[5] H. Poincaré, *Méth. Nouv.*, **1** (1892), chap. III; G. D. Birkhoff, loc. cit.[1]

[6] H. Poincaré, op. cit., **2** (1893), chap. XVII. The figure given by Poincaré assumes that p is positive. However, the case of a negative p may be reduced to the case of a positive p, as seen from (6) by writing $v + 1/2\pi$ for v.

[7] M. J. O. Strutt, *Math. Ann.*, **99**, 625–628 (1928).

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[9] H. Poincaré, op. cit., **3**, 346–351 (1899).

[10] H. Poincaré, op. cit., **3**, 343–344 (1899).

[11] Cf. E. Hölder, *Sächs. Sitzber.*, **83**, 179–184 (1931).

[12] G. D. Darwin, *Scientific Papers*, **4** (1911), Part I. Cf. H. Poincaré, op. cit., **3**, 352–361 (1899).

ON THE STRUCTURE OF NATIVE, DENATURED, AND COAGULATED PROTEINS

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In this paper a structural theory of protein denaturation and coagulation is presented. Since denaturation is a fundamental property of a large group of proteins, a theory of denaturation is essentially a general theory of the structure of native and denatured proteins. In its present form our theory is definite and detailed in some respects and vague in others; refinement in regard to the latter could be achieved on the basis of the results of experiments which the theory suggests. The theory (some features of which have been proposed by other investigators) provides a simple structural interpretation not only of the phenomena connected with denaturation and coagulation which are usually discussed (specificity, solubility, etc.) but also of others, such as the availability of groups, the entropy of denaturation, the effect of ultra-violet light, the heat of activation and its dependence on pH, coagulation through dehydration, etc.

I. The experimental basis upon which the present theory rests will be briefly described.

1. The most significant change that occurs in denaturation is the loss of certain highly specific properties by the native protein. Specific differences between members of a series of related native proteins and specific enzymatic activities of native proteins disappear on denaturation, as the following observations demonstrate:

(*a*) Many native proteins can be crystallized and the crystal form is characteristic of each protein. No denatured protein has been crystallized.

(*b*) Most native proteins manifest in their immunological properties a high degree of specificity, which is diminished by denaturation.[1]

(*c*) The native hemoglobins of closely related animal species can be distinguished from each other by differences in crystal form, solubility,[2] gas affinities, positions of absorption bands, and other properties.[3] On the other hand, in the denatured hemoglobins some of these properties, the positions of the absorption bands, for example, can be subjected to precise measurement, and it is found that differences between the various hemoglobins have disappeared.[4]

(*d*) A number of enzymes have recently been isolated as crystalline proteins. When these proteins are denatured their enzymatic activity vanishes. In pepsin and trypsin, where an especially careful study has been made of this phenomenon, there is a close correlation between loss of activity and formation of denatured protein.[5]

2. Striking changes in the physical properties of a protein take place during denaturation. At its isoelectric point a denatured protein is insoluble, although the corresponding native protein may be quite soluble. It was the loss of solubility that first drew attention to the phenomenon of denaturation, and denaturation is now usually defined by the change in solubility. The denatured protein after precipitation has taken place is called a coagulated protein, the process of coagulation being considered to include both denaturation and aggregation of denatured protein in the form of a coagulum.[6] If the denatured protein is dissolved, by acid, alkali, or urea, the solution is found to be far more viscous than a solution of native protein of the same concentration.[7]

3. Changes in the availability of sulfhydryl, disulfide, and phenol groups appear as a consequence of denaturation. All of the SH and S-S groups found in a protein after hydrolysis can be detected in a denatured protein even before hydrolysis, while in the corresponding native protein only a fraction of these groups is detectable. In native egg albumin no SH or phenol groups are detectable. In other native proteins (hemoglobin, myosin, proteins of the crystalline lens, for example) some groups can be detected; new groups appear when the protein is made more alkaline, although not alkaline enough to cause denaturation, and then disappear when the original pH is restored. In all the different ways of coagulating a protein a close correlation between appearance of groups and loss of solubility has until recently been observed.[8] Now, however, it has been found that when myosin is rendered insoluble by drying (or when water is removed by freezing) there is no change in availability of its SH groups.[9] Furthermore, if the insoluble myosin is treated with a typical denaturing agent, such as heat or acid, all of the SH groups in the protein become available, although no change in solubility is observed. Hitherto protein coagulation due to dehydration has not been distinguished from coagulation

caused by other agents, but the tests for availability show that in coagulation by dehydration the change in protein constitution is distinctly different from that caused by any of the known protein coagulating agents. In our theory of coagulation both types of change will be considered. Probably both types occur biologically. It has been suggested that when light converts visual purple, a conjugated protein, into visual yellow the former is denatured in much the same way as it is by heat, alcohol, or acid.[10] And it has been shown that in the course of fertilization and in the rigor of muscle a coagulation of protein similar to that caused by dehydration takes place.[11,12]

4. In a list of the large number of different agents, with apparently little in common, that cause denaturation are heat, acid, alkali, alcohol, urea, salicylate, surface action, ultra-violet light, high pressure. The temperature coefficient of heat denaturation of many proteins (egg albumin, ferrihemoglobin, trypsin, etc.) is about 600 for a rise in temperature of ten degrees, and from this the energy of activation can be calculated. On either side of a point between the isoelectric point and neutrality the temperature coefficient of denaturation by heat is diminished.[13] A dry preparation of egg albumin is not readily denatured by heat.[6]

5. The denaturation of certain proteins, notably hemoglobin, serum albumin, and trypsin, is reversible.[14] From the effect of temperature on the equilibrium constant, the heat of reaction and the entropy change can be calculated. In the denaturation of hemoglobin by salicylate and in the denaturation of trypsin by heat or acid, the equilibrium between native and denatured protein is not affected by changes in the total concentration of protein present,[15] from which it can be inferred that in the denaturation of hemoglobin and trypsin by these agents no change in molecular weight takes place. Since typical denaturation can occur without change in the molecular weight, it is important to distinguish between denaturation and the changes in particle size observed by Svedberg. Although denaturation can occur without change in molecular weight, under certain conditions, as in the denaturation of myosin by urea, denaturation may be accompanied by depolymerization. Weber found the molecular weight of myosin to be of the order of a million and that of myosin in urea to be thirty-five thousand.[16] On the other hand, it is unlikely that depolymerization is always accompanied by denaturation, for under some of the conditions of depolymerization described by Svedberg it is improbable that denaturation (loss of specificity, loss of solubility, or appearance of previously inaccessible groups) takes place. In the case of hemocyanin, for example, decomposition into products $^1/_2$ and $^1/_{16}$ of the size of the original molecule readily occurs, and these smaller particles seem to have the properties of native hemocyanin.[17]

6. The shape of the native protein molecule appears to have little sig-

nificance for an understanding of denaturation. Denaturation occurs in the spherical molecules of egg albumin and hemoglobin, in the elongated particles of soluble myosin[18] and (as indicated by SH groups becoming detectable) even in myosin that has been formed into insoluble fibres by drying.[9]

7. In certain conjugated proteins stability of the protein and presence of the prosthetic group are related. In hemoglobin, the yellow oxidizing ferment of Warburg, and visual purple, it is necessary to denature the protein in order to detach the prosthetic group with the use of present methods.[10,19] After removal of the prosthetic group it is possible to reverse the denaturation of globin and the protein of the oxidizing ferment, but these native proteins are more unstable (with respect to denaturation) than they are when conjugated with their prosthetic groups. In hemoglobin the ease of denaturation depends upon the state of the prosthetic group. Carbon monoxide hemoglobin, for example, is less readily denatured by heat, acid, or alkali than are oxyhemoglobin and ferrihemoglobin.[20] In visual purple presence of the prosthetic group causes the protein to be denatured by visible light. Denaturation in this case appears to be reversible.[10]

II. Our conception of a native protein molecule (showing specific properties) is the following. The molecule consists of one polypeptide chain which continues without interruption throughout the molecule (or, in certain cases, of two or more such chains); this chain is folded into a uniquely defined configuration, in which it is held by hydrogen bonds[21] between the peptide nitrogen and oxygen atoms and also between the free amino and carboxyl groups of the diamino and dicarboxyl amino acid residues.

We shall not enter into a long discussion of the precise configurations of native proteins, about which, indeed, little reliable information is available. From the x-ray investigations of Astbury[22] and his collaborators it seems probable that in most native proteins the polypeptide chain, with the extended or one of the contracted configurations discussed by Astbury, folds back on itself in such a way as to form a layer in which peptide nitrogen and oxygen atoms of adjacent chains are held together by hydrogen bonds; several of these layers are then superposed to form the complete molecule, the bonds between layers (aside from the continuation of the polypeptide chain from one layer to the next) being hydrogen bonds between side-chain amino and carboxyl groups. In general not all of the side chain groups will be used in forming bonds within the molecule; some will be free on the surface of the molecule.

The importance of the hydrogen bond in protein structure can hardly be overemphasized. No complete review of the large amount of recent work on this bond is available; we shall mention only the most striking of its properties.[21] The hydrogen bond consists of a hydrogen atom which bonds two electronegative atoms together (F, O, N), the hydrogen atom lying between the two bonded atoms. The bond is essentially electrostatic in nature. The bonded atoms are held more closely together than non-bonded atoms, the N-H-O distance being about 2.8 Å. The energy of a strong hydrogen bond is 5000 to 8000 cal. per mole, the lower value being approximately correct for an N-H-O bond as

in proteins. Side-chain bonds in proteins we consider to involve usually an amino and a carboxyl group, the nitrogen atom forming a hydrogen bond with each of two oxygen atoms and holding also one unshared hydrogen atom. In acid solutions hydrogen bonds may be formed between two carboxyl groups, as in the double molecules of formic acid.[23]

The characteristic specific properties of native proteins we attribute to their uniquely defined configurations.

The denatured protein molecule we consider to be characterized by the absence of a uniquely defined configuration. As the result of increase in temperature or of attack by reagents (as discussed below) the side-chain hydrogen bonds are broken, leaving the molecule free to assume any one of a very large number of configurations. It is evident that with loss of the uniquely defined configuration there would be loss of the specific properties of the native protein; it would not be possible to grow crystals from molecules of varying shapes, for example, nor to distinguish between closely related proteins when the molecules of each protein show a variability in configuration large compared with the differences in configuration of the different proteins.

Strong support of this view of the phenomenon of denaturation is provided by the known difference in entropy of native and denatured proteins, which is about 100 E. U. for trypsin, the entropy of the denatured form being the greater.[5] This very large entropy difference cannot be ascribed to a difference in the translational, vibrational, or rotational motion, but must be due to a difference in the number of accessible configurations. It corresponds to about 10^{20} accessible configurations for a denatured protein molecule. The large entropy of denaturation thus shows clearly that the phenomenon of denaturation consists in the change of the molecules of the native protein to a much less completely specified state.

The magnitude of the heat change, entropy change, and activation energy of denaturation (about 30,000 cal./mole, 100 E. U., and 150,000 cal./mole, respectively, for trypsin) can be interpreted in terms of our hydrogen-bond picture of the protein molecule. We consider the native protein molecule to be held in its definite configuration by side-chain hydrogen bonds, about fifty in number for this protein (corresponding to about twenty-five amino and twenty-five carboxyl side chains), each with a bond energy of about 5000 cal./mole, as in simpler systems. The activation energy of 150,000 cal./mole shows that in order for the molecule to lose its native configuration about thirty of the bonds must be broken. Some of the side-chain groups then again form hydrogen bonds; the heat of denaturation shows that on the average there are about six fewer such bonds in the denatured molecule than in the native molecule. (The activation energy for transition of a denatured protein molecule from one configuration to another is without doubt smaller than the activation energy of denaturation, as it

involves breaking a smaller number of hydrogen bonds, so that at a temperature at which the rate of denaturation is appreciable the denatured molecule would run rapidly through its various configurations.) The magnitude of the entropy of denaturation also fits into our picture; it corresponds to the number of configurations obtained by forming hydrogen bonds at random between about twenty amino side chains and twenty carboxyl side chains.

The reagents which cause denaturation are all substances which affect hydrogen-bond formation. Alcohol, urea, and salicylate are well-known hydrogen-bond-forming substances; they form hydrogen bonds with the protein side chains, which are thus prevented from combining with each other and holding the protein in its native configuration. Acids act by supplying protons individually to the electronegative atoms which would otherwise share protons, and bases by removing from the molecule the protons needed for hydrogen-bond formation. This conception provides an explanation of the facts that the isoelectric point of a protein shifts toward the neutral point on denaturation[24] and that the pH at which the activation energy for denaturation has its maximum value is in general not at the isoelectric point of the native protein, but between this point and the neutral point.[25] In the native protein molecule of the usual type some amino and carboxyl side-chain groups are paired together by forming hydrogen bonds. The acid-base properties of the molecule are in the main determined by the groups which are left free. On denaturation some of the paired groups are freed, amino and carboxyl in equal numbers, and in consequence the isoelectric point of the denatured protein is shifted toward neutrality. We have pictured the process of denaturation as involving the rupture of a large number of hydrogen bonds, to form a labile activated molecule. This labile molecule is stabilized by action of base on its free carboxyl groups or of acid on its free amino groups, the activation energy thus having a maximum at a pH value between the neutral point and the isoelectric point of the native protein.

The action of ultra-violet light must be different. It is not possible to formulate a reasonable mechanism whereby a quantum of light can break twenty or thirty hydrogen bonds. Instead the light must attack the molecule in a different place, probably breaking the main polypeptide chain after absorption in a tyrosine or other phenolic residue, as suggested by Mitchell.[26] That this occurs is indicated by the observation[27] that after illumination with ultra-violet light in the cold denaturation occurs only on warming, though then at a lower temperature than without illumination; it is clear that illumination in the cold causes a break in the molecule, which, however, is restrained to configurations near to its native configuration by the side-chain hydrogen bonds. (That some loosening of the molecule occurs is shown by the observation of an increase in the available

groups in egg albumin after illumination in the cold.) On warming, these bonds are broken; because of the break in the molecule, however, it can be denatured "in parts," and hence at a lower temperature than before illumination. We predict that it will be found that denaturation by illumination with ultra-violet light is in general not reversible.

In a conjugated protein the prosthetic group plays a part in holding the molecule in the native configuration (hemoglobin, yellow oxidizing ferment, visual purple). It is possible in such a protein for reversible denaturation to take place after absorption of light by the prosthetic group, no permanent damage being done to the molecule.

In a protein coagulum side-chain hydrogen bonds hold adjacent molecules together. Native proteins do not coagulate because most of the side chains are in protected positions inside of the molecule; denatured proteins (at the isolectric point) do coagulate because they have a larger number of free side chains and because in the course of time, as the molecule assumes various configurations, all of the side chains become free. The increase of viscosity of protein solutions on denaturation we attribute to the change from the compact configuration of the native protein molecules to more extended configurations.

A native protein molecule of small molecular weight may have free side chains so arranged as to permit it to combine with similar molecules to form a polymer with properties differing little from those of the small molecules. The observations of Svedberg and his collaborators indicate that this is the case for hemocyanin, casein, and certain other proteins.

Our theory of denaturation leads to definite predictions regarding the availability of groups to attack by reagents. In the large compact molecule of a native protein, of the order of magnitude of 50 Å in diameter and having the same structure as every other molecule of the protein, all groups would be protected from attack by reagents except those on the surface or near the surface. After denaturation of the protein, however, the molecule (in solution) would in the course of time assume various configurations, and every group in the molecule would become available to attack. These statements are in complete agreement with the experimental results regarding sulfhydryl, disulfide, and phenol groups mentioned above. The observation that the number of available groups is increased when the solution is made alkaline, though not alkaline enough to cause denaturation, shows that under these conditions some of the hydrogen bonds in the protein molecule are broken, causing it to assume a configuration somewhat more open than its original configuration.

Although a denatured protein molecule in a coagulum is not free to assume all configurations, being restrained by bonds to its neighbors, in general its configuration will be so open as to make all groups accessible to attack. However, as mentioned above, there is no change in the avail-

ability of groups in myosin when it is rendered insoluble by drying or when water is removed by freezing. We interpret this as showing that *coagulation of this type is not accompanied by denaturation; that is, the molecules do not lose their uniquely defined configurations.* On dehydration of the native protein the surface side chains of adjacent molecules form bonds sufficiently strong to produce a coagulum of native protein molecules. It would be expected that the mechanical bolstering effect of adjacent molecules in this coagulum would aid the molecules to retain their native configurations; this is observed to be the case for egg albumin and some other proteins, which in this state are denatured by heat only at a temperature considerably higher than before dehydration.[6]

Some features of the theory of protein structure discussed above have been suggested before. Many investigators have correlated the specific properties of native proteins with definitely specified molecular configurations and the loss of specific properties on denaturation with change in configuration. Astbury and his collaborators in particular, in discussing their x-ray investigations, which have provided so much valuable information on protein structure, have stated[22] that dehydration and temperature denaturation lead to destruction of the original special configuration of the native protein, giving a débris consisting simply of peptide chains. Our picture agrees with theirs except in regard to the effect of dehydration, which we believe to consist primarily in the coagulation of molecules with essentially unchanged structure. The hydrogen bonds which we postulate to exist between side-chain carboxyl and amino groups (each nitrogen atom attached by hydrogen to two oxygen atoms, with the distances N-H-O equal to 2.8 Å) are a refinement of the side-chain salt-like linkages discussed by Astbury. Our picture of the spreading of protein films on water, involving the unfolding of compact molecules to layers one amino-acid-residue thick, is not essentially different from that of Gorter and Neurath, who have suggested unfolding in connection with film formation, though not with denaturation in general.[28]

In this paper we have discussed in some detail a very drastic change in configuration of protein molecules, that connected with denaturation. We have pointed out that the large entropy of denaturation provides strong support for our suggested structures of native and denatured protein molecules, and that many other phenomena can also be interpreted in a simple way from this point of view. There also has been put forth recently some evidence that small changes in configuration of proteins occur, which play an important part in protein behavior. Thus Northrop and his collaborators have prepared an enzymatically inactive protein which on slight hydrolysis by trypsin is transformed into native trypsin,[29] and another which is similarly transformed by pepsin into native pepsin,[30] and Gorter[31] has shown that myosin spreads on water only after slight hydrolysis. The

structural interpretation of these phenomena can be made only after further experimental information is available.

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