherent molar entropy of the R—A molecule when it is surrounded with water and has been called the *unitary entropy* by Gurney (1953). (The term, $-R \ln x_A$, is called the *cratic* contribution to the entropy. The unitary entropy is also sometimes called the *contact entropy*.) Similar unitary entropies may be defined for R′—B(aq) and for the complex R—A $\cdots$ R′—B(aq); they will be represented by S_B^0 and S_{AB}^0, respectively. Now the standard entropy change in reaction (I), ΔS, is given by

$$\Delta S = S_{AB} - S_A - S_B = S_{AB}^0 - S_A^0 - S_B^0 + R \ln x_0$$

where x_0 is the mole fraction when the concentration is one mole per liter of water. We may therefore define

$$\Delta S_u = S_{AB}^0 - S_A^0 - S_B^0 = \Delta S - R \ln x_0$$

as the unitary entropy change for the reaction, which depends only on those factors which involve the interaction of the R—A and R—B molecules with the solvent and with each other, and not on the contribution due to randomness of the mixing with the solvent. If concentrations are expressed as molarities or as molalities, and if ΔS was determined from measurements at sufficiently high dilution, we must take $x_0 = 1/55.6$, since one liter contains 55.6 moles of water. This gives $-R \ln x_0 = 7.98$ entropy units, so that

$$\Delta S_u = \Delta S + 7.98$$

A. Hydrogen Bonds between Peptide Linkages

The hydrogen bonds between the oxygen atoms of the carbonyl groups and the hydrogen atoms of the amide groups of peptide linkages are assumed to play a basic role in determining the pattern of folding of polypeptide chains in most of the structures that have been proposed to account

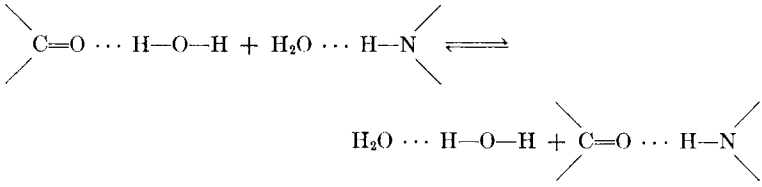
for the X-ray diffraction patterns of fibrous proteins (Astbury, 1940; M. L. Huggins, 1943; Pauling *et al.*, 1951; and Rich and Crick, 1955), and there is every reason to believe that this bond is also an important factor in many globular proteins, if not in all of them. The thermodynamic properties of this bond in water have been discussed by J. A. Schellman (1955a), who employed aqueous urea solutions as his model system. J. A. Schellman showed that the deviations of dilute urea solutions from ideality (which are very accurately known) can be explained if one assumes that there is an equilibrium,

$$2 \text{ urea (aq)} \rightleftharpoons (\text{urea})_2 \text{ (aq)} \tag{1}$$

He also showed that there is probably further polymerization to give trimers, tetramers, etc., at higher urea concentrations. The thermodynamic constants for reaction (1) at 25°C were found to be (all concentrations being expressed in moles per liter)

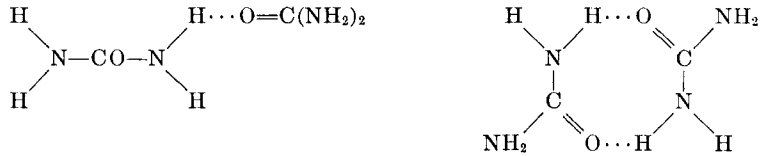
$$\begin{aligned} K &= 4.1 \times 10^{-2} \\ \Delta F &= 1890 \text{ cal} \\ \Delta H &= -2090 \text{ cal} \\ \Delta S &= -13.4 \text{ e.u.} \\ \Delta S_u &= -5.4 \text{ e.u.} \\ \Delta V &= 3.2 \text{ ml} \end{aligned}$$

[The magnitude of the volume change has been determined by J. A. Schellman (private communication) and by the author from the data of Gucker *et al.* (1938) on the concentration dependence of the apparent molal volume of urea in water.] J. A. Schellman ascribed the polymerization to hydrogen bond formation involving the carbonyl and amide groups of urea



(2)

Since, if this be the case, it should be possible to form two kinds of urea dimers shown below,



(linear) (cyclic)

Two types of urea dimers

the thermodynamic properties of reaction (2) will not be exactly the same as those of reaction (1). If f_1 is the fraction of linear dimers present (containing a single N—H $\cdots$ O=C bond) and f_2 is the fraction of cyclic dimers, and if h is the enthalpy change of reaction (2), then the enthalpy change in reaction (1) is

$$\Delta H = (f_1 + 2f_2)h = (1 + f_2)h \tag{3}$$

Now

$$f_2/f_1 = \exp\,[-(h - T\Delta S_d)/RT] \tag{4}$$

where ΔS_d is the entropy change on formation of the cyclic dimer from the linear one. J. A. Schellman estimates that ΔS_d is between -3 and -6 e.u. Using these values and the observed value of ΔH in equations (3) and (4), it is found that $h = -1300$ to -1500 cal, with $f_2/f_1 = 1.9$ to 0.59. Similarly, if v is the volume change accompanying reaction (2) and ΔV is the volume change accompanying reaction (1), then if we assume that the volume change on formation of the cyclic dimer is double that resulting from formation of the non-cyclic one,

$$\Delta V = (f_1 + 2f_2)v = (1 + f_2)v \tag{5}$$

which gives $v = 2.1$ ($\pm$0.2)ml. If s_u is the unitary entropy change in reaction (2) and ΔS_u is the unitary entropy change in reaction (1),

$$\Delta S_u = s_u + f_2\Delta S_d \tag{6}$$

which gives $s_u = -3.3(\pm 0.1)$e.u. [Note that ΔS_d as defined in equation (4) includes the contribution of s_u from the second hydrogen bond in the cyclic dimer, so (6) is slightly different in form from (3) and (5).] Finally, writing $f_u = h - Ts_u$, we find at 25°C using the above values of h and s_u that $f_u = -400 \pm 100$ cal.

These values of h, s_u, f_u, and v are, of course, to be regarded as rough, though reasonable, estimates of the thermodynamic properties of reaction (2) as it might be involved in the unfolding of a polypeptide chain in water, since the urea-water model is undoubtedly an imperfect duplicate of the peptide hydrogen bond system in proteins. From the rather small value of f_u for the formation of the hydrogen bond between urea molecules, J. A. Schellman (1955b) concluded that "hydrogen bonds, taken by themselves, give a marginal stability to ordered structures, which may be enhanced or disrupted by interactions of side chains."

B. Hydrophobic Bonds

Nearly all proteins contain a relatively high proportion of amino acids with non-polar side chains, such as the isopropyl of valine, the secondary and iso-butyl groups of the leucines and the benzyl of phenylalanine. In

the great majority of proteins 20–30 % of the amino acids are either valine, leucine, isoleucine or phenylalanine. If proline, alanine and tryptophan are also included among the non-polar amino acids, then the percentage becomes 35–45 %. Since the non-polar side chains have a low affinity for water, those polypeptide chain configurations in proteins which bring large numbers of these groups into contact with each other, and hence tend to remove them from the aqueous phase, will be more stable than other configurations, other things being equal. One can consider that the side chains of the above-mentioned amino acids (and perhaps others) will form intramolecular "micelles" analogous to the micelles known to occur in aqueous solutions of soaps and detergents. This tendency of the non-polar groups of proteins to adhere to one another in aqueous environments has been referred to as *hydrophobic bonding*. The hydrophobic bond is probably one of the more important factors involved in stabilizing the folded configuration in many native proteins. It is therefore pertinent to discuss the thermodynamics of the hydrophobic bond in some detail with particular reference to proteins. The most appropriate model systems for this discussion are solutions of hydrocarbons in water; aqueous solutions of other small organic molecules containing non-polar groups, such as alcohols, ethers, esters, amines and alkyl halides, may also be expected to supply useful information. Such systems have been discussed in detail by many authors, particularly by Frank and Evans (1945), and the discussion that follows is based on this work.

Consider the two reactions:

$$\text{Hydrocarbon in non-polar solvent (mole fraction x)} \rightarrow \text{hydrocarbon in water (mole fraction x)} \qquad \text{(A)}$$

$$\text{Pure liquid hydrocarbon} \rightarrow \text{hydrocarbon in water (mole fraction x)} \qquad \text{(B)}$$

In reaction (A) the cratic contribution to the entropy change will be identical in the two solutions, and if the solutions are sufficiently dilute so that the interactions between the hydrocarbon molecules can be neglected, the entropy change will be identical with the unitary entropy change in bringing a hydrocarbon molecule from a non-polar environment into an aqueous environment. In reaction (B) the cratic terms do not cancel and the entropy change of the reaction, ΔS, is related to the unitary entropy change, ΔS_u, by

$$\Delta S_u = \Delta S + R \ln x$$

Table III shows the changes in the enthalpy and in the unitary free energy and entropy when various simple aliphatic and aromatic hydrocarbons are transferred from a non-polar environment to an aqueous one at

25°C. It is seen that this process is invariably exothermic for the aliphatic compounds, and nearly athermal for the aromatic ones; furthermore, it is invariably accompanied by a very large decrease in the entropy. It is therefore clear that the low affinity of these substances for water [positive unitary free energy change for reactions (A) and (B)] is not caused by an unfavorable energetic situation (such as would arise if hydrogen bonds were broken on the introduction of the non-polar molecule into water). It

TABLE III

Thermodynamic Changes in the Transfer of Hydrocarbons from a Non-Polar Solvent to Water[a]

Process	Temp. (°K)	ΔS_u (e.u.)	ΔH (cal/ mole)	ΔF_u (cal/ mole)	$\Delta H/\Delta S_u$ (°K)
CH_4 in benzene → CH_4 in H_2O	298	−18	−2800	+2600	155
CH_4 in ether → CH_4 in H_2O	298	−19	−2400	+3300	126
CH_4 in CCl_4 → CH_4 in H_2O	298	−18	−2500	+2900	140
C_2H_6 in benzene → C_2H_6 in H_2O	298	−20	−2200	+3800	110
C_2H_6 in CCl_4 → C_2H_6 in H_2O	298	−18	−1700	+3700	94
C_2H_4 in benzene → C_2H_4 in H_2O	298	−15	−1610	+2920	107
C_2H_2 in benzene → C_2H_2 in H_2O	298	−7	−190	+1870	27
Liquid propane → C_3H_8 in H_2O	298	−23	−1800	+5050	78
Liquid *n*-butane → C_4H_{10} in H_2O	298	−23	−1000	+5850	44
Liquid benzene → C_6H_6 in H_2O	291	−14	0	+4070	0
Liquid toluene → C_7H_8 in H_2O	291	−16	0	+4650	0
Liquid ethyl benzene → C_8H_{10} in H_2O	291	−19	0	+5500	0
Liquid *m*- or *p*-xylene → C_8H_{10} in H_2O	291	−20	0	+5800	0

[a] Data from Frank and Evans (1945), Bohon and Claussen (1951), Claussen and Polglase (1952), American Petroleum Institute (1953), and Din (1956).

is, rather surprisingly, associated entirely with a highly unfavorable unitary entropy change.

The entropic origin of the low affinity of non-polar groups for water has been long recognized (Butler, 1937; Eley, 1939; Edsall and Scatchard, 1943; Frank and Evans, 1945). This large entropy effect in water is characteristic not only of hydrocarbons, but also of many other organic molecules containing non-polar groups attached to polar groups, as may be seen from the following facts:

(1) Mixtures of the lower aliphatic alcohols (methanol, ethanol, propanol) with water all show positive deviations from Raoult's law, indicating that there is an increase in the unitary free energy when the alcohol molecule is transferred from the pure alcohol (less polar) phase to water ($\Delta F_u > 0$). Yet it is well known that the addition of alcohols to a large excess of water

is exothermic ($\Delta H < 0$). This can only mean that the unitary entropy decreases on such a transfer ($\Delta S_u = (\Delta H - \Delta F_u)/T < 0$).

(2) The solubilities of a great many liquid aliphatic derivatives (for example, diethyl ketone, diethyl ether, diethyl sulfide, ethyl acetate, *n*-butanol, heptaldehyde, ethyl bromide, *n*- and *i*-propyl chlorides and bromides) in water decrease as the temperature is raised. This means, by LeChatelier's principle, that $\Delta H < 0$ for the transfer of a molecule from the non-aqueous phase to water. The fact that these substances do not mix with water in all proportions shows, however, that $\Delta F_u > 0$. Therefore the unitary entropy of mixing with excess water must be negative.

(3) A lower consolute temperature is observed for mixtures of certain aliphatic derivatives with water not far above room temperature (nicotine at 60°C, triethyl amine at 18.5°C, *sec*-butanol under pressure, methylethyl ketone under pressure). These systems separate into two phases on warming, indicating that ΔF_u becomes more positive at higher temperatures. This behavior means that both ΔH and ΔS_u are negative.

(4) It has been observed (Goddard *et al.*, 1957) that the formation of micelles from detergent molecules in water is accompanied by very small heat effects. That is, the slight tendency for the reaction,

micelle in water → isolated detergent molecules in water

to go to the right except at very low concentrations of detergent does not originate from a large and positive value of ΔH. There must be, therefore, a large negative value of ΔS_u, presumably arising when the non-polar residues of the detergent leave the non-polar environment of the micelle for the aqueous environment.

The probable origin of the large negative unitary entropy change and the small negative enthalpy change was first clearly stated by Frank and Evans (1945). It had been shown unequivocally by Butler (1937) that these heat and entropy effects are caused by changes in the aqueous phase when the non-polar molecules were added, rather than by changes in the non-polar phase. (This follows from the fact that the process

non-polar molecule in hydrocarbon environment → non-polar molecule in vapor

gives a unitary entropy change which obeys Trouton's rule, whereas the process,

non-polar molecule in aqueous environment → non-polar molecule in vapor

gives a unitary entropy change which deviates widely from Trouton's rule.) Frank and Evans concluded that when a non-polar molecule is present in water, the water molecules in the immediate vicinity must arrange themselves into a quasi-crystalline structure in which there is less randomness and somewhat better hydrogen bonding than in ordinary liquid water at

the same temperature. They called these structures "icebergs," although they were careful to point out that the structure present in the icebergs need not be the same as that in ordinary ice, and that different structures might be found in the icebergs of different systems.

Some insight into the possible nature of the icebergs can be gained from a study of the crystalline hydrates that form at elevated pressures and near room temperature between water and methane, ethane and propane (Stackelberg, 1949; Stackelberg and Müller, 1954; Deaton and Frost, 1949). These crystals are examples of the so-called clathrate (or inclusion, or cage) compounds (Cramer, 1954). According to Claussen (1951), Stackelberg and Müller (1951a, b; 1954) and Müller and Stackelberg (1952) the water molecules in these hydrates are hydrogen bonded together to form large polyhedral structures, which, in turn, are held to one another by further hydrogen bonds. A spongelike arrangement results; the interiors of the polyhedra form the holes of the sponge, and the hydrocarbon molecules fit into the holes. It seems reasonable to suppose that the structures of the Frank-Evans icebergs might resemble the polyhedral structures in the crystalline hydrates. It should be noted that the structures of these hydrates are entirely different from that of ice.

The entropy lost in the freezing of water to ordinary ice is 5.3 cal/deg mole, and the molar entropy loss by each molecule of water on freezing into an iceberg might be expected to be of a similar order of magnitude. Since the unitary entropy loss per mole of non-polar material on entering an aqueous phase is of the order of 20 cal/deg mole, it is clear that if the Frank-Evans icebergs are really highly crystalline, they must contain no more than half a dozen or so water molecules. It is more likely, however, that the crystallinity is somewhat less perfect and that correspondingly more water is involved—say one or two dozen molecules. The small value of the enthalpy change on introducing a hydrocarbon into water (1000 to 2000 cal/mole of hydrocarbon) as compared with the latent heat of fusion of ordinary ice (1440 cal/mole of water) also strongly indicates that the hydrogen bonding in the icebergs cannot be much better than that in liquid water, and can hardly be as good as that found in ice. This again points to the imperfect crystallinity of the icebergs. In any case, it is very unlikely that large regions of crystalline order are produced in the icebergs. It is difficult to believe that an appreciable amount of ordering can occur in a region thicker than two molecules of water around a given hydrocarbon molecule.

One piece of evidence indicating that the structures present in icebergs are quite different from those in ordinary ice is the fact that the ratio $\Delta H_{fus}/\Delta S_{fus}$ for ordinary ice has the value 273°K, whereas the ratio $\Delta H/\Delta S_u$ for icebergs (see Table III) has very much lower values. A much stronger

indication of a profound difference, however, is to be found in the volume changes accompanying iceberg formation (see Table IV). Whereas the formation of ordinary ice from water results in an *increase* in volume of 1.6 ml/mole, the formation of icebergs results in a large *decrease* in volume. Decreases in volume similar in order of magnitude to those given in Table IV are observed when the lower alcohols are added to water. For substances such as *n*-butanol, methyl ethyl ketone and diethyl ketone the solubilities in water are increased by applying hydrostatic pressure. This shows that for these compounds, too, there is a decrease in volume on transfer from a non-polar phase to the aqueous phase.

The unusual heat, entropy and volume effects described above become less unusual as the temperature is raised to 50°C or 100°C; ΔH becomes less negative and even changes sign, and ΔS_u, perforce, also becomes less nega-

TABLE IV

Volume Changes in the Transfer of Hydrocarbons from Non-Polar Solvents to Water at 25°C[a]

Process	ΔV (ml/mole)
CH_4 in hexane → CH_4 in H_2O	−22.7
C_2H_6 in hexane → C_2H_6 in H_2O	−18.1
C_3H_8, pure liquid → C_3H_8 in H_2O at 10 atm.	−21
C_6H_6, pure liquid → C_6H_6 in H_2O	−6.2

[a] Data from Masterton (1954) and American Petroleum Institute (1953).

tive. This means that the specific heat of the water in the icebergs is much higher than the specific heat of ordinary water (ΔC_p values of the order of 50 cal/per degree per mole of hydrocarbon are commonly observed at 25°C). The large specific heat differences undoubtedly arise from melting of the icebergs as the temperature is raised. The volume change also becomes much less strongly negative as the temperature is raised (Masterton, 1954). The melting of the icebergs has the consequence that at temperatures above 50–100°C the solutions tend to become more nearly ideal; this is responsible for the appearance of the upper consolute temperature in mixtures of water with substances such as nicotine, triethyl amine and methyl ethyl ketone, which separate into two phases at a lower temperature.

Aromatic compounds show all of these effects, but to a considerably lesser degree than do aliphatic compounds. For instance, benzene, toluene, ethyl benzene and the xylenes all show a minimum in solubility in water at 18°C (Bohon and Claussen, 1951), indicating that $\Delta H = 0$ for reaction (B) at this temperature and $\Delta H > 0$ above this temperature. The unitary entropy of solution of aromatic hydrocarbons in water, ΔS_u, is nevertheless

strongly negative (see Table III), though not so negative as found for the aliphatic compounds mentioned above.

There is also reason to believe that for aliphatic hydrocarbons with very long chains, negative values of ΔH and ΔS_u for reactions (A) and (B) would not be found, though direct experimental evidence here is lacking. When dissolved in water, long chain hydrocarbon molecules would undoubtedly roll up into molecular oil droplets which would act like macroscopic droplets if the chains were sufficiently long. Reactions (A) and (B) would then be governed by the interfacial tension between the hydrocarbon and water, γ, with

$$\Delta F_u = A\gamma$$

$$\Delta S_u = -A\,d\gamma/dT$$

$$\Delta H = A[\gamma - T(d\gamma/dT)]$$

where A is the surface area of the drop. It is observed that the interfacial tensions of hydrocarbons and water have a slight negative temperature coefficient ($d\gamma/dT < 0$); this indicates that ΔS_u should in this case be slightly positive. It is found that $[\gamma - T(d\gamma/dT)]$ is also positive. It should be noted, however, that $d\gamma/dT$ is much less negative for hydrocarbon/water interfaces than for air/hydrocarbon or air/water interfaces, so it is possible that "iceberg" formation does occur to a limited extent even at hydrocarbon/water interfaces. Just how long a hydrocarbon chain would have to be in order for ΔS_u to become positive is not known; since ΔS_u is still negative for the disruption of soap micelles, when the chains are eight to ten carbon atoms long, the inversion of sign probably occurs for chains longer than this.

From the above considerations we may draw the following somewhat tentative conclusions about the thermodynamic properties of hydrophobic bonds involving the non-polar side chains found in proteins:

(1) These bonds are stabilized largely be entropy effects. For each non-polar aliphatic side chain which leaves the aqueous environment and enters a non-polar region of the protein a gain in entropy of the order of 20 entropy units may be expected. This value may be more or less independent of the length of the side chain in alanine, valine and the leucines, if we may extrapolate from the behavior of methane, propane and butane. For phenylalanine the entropy gain is probably about half as great.

(2) The transfer of an aliphatic side chain from water to a non-polar region in the protein is endothermic to the extent of 1000 to 2000 cal per mole of groups. For phenylalanine the transfer is more nearly athermal.

(3) The free energy change in the transfer from water to a non-polar environment is exergonic to the extent of about 3000 to 5000 cal per mole of groups at room temperature.

(4) Hydrophobic bonds involving aliphatic side chains are more stable at room temperature than they are at 0°C because of the endothermicity of the transfer of non-polar groups from water to a non-polar environment.

(5) The transfer ought to be accompanied by an expansion of something like 20 ml per mole of aliphatic side chains. For phenylalanine the volume change resulting from the transfer will be somewhat smaller.

These conclusions are tentative because they are based on the behavior of small hydrocarbon molecules in which no polar groups are present. The general similarity to the behavior of alcohols and other organic derivatives, however, makes it likely that the above estimates are correct as to order of magnitude. Further study of this question is clearly called for, but it is not an easy matter to find suitable data in the literature.

The observation that hydrophobic bonds are more stable at higher temeratures may possibly account for the marked negative temperature coefficients of the rates of denaturation of some proteins by urea below room temperature (Jacobsen and Christensen, 1948; Simpson and Kauzmann, 1953). If hydrophobic bonds are weakened at 0°C, if urea brings about denaturation by breaking interpeptide hydrogen bonds, and if both hydrogen bonds and hydrophobic bonds are required to maintain the native structure, then any weakening of the hydrophobic bonds (such as by lowering the temperature) will make it easier for urea to bring about denaturation.

There is a possibility that urea has a small direct effect in weakening hydrophobic bonds. Simko (unpublished observation in our laboratory) has found that the solubility of diethyl ketone is 40% greater in 8 *M* urea than in water, and Schlenk (1949) found that the solubility of *n*-valeric acid is markedly increased by saturating the water with urea. (At 25°C, 100 parts of water dissolves 12 parts of valeric acid, and the two liquids become miscible in all proportions if the water is saturated with urea.) The effects of urea on the solubility of non-polar compounds may well be associated with the ability of urea to form crystalline clathrates with hydrocarbons and straight chain aliphatic derivatives (Schlenk, 1949; Cramer, 1954; and Waugh, 1954). The insolubility of denatured proteins in water is undoubtedly caused by intermolecular hydrophobic bond formation as well as by intermolecular hydrogen bond formation and intermolecular disulfide bond formation. The fact that denatured proteins dissolve readily in strong urea solutions might be taken as an indication that urea is able to weaken hydrophobic bonds as well as hydrogen bonds.

Although not directly concerned with the question of denaturation, it is appropriate at this point to call attention to the thermodynamic evidence for the possible involvement of hydrophobic bonding in protein aggregation and binding reactions. Measurements of the thermodynamic changes

accompanying such reactions frequently show that the reactions are accompanied by small enthalpy changes and large entropy changes. Some examples are shown in Table V. In many instances these reactions go in

TABLE V

Thermodynamic Changes for Some Typical Protein Reactions in Water

Reaction	ΔH (cal)	ΔS_u (e.u.)	Reference
Several divalent haptens + rabbit anti-arsenilic acid antibody	−800 (±2000)	+30 (±9)	Epstein *et al.* (1956)
Bovine serum albumin + rabbit anti-bovine serum albumin antibody	0 (±2000)	+28 (±8)	Singer and Campbell (1955a)
Ovalbumin + rabbit anti-ovalbumin antibody	0 (+2000)	+28 (±8)	Singer and Campbell (1955b)
Benzene-arsonic-acid-azo-bovine serum albumin + rabbit anti-benzene-arsonic-acid-antibody	0 (±2000)	+26 (±8)	Baker *et al.* (1956)
Insulin monomer → dimer	+6700 (±1500)	+14 (±6)	Doty and Myers (1952)
Insulin dimer → trimer	+8400 (±300)	+17 (±2)	
Bovine serum albumin +			
caprylate	0	+17	Boyer *et al.* (1947)
octyl sulfate	0	+24.7	Karush and Sonenberg (1949)
decyl sulfate	−2000	+21.3	
dodecyl sulfate	0	+32.0	
chloride ion[a]	+430 (±540)	+15.5	Scatchard *et al.* (1950)
methyl orange	−2100	+22.5	Klotz and Urquhart (1949a)
azosulfathiazole	−2000	+17.1	Klotz and Urquhart (1949a)
p-(2-hydroxy-5-methyl-phenyl-azo)-benzoic acid;			
site group 1	−3930	+16.7	Karush (1950)
site group 2	−3500	+11.3	
cupric ion	+2780 to 3680	+26 to 37	Klotz and Curme (1948)

[a] Human serum albumin.

the direction they do because of their entropy change, which at room temperature greatly outweighs the enthalpy effect ($T\Delta S_u > \Delta H$). In some instances (for instance, in the bonding of organic dyes and detergents by serum albumin), non-polar groups are known to be present on one of the reactants. In such cases there is a strong possibility that hydrophobic bonding may be the principal factor in bringing about the combination.

It seems likely that the observed large entropy changes may come about through the transfer of non-polar groups from the aqueous phase to a non-polar environment within or on the protein. A further observation consistent with this view is the volume increase of 6.7 ml per mole of sodium dodecyl sulfate when it is bound by bovine serum albumin (Rosenberg and Klotz, 1955).

Table V also shows that protein aggregation reactions (such as the polymerization of insulin and antibody-antigen reactions) are frequently accompanied by a large increase in the unitary entropy and by small enthalpy changes. In some instances, aggregation even appears to be endothermic, which means that the reaction takes place entirely as a result of an increase in the unitary entropy and in opposition to both the cratic entropy and the enthalpy. This appears to be the case in the recent observation of Lauffer *et al.* (1958), who found that at pH 6.5 and ionic strength 0.1 the protein of tobacco mosaic virus is reversibly aggregated into rods at room temperature and disaggregated at 0°C. Similar reversible disaggregation at low temperatures has been observed by Murayama (1956) with sickle cell hemoglobin and by Fessler (1957) with collagen. All of these reactions could well be brought about by small patches of non-polar side chains on the surfaces of the non-aggregated protein. If two patches on different protein molecules had similar dimensions and could come into contact, intermolecular hydrophobic bonds could be established, and the large increase in the unitary entropy as well as the small (and even positive) enthalpy changes on aggregation would result.

The large entropy effects which are responsible for these aggregation and binding reactions have been noticed before, but have generally been ascribed to the close approach of positively and negatively charged groups in the aggregated or bound protein (see Section C, below, on salt linkages). In the case of antibody-antigen reactions, studies with low molecular weight haptens indicate rather strongly that electrostatic effects play a dominant role in the interaction, because it is found that unless charged groups are present on a hapten, antigenicity is liable to be feeble. Furthermore, electrolytes are able to dissociate the antibody-antigen complex. Thus in this one type of interaction electrostatic forces may well predominate.[3] In the other types of protein interactions, however, hydrophobic bonds appear to give just as good a basis for interpretation as do electrostatic bonds.

It has often been suggested that native proteins are surrounded by ordered layers of water. The stability of native proteins and many of their

[3] *Note added in proof.* I have been informed by Professors F. Haurowitz and F. Karush that hydrophobic bonds are undoubtedly of importance in many antibody-antigen reactions. Instances in which hydrophobic bonds, rather than electrostatic ones, probably predominate will be found in papers by Karush (1958b) and Beiser *et al.* (1959).

unusual properties have been ascribed to this "frozen water" (e.g., p. 31 of F. H. Johnson *et al.*, 1954; Jacobson *et al.*, 1954; Szent-Györgyi, 1957; Klotz, 1958). The reader might be tempted to identify the "icebergs" discussed above with these frozen water layers. There are, however, grave difficulties in justifying the assumption that extensive regions of frozen water arise *in this way* in the vicinity of native proteins. From all that is known about the icebergs that are believed to form around small non-polar molecules in water, these icebergs are distinguished by their very low thermodynamic stability. As we have seen, their formation leads to a very large decrease in entropy without a compensating loss in enthalpy. Therefore, insofar as icebergs do arise in this way at hydrophobic regions on the surfaces of native proteins, they must lead to instability. Native proteins would be expected to prefer to expose to the ambient water those side chains which do not tend to form icebergs. If icebergs did happen to form on limited regions of the surface, a tendency to aggregate would result, and the protein might even become insoluble in water. Iceberg formation will undoubtedly increase on denaturation, rather than the contrary, because hydrophobic bonds will have been broken.

The extensive hydration of native proteins that is inferred from hydrodynamic measurements cannot possibly arise from the same forces as those that produce the Frank-Evans icebergs. There is no justification for using the iceberg concept as a basis for the hypothesis that protein molecules are surrounded and stabilized by regions of ordered water molecules.

C. Salt Linkages (Ion Pair Bonds) and Other Electrostatic Forces

It has been suggested frequently that an important factor in maintaining the structure of native proteins is the electrostatic attraction between the positively charged amino and guanidino groups and the negatively charged carboxyl groups that are so abundant in proteins. Jacobsen and Linderstrøm-Lang (1949) have given various reasons for believing that in typical proteins only a small fraction of the charged groups can be involved in bonding of this kind, but it is distinctly possible that some of these bonds may be present in certain proteins.

The thermodynamic properties of salt linkages would be expected to be characterized by large volume and entropy effects and, often, by small heat effects. The formation of a salt link in water can be indicated by the reaction

$$R{-}A^-(aq) + R'{-}B^+(aq) \rightarrow R{-}A^- \cdots B^+{-}R'$$

When they are separated, the charged groups, A^- and B^+, are surrounded by strongly oriented water molecules which are highly compressed by electrostrictive forces arising from the large electric fields in the vicinity of the

charges. When oppositely charged groups come into contact, the electric fields no longer pass so extensively through the water, so that the solvent molecules are much less strongly oriented and compressed. A large increase in entropy and volume will result. These effects are very clearly seen in simple reactions between cations and anions, such as those shown in Table VI. In many of these reactions the enthalpy change is quite small and the reaction is driven entirely by the entropy change. The weakness of acetic acid and the basicity of ammonia are, for instance, almost entirely entropy effects.

The thermodynamic consequences of bringing, say, a charged amino group and a charged carboxyl group into contact in water can be roughly estimated by means of Born's theory of ion-solvent interactions. The ions are regarded as spheres immersed in a uniform dielectric. The unitary free

TABLE VI

Thermodynamic Changes for Some Simple Reactions Involving the Neutralization of Ions at 25°C.

Reaction	ΔH^a (cal.)	$\Delta S_u{}^a$ (e.u.)	ΔV^b (ml)
Acetate$^-$ + H_3O^+ → acetic acid + H_2O	+400	+30.1	+11
H_3O^+ + OH^- → 2 H_2O	−13360	+35.2	+21
NH_4^+ + OH^- → NH_3 + H_2O	−940	+21.5	+28

[a] F. D. Rossini (1952).

[b] Linderstrøm-Lang and Jacobsen (1941).

energy change resulting when the two ions are brought together is then roughly

$$\Delta F_u = -\frac{e_1 e_2}{D}\left[\frac{1}{r_1} + \frac{1}{r_2}\right]$$

where e_1 and e_2 are the charges on the ions, r_1 and r_2 are the ionic radii and D is the dielectric constant of the solvent (here, water). Assuming that the ionic radii are independent of the temperature, the unitary entropy change for the process is

$$\Delta S_u = -d\Delta F_u/dT = -\frac{e_1 e_2}{D^2}\left[\frac{1}{r_2} + \frac{1}{r_2}\right]\frac{dD}{dT} = \Delta F_u \frac{1}{D}\frac{dD}{dT}$$

and the enthalpy change is

$$\Delta H = -T^2\, d(\Delta F_u/T)/dT = -\frac{e_1 e_2}{D^2}\left[\frac{1}{r_1} + \frac{1}{r_2}\right]\frac{dDT}{dT} = \Delta F_u \frac{1}{D}\frac{dDT}{dT}$$

For water both D and the product DT decrease with increasing temperature, and it is found that at 25°C

$$-T\Delta S_u = 1.37\Delta F_u$$

$$\Delta H = -0.37\Delta F_u$$

Thus, insofar as this simple electrostatic model is correct, the driving force for the formation of a salt linkage lies entirely in the entropy change; the enthalpy change *opposes* the formation of the salt linkage. In proteins an additional factor arises in that the charged groups must probably be regarded as attached to regions whose dielectric constant is less than that of water and independent of the temperature. This markedly increases the strength of the salt link (J. A. Schellman, 1953) and will probably make ΔH somewhat negative and ΔS_u somewhat less positive. The assumption that water may be treated as a continuous dielectric in the vicinity of an ion will also change the numerical values (though probably not the orders of magnitude) estimated for ΔH and ΔS_u .

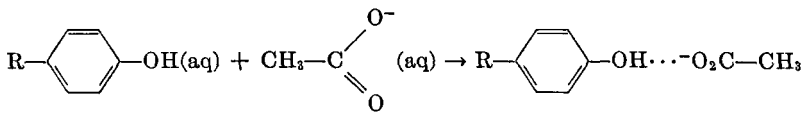
It is evident that both salt linkages and hydrophobic bonds are stabilized predominantly by entropy effects rather than by energy effects. The two types of bonds would, however, be expected to respond very differently both to the addition of electrolytes to the aqueous medium and to the addition of non-polar substances to the medium. Salt linkages should be strengthened by lowering the dielectric constant of the medium (which will follow from the addition of a non-polar substance such as dioxane to the aqueous environment of the protein). Hydrophobic bonds, on the other hand, are weakened by adding a non-polar substance to the aqueous phase. Electrolytes tend to strengthen hydrophobic bonds (they decrease the solubility in water of non-polar materials) whereas they weaken salt linkages (by producing a stabilizing Debye-Hückel atmosphere around the charged groups when they are in the dissociated form).

It is commonly observed that solvents such as dioxane, the alcohols and acetone, which are much less polar than water, act as strong denaturing agents toward proteins, whereas many electrolytes (for instance, ammonium sulfate) are powerful inhibitors of denaturation. These observations would seem to show that hydrophobic bonds are much more important than salt linkages in maintaining the stability of most native proteins. Similarly, the effects of salts and non-polar solvents on the aggregation of proteins provide important clues to the origin of the predominant forces responsible for aggregation. In this connection it is interesting to note that insulin is completely dissociated in 40% dioxane (Fréderiqu, 1956, 1957). Furthermore, the binding of organic dyes by serum albumin is markedly reduced by the addition of dioxane (Fréderiqu, 1955, 1956). In both instances it appears that hydrophobic bonds are of primary importance.

If a protein molecule is covered with dissociable groups, say A, which are present partially in a charged form and partially in an uncharged form, and if oppositely charged groups, B, are also present, then there will be a tendency at a given pH for the A groups which are close to the B groups to exist in a charged form. This effect—which is in a sense a polarization of the dissociable protons—gives rise to intermolecular and intramolecular forces which have been discussed by Kirkwood and Shumaker (1952; see also Hill, 1956b). The fluctuating charge force will be present when groups are partially in the dissociated form and therefore acts only in limited ranges of pH.

D. Hydrogen Bonds Other Than Those between Peptide Links

There have been numerous suggestions that various types of hydrogen bonds other than those between peptide linkages might make important contributions to the stabilities of native proteins. The strengths of hydrogen bonds involving carboxylate ions have been investigated by Tanford (1954) and by Wetlaufer (1956) using simple model systems. Wetlaufer studied the hydrogen bond between the phenolic hydroxyl of tyrosine and the carboxylate of acetate ion by comparing the effects of sodium chloride and sodium acetate on the solubility of tyrosine in water. Both salts decrease the solubility, presumably because of the salting-out action, but sodium acetate decreases the solubility to a slightly lesser degree. (Furthermore, sodium acetate actually increases the solubility of phenol in water.) If the difference between the effects of acetate and chloride is ascribed to the formation of a complex between the acetate ion and tyrosine, then the equilibrium constant for the reaction



must be less than 0.1 liters/mole and ΔF_u must be more positive than $-1{,}000$ cal. It appears from this model system that the tyrosine-carboxylate hydrogen bond can be no stronger than the hydrogen bond between two peptide groups, and that it might be weaker.

Tanford (1954) compared the effects of potassium ion, ammonium ion and guanidinium ion on the pH of sodium acetate-acetic acid buffers in order to see if there might be a specific interaction between acetate ion and ammonium or guanidinium ions. He concluded that the ammonium ion and the carboxylate ion might combine with an association constant which could be as great as 0.5 liters per mole, whereas the association between guanidinium and carboxylate could not be as strong. This means that ΔF_u must be more positive than $-2{,}000$ cal for these reactions.

Katchalsky *et al.* (1951) showed that the concentration dependence of

the conductance of carboxylic acids in water indicates the existence of dimers of the undissociated acids in aqueous solutions. (Such dimers are, of course, well known in the vapor). The association constant for the reaction

$$2\ \text{R—COOH(aq)} \rightleftharpoons (\text{R—COOH})_2\text{(aq)}$$

was found to be 0.04 for formic acid, 0.36 for butyric acid and 0.75 for benzoic acid (all concentrations in moles per liter). The rather strong dependence of the constant on the nature of the group R may indicate that hydrophobic bonds as well as hyrogen bonds play a role in the dimerization. In any case, it would appear that the unitary free energy for the formation of the bond is negative and has a magnitude of the order of one or two thousand calories. Arnold and Overbeek (1950) believed that the titration curve of polymethacrylic acid could best be interpreted if it were assumed that no hydrogen bonding takes place between undissociated carboxyl groups. On the other hand, Silverberg *et al.* (1957) conclude from studies of light scattering and viscosity that intramolecular hydrogen bonding does occur in polymethacrylic acid.

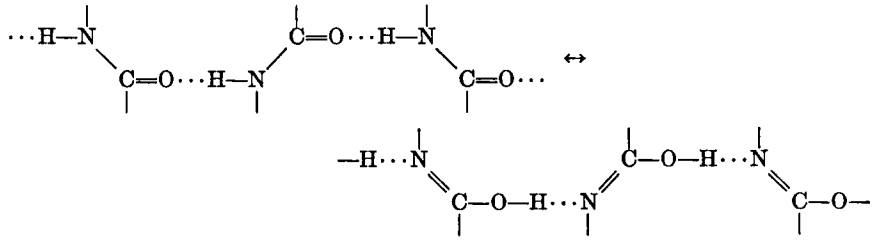
The hydrogen bonds described above evidently have negative unitary free energies of formation of no more than one or two thousand calories at 25°C. Such bonds can modify the properties of dissociable groups to an appreciable extent (for instance, they might alter the pK by one or two units). These modifications have been discussed by Laskowsky and Scheraga (1954, 1956), and experimental studies have been made by Scheraga (1957), Laskowski *et al.* (1956), Shugar (1952), Tanford and Roberts (1952), Tanford *et al.* (1956), and others. It does seem unlikely that hydrogen bonds other than those involving peptide linkages can make a major contribution to the stability of most native proteins; there are generally relatively few of the other types of groups present, and none of them appear to be especially strong. Carboxyl-carboxyl hydrogen bonds would, of course, only be expected at acid pH.

Hydrogen bonds other than those discussed above have also been suggested as important for proteins. Klotz and Urquhart (1949b) have proposed that there is a hydrogen bond between the hydroxyl of threonine or serine and the carboxylate ion. Other workers have proposed bonds involving the thiol group of cysteine. Very little is known about the strength of these bonds in water but there is no reason to believe that they would have unitary free energies of formation more negative than one or two thousand calories.

E. Stabilization by Electron Delocalization

It has been suggested by M. L. Huggins (1943), Simanouti and Mizushima (1948), Evans and Gergely (1949) and others that when the poly-

peptide chain is folded in certain ways delocalization is possible across the hydrogen bonds that join the peptide groups. One way of describing the delocalization is by means of the resonating forms shown below.



Resonating forms of peptide bonds

where the peptide groups may belong to the same polypeptide chain (Simanouti and Mizushima) or they may belong to different polypeptide chains (Evans and Gergely).

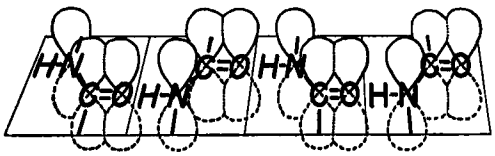


FIG. 1. Positions of the pi orbitals of peptide bonds in the Evans-Gergely treatment of electron delocalization.

Evans and Gergely (1949) have made an estimate of the effects of delocalization involving many peptide links using a molecular orbital approach. Pi atomic orbitals with axes perpendicular to the plane of the peptide groups were assumed to be available on the nitrogen, carbon, and oxygen atoms of each peptide bond (Fig. 1) and molecular orbitals were constructed from them for an infinite number of peptide linkages arranged in a linear sequence as indicated in Fig. 1. Estimates were made of the magnitudes of the coulombic integrals of the pi orbitals and of the exchange integrals between adjacent pi orbitals. (In making these estimates it was assumed that the axes of the pi orbitals in adjacent peptide links were parallel—an assumption which is not fulfilled in the α-helix, for instance.) Because of the great distance between the pi orbital on the carbonyl oxygen and the pi orbital on the nitrogen atom of the adjacent peptide link, the exchange integral between these two orbitals is much smaller than those between the other adjoining pi orbitals. (The value of this integral is further reduced if adjacent peptide links are not co-planar.) As a consequence of this small exchange integral, the energies which are obtained for the molecular orbitals of the system fall into three widely separated bands. The two lowest

bands are occupied by the electrons of the pi bonds of the peptide groups and the highest band is empty when the molecule is in its ground state. It was found that the average energy of the electrons in the two filled bands is lower by 500 to 1,000 calories per peptide link than the electronic energy in an isolated peptide group. Evans and Gergely suggest that this stabilization by delocalization through the pi orbitals might make an important contribution to the energy of polypeptide chain configurations in which peptide links are hydrogen bonded to one another in suitable fashion.

The magnitude of the average stabilization per peptide group caused by delocalization depends on the number of peptide groups that are hydrogen bonded to one another, being larger as the number of groups is increased. That is, for the system

$$(\cdots \text{H—N—C=O} \cdots)_n$$

the total stabilization increases more rapidly than in direct proportion to n. Even for $n = 2$ a considerable amount of stabilization is present, however, and rough calculations by the writer indicate that the extra stabilization per hydrogen bond brought about by delocalization is about half as great for the case $n = 2$ as it is for the case $n = \infty$. Evidently the energy of a single hydrogen bond estimated in Section A, above, already includes an appreciable contribution from delocalization, and if this hydrogen bond energy is used for structures in which a large number of peptide bonds are hydrogen bonded to one another, the additional stabilization can amount to only about half of the value suggested by Evans and Gergely (i.e., 250 to 500 cal. per peptide residue).

In the α-helix the axes of the pi orbitals in a given peptide link are rotated by about 65.4° with respect to the axes of the pi orbitals on the two peptide linkages to which it is hydrogen bonded. This has the result that the stabilization brought about by pi electron delocalization is reduced by a factor of $\cos 65.4° = 0.418$ in the α-helix as compared with configurations in which the pi orbital axes are parallel throughout the molecule. Consequently the average hydrogen bond strength in an infinitely long α-helix is about the same as the hydrogen bond strength in a dimer in which the peptide links are coplanar. The average hydrogen bond strength in very short helices (say two or three turns) should be 100 to 200 cal smaller than the average hydrogen bond strength in an infinite helix, however. This should have the effect of sharpening slightly the thermodynamic transition between the helical form and the random coil form of a polypeptide, as discussed by J. A. Schellman (1955b), Peller (1957, 1959), Zimm and Bragg (1958), Gibbs and DiMarzio (1958), Rice and Wada (1958), and Hill (1959).

Since it does not make use of the orbital present on the hydrogen atom of the peptide link, the treatment of Evans and Gergely is not the molecular

orbital equivalent of the resonance between valence bond structures mentioned at the beginning of this section. It seems possible that a different molecular orbital treatment based on the sigma orbitals of the peptide group might lead to an additional stabilization due to delocalization that is not included in the model treated by Evans and Gergely. In this approach the stabilization would not depend on the coplanarity of adjacent peptide linkages and there would be no reduction in the stabilization of α-helices of the 65.4° angle mentioned above. On the other hand, it should be realized that delocalization through sigma orbitals can occur between the peptide link and any other molecule to which it is hydrogen bonded; in contrast to pi orbital delocalization, it is not restricted to peptide-peptide hydrogen bonds. Stabilization by delocalization through sigma bonds is therefore probably only slightly greater when the polypeptide chain is hydrogen bonded intramolecularly in folded configurations than when the chain is hydrogen bonded to the solvent in randomly coiled configurations.

F. Dispersion Forces (London Forces)[4]

London showed that if a pair of groups, A and B, are brought together from infinity to a distance R, their energy is lowered by an amount

$$K \frac{I_A I_B \alpha_A \alpha_B}{(I_A + I_B) R^6}$$

where I_A and I_B are the ionization energies of A and B, α_A and α_B are their polarizabilities and K is a universal positive constant. If the two groups can be treated as hard spheres of radius r_A and r_B interacting exclusively by dispersion forces then the energy of the reaction

$$\text{A—A} + \text{B—B} \rightarrow 2\ \text{A—B}$$

is given by

$$\Delta E = K \left[\frac{I_A \alpha_A^2}{2^7 r_A^6} + \frac{I_B \alpha_B^2}{2^7 r_B^6} - \frac{2 I_A I_B \alpha_A \alpha_B}{(I_A + I_B)(r_A + r_B)^6} \right]$$

If we let $r_A/r_B = \rho$ and $I_A/I_B = \sigma$ and if we define the positive quantity

$$P = (K I_A / 2^7 r_A^6) \left[\alpha_A - \frac{\sqrt{\sigma}\, \alpha_B}{\rho^3} \right]^2$$

[4] The so-called van der Waals' forces between molecules may be the result of several factors, of which hydrogen bonds and dispersion forces are but two. Since these factors are discussed separately here, van der Waals' forces are not treated under that designation. The term, as employed by protein chemists in discussing secondary bonds in protein molecules, presumably refers to the dispersion forces considered in this section (see, for instance, Waugh, 1954).

we find that

$$\Delta E = P + \frac{KI_A\alpha_A\alpha_B}{2^6\rho^3 r_A^6}\left[1 - \frac{2^7\rho^3\sqrt{\sigma}}{(1+\rho)^6(1+\sigma)}\right]$$

It is readily shown that the quantity $(2^7\rho^3\sqrt{\sigma})/[(1+\rho)^6(1+\sigma)]$ is never greater than unity for any values of ρ and σ between 0 and ∞. Therefore, ΔE must always be positive, and the dispersion forces acting between groups will tend to bring identical groups into contact. This conclusion is substantiated by experimental observation, since heat is almost invariably abosorbed on mixing any pair of liquids whose interactions would be expected to be predominantly of the dispersion type. (For instance, benzene

TABLE VII

Molar Heats of Solution of Hydrocarbons in Large Excess of Hydrocarbon Solvents

Substance	Solvent	ΔH (cal/mole)
Benzene[a]	*n*-Hexane	725
Benzene[b]	Cyclohexane	780
n-Hexane[a]	Benzene	1120
n-Hexane[b]	*n*-Hexadecane	111
Cyclohexane[a]	*n*-Hexane	130
Cyclohexane[b]	Benzene	801
Toluene[c]	Benzene	72

[a] Wolf *et al.* (1935).
[b] Scatchard *et al.* (1952).
[c] Baud (1915).

with either hexane, toluene, carbon tetrachloride or carbon disulfide; heptane with either benzene, carbon tetrachloride or carbon disulfide; *n*-hexane with either cyclohexane or *n*-hexadecane; and oxygen with either krypton or xenon. Toluene and carbon tetrachloride appear to be exceptional, since a small amount of heat is evolved when they are mixed.) The amounts of energy involved are in some instances appreciable, as can be seen from Table VII. Evidently the transfer of an aromatic group from an aromatic environment to an aliphatic one can raise the enthalpy by 700 or 800 cal per mole of groups, and the transfer of an aliphatic group from an aliphatic environment to an aromatic one can raise the enthalpy by 800–1,000 cal per mole of groups. There are indications, however, that in systems of this kind the unitary entropy term acts in the opposite direction to the enthalpy term. As a result, although the unitary free energy changes have the same sign as the enthalpy changes, they are only about one-half to two-thirds as large in magnitude (see Scatchard *et al.*, 1939a, b, 1940).

This result may be of importance for proteins in connection with the detailed structure and composition of the non-polar micelles which arise from hydrophobic bonding. One would expect that, for instance, the aromatic rings of phenylalanine and tryptophan will prefer to be close to one another, and the aliphatic side chains of leucine, valine and alanine will also tend to cluster together. Therefore, those chain configurations which permit segregation of aliphatic groups from aromatic groups ought to be appreciably more stable (other things being, of course, equal) than configurations which do not allow segregation.

G. Effects of Disulfide Groups and Other Cross Linkages on the Stability of the Folded Form of a Polypeptide Chain

The introduction of intramolecular cross linkages (such as those provided by cystine or the phosphodiester links discovered by Perlmann (1955)) may have two opposing effects on the stability of a given folded form of a polypeptide chain. In the first place, the cross linkages may be so located in the chain as to make it impossible for the chain to fold at all into a configuration which would otherwise be very stable. This effect would, of course, be highly specific, depending on the positions of the cross-linking points along the chain, as well as on the configuration that is excluded. On the other hand, cross-linkages reduce the total number of configurations available to a chain, so that a smaller amount of entropy is gained in going from some particular folded configuration to the random coil form of the chain. Consequently, if a configuration does happen to be possible when some pattern of cross-linkages is introduced, then that configuration is stabilized by the cross-linkages (J. A. Schellman, 1955b). The statistical theory of the random coil shows that if a single cross-link is formed between two segments of a chain which are separated by n segments, the number of configurations is reduced by a factor proportional to $n^{-3/2}$ (Kuhn and Majer, 1956). The entropy difference between the folded state and the random coil is therefore reduced by $\frac{3}{2}R \ln n$ per mole of cross linkages. If $n = 100$, the free energy of the folded state relative to the random coil at 300°K is lowered by 4,100 cal. When more than one cross linkage is introduced into each molecule, the factor by which the number of configurations is reduced is more complicated; if there are not too many cross-linkages, the factor may, however, be evaluated without much difficulty (Kauzmann, 1959).

In this section we have discussed seven different types of intramolecular bonds which might be expected to influence the polypeptide chain configuration of proteins. It is appropriate to conclude with the discussion of the probable relative importance of the different kinds of bonds. Because of the large number of peptide groups and hydrophobic groups in nearly all

proteins, it is likely that hydrogen bonds between peptide links and hydrophobic bonds are by far the most important in determining the over-all configuration of the protein molecule. Yet many properties of proteins certainly depend on the configurations present in localized regions of the molecule, and these configurations might very well be determined by some of the less abundant types of bonds. Furthermore, it could well be that peptide hydrogen bonds and hydrophobic bonds are barely sufficient to stabilize the native configuration of some proteins, and that a relatively few of the less abundant bonds contribute the decisive increment that stabilizes the native configuration. Therefore, it is not safe to say that any of the bonds are "less important" than others.

The fact that electrolytes generally fail to act as denaturing agents is probably an indication that salt linkages are not prominent contributors to the stability of proteins. The denaturing tendencies of detergents, of interfaces, and of organic solvents, such as acetone, dioxane and alcohol, indicate the wide importance of hydrophobic bonds, since they should be weakened by these reagents. The denaturing action of urea and guanidine salts on proteins is usually considered to result from their ability to attack hydrogen bonds, although as has been mentioned, it is conceivable that they also weaken hydrophobic bonds.

Mention should be made of the possibility that new kinds of bonds, hitherto unrecognized in simple systems, might be present in proteins. It is also conceivable that in proteins the forces discussed in this article act in an unusual fashion that is not adequately represented by the simple model systems described here. There is, however, every indication that the behavior of large molecules (especially synthetic polymers and polypeptides) can be accounted for completely in terms of the same forces as those that act in ordinary chemical processes. It seems fruitless, therefore, to speculate about these possibilities at this time, even though we should be careful not to close our eyes to them.

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