

## ON SOME GENERAL PROPERTIES OF PROTEINS.

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In this paper we shall consider some general properties of proteins—their denaturation, coagulation, structure, and specificity—on the basis of our knowledge of these properties of hemoglobin (1).

### I.

#### *The Coagulation of Proteins.*

The coagulation of proteins has been shown to consist of two distinct processes (2, 3). In the first of these the protein is denatured, and in the second the denatured protein is agglutinated or flocculated. Chick and Martin (3) and more recently Lepeschkin (4) have investigated these two processes separately, and it is to them that we owe much of our knowledge in this field. Most of the work has been done on egg albumin. The process of denaturation is greatly accelerated by a rise in temperature, by an increase in hydrogen ion concentration (when the medium is acid), by an increase in hydroxyl ion concentration (when the medium is alkaline), and by alcohol. In general acids increase the rate of denaturation much more than do bases. If the hydrogen ion concentration is kept constant, denaturation proceeds as a monomolecular reaction. The temperature coefficient of the reaction is 1.91 for 1°C. or about 635 for 10°C. The velocity of most chemical reactions is increased two to three times by an increase of temperature of 10°C. The temperature coefficient of denaturation is perhaps the greatest of any reaction that has yet been measured.

Very little is known about the chemical changes occurring in a protein during denaturation. Sørensen does not believe that the

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protein on denaturation gives off any ammonia or other nitrogenous substances (5). "Under these circumstances then, it seems to us most natural to assume that the actual denaturation itself by heating is, like the denaturation with alcohol, not accompanied by any decomposition, but that the decomposition is a secondary process, whereby the egg albumin during and after denaturation is decomposed by the action of hot water." He did, however, show that in denaturation by heating and by alcohol the protein gives off water. So far practically no explanation has been given of denaturation. It is, however, obvious that the protein is radically changed. Whereas it was formerly a hydrophilic colloid it now has many of the properties associated with hydrophobic colloids. Denatured proteins, though very soluble in acids and bases, are readily flocculated at the isoelectric point. The addition of salt extends the limits for complete flocculation.

## II.

### *The Coagulation of Hemoglobin.*

The coagulation of hemoglobin is in practically all respects like that of any other protein. Chick and Martin found that dry crystals of egg albumin remained unchanged after 5 hours heating at 120°C. and that dry crystals of methemoglobin were unchanged by subjection for 4 hours to a temperature of 110°C. Hartridge<sup>1</sup> (6) found that the denaturation of *methemoglobin*, like that of egg albumin, is a monomolecular reaction and that the temperature coefficient has the same enormously high value, 1.93 for 1°C. Hemoglobin is first denatured and then coagulated by alcohol. Both acids and bases may be said to denature hemoglobin, for when the solution is later brought to what appears to be the isoelectric point flocculation occurs. The denaturation of oxyhemoglobin has been investigated by Chick and Martin and by Hartridge and that of carbon

<sup>1</sup> The reader of Hartridge's paper will find that when Hartridge tried to measure the rate of coagulation of "nitric oxide hæmoglobin" he was unable to do so because this compound changed into methemoglobin when warmed. This puzzling result is explained in a recent paper on the combination of nitric oxide with hemoglobin (7).

monoxide hemoglobin by Hartridge. It was found in all cases that denaturation is a monomolecular process, but the temperature coefficients of the reactions were found to be much less than the values found for egg albumin and methemoglobin. The rate of denaturation of  $\text{HbO}_2$  was increased 1.3 times for a rise of  $1^\circ$  and that of  $\text{HbCO}$  1.18 times. These values are much closer to those found for ordinary chemical reactions. It might seem that the process of denaturation of  $\text{HbO}_2$  and  $\text{HbCO}$  was different from that of egg albumin and methemoglobin. But if these experiments are repeated and the changes carefully observed, it appears that Chick and Martin and Hartridge were in reality not measuring the rate of denaturation of  $\text{HbO}_2$  or  $\text{HbCO}$  but rather the rate of conversion of  $\text{HbO}_2$  or  $\text{HbCO}$  into methemoglobin. Spectroscopic examination of the solutions showed us unmistakably that when  $\text{HbO}_2$  or  $\text{HbCO}$  is heated methemoglobin slowly appears and this then coagulates. The "denaturation" of  $\text{HbO}_2$  or  $\text{HbCO}$  consists then of two consecutive reactions, and if the temperature coefficient of the whole process is measured, the result will clearly be the temperature coefficient of the slower reaction. The monomolecular reaction, the temperature of which was measured, was, then, the change from  $\text{HbO}_2$  or  $\text{HbCO}$  to methemoglobin. A detailed analysis of the results of Chick and Martin and of Hartridge confirms this view. Hartridge found that for a given velocity of denaturation he had to heat the  $\text{HbCO}$  to a higher temperature than the  $\text{HbO}_2$ . This is to be expected since it is more difficult to convert  $\text{HbCO}$  than  $\text{HbO}_2$  into methemoglobin. Moreover, the temperature coefficient of "denaturation" of  $\text{HbO}_2$  was over 1.3 while that of  $\text{HbCO}$  was only 1.18. It is probable that the true temperature coefficient of the conversion of  $\text{HbO}_2$  into methemoglobin is lower than 1.3. It would be expected that a value as high as 1.3 would be obtained because in the test-tube there would actually be a mixture of  $\text{HbO}_2$  and methemoglobin so that there would result a value intermediate between the temperature coefficients of the reaction from  $\text{HbO}_2$  to methemoglobin and of the denaturation of the latter. In the case of  $\text{HbCO}$  such a mixture would not so easily occur, and therefore 1.18 is probably very close to the true value for the temperature coefficient of the change from  $\text{HbCO}$  to

methemoglobin.<sup>2</sup> In this way we can easily explain why Chick and Martin and Hartridge found that the temperature coefficients of "denaturation" of HbO<sub>2</sub> and HbCO were so much lower than that of egg albumin. In studying the denaturation of hemoglobin it is therefore on methemoglobin that we should fix our attention, and when we do so we find that the process of denaturation of methemoglobin seems to be the same as that of egg albumin.

### III.

#### *The Products of Hemoglobin Coagulation.*

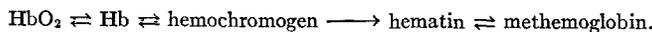
When egg albumin is coagulated, denatured egg albumin is first formed. What substances correspond to denatured egg albumin when hemoglobin is coagulated? Hoppe-Seyler (8) found that when reduced hemoglobin is coagulated hemochromogen is formed, and that when HbCO is heated in the absence of oxygen CO hemochromogen is formed. We found that when HbO<sub>2</sub>, HbCO, or methemoglobin is coagulated in the presence of oxygen a brown coagulum is obtained. If some sodium hydrosulfite is put on this and the coagulum is then examined with the microspectroscope it appears that the coagulum was hematin since the reduced product is hemochromogen. If the alcohol coagulum is washed with water and reduced with sodium hydrosulfite it is found that here too the coagulum consists of hemochromogen. Indeed Hoppe-Seyler (8) noticed that the red color of muscles preserved in alcohol in museum jars is due to hemochromogen. When hemoglobin then is coagulated the body corresponding to denatured egg albumin is either hemochromogen or hematin.

### IV.

#### *The Nature of Hemochromogen and Hematin.*

It has recently been shown (1) that hemochromogen is not merely the iron-pyrrol complex of hemoglobin, but that it is a conjugated

<sup>2</sup> We have recently (1) presented a theory of methemoglobin formation. It was supposed that methemoglobin was formed by the following process.



We still think that this mechanism is possible, but the considerations set forth above show that hemoglobin can be more directly converted into methemoglobin.

protein consisting of hem (the iron-pyrrol complex) and globin. Hemochromogen probably has a molecular weight of about 17,000<sup>3</sup> (1). Hemoglobin, whose molecular weight is 67,000 (9) is formed by the polymerization of four molecules of hemochromogen. If this is true, then the process of formation of hemochromogen from hemoglobin or of hematin from methemoglobin is simply a process by which a large protein molecule is depolymerized into four smaller ones. It has been shown above that this process is completely analogous to the process of denaturation of egg albumin, that it is, indeed, the process of denaturation of hemoglobin. On the other hand, hemochromogen has all the properties of a denatured protein. It plays the same rôle in the coagulation of hemoglobin that denatured egg albumin does in the denaturation of egg albumin. Both denatured egg albumin and hemochromogen are formed by heat, alcohol, acids, and alkalies. In both cases acids are more effective than alkalies. Like denatured egg albumin hematin is very soluble in acids and alkalies, but flocculated at the isoelectric point. Just as a solution of denatured egg albumin is much more viscous than the solution of native egg albumin from which it is prepared, so a solution of hemochromogen is much more viscous than the solution of hemoglobin from which it is prepared. A concentrated solution of denatured egg albumin sets to a gel; similarly if alkali is added to a fairly concentrated solution of reduced hemoglobin the hemochromogen formed sets to a gel. Hemochromogen can thus be regarded as denatured hemoglobin. Since the processes of denaturation of egg albumin and hemoglobin are so similar, it is highly probable that exactly the same thing happens in both cases. It would seem, therefore, that when egg albumin is denatured it is depolymerized. Just as hemoglobin can be regarded as a polymer of its denatured form (hemochromogen), so native egg albumin is probably a polymer of denatured egg albumin. In any case, entirely independent of any molecular weight measurements, of any polymerization theory is the general argument that whatever the essential difference between hemoglobin and hemochromogen there is that same difference between the native and the denatured forms of egg albumin.

<sup>3</sup> This value rests on a *preliminary* osmotic pressure determination on acid hematin which Adair made at our suggestion. The exact value may prove to be different, but it is probably not very different.

## V.

*The Nature of Proteins.*

Since the coagulable proteins behave in these respects like egg albumin and hemoglobin the same views can be extended to them. Thus, among others, we can regard the albumins and globulins as polymerized proteins. Plant proteins such as edestin (10) form no exception. Even such a peculiar protein as the Bence-Jones protein is of this nature (11). The problem that lies before us is to determine the molecular weights of the various denatured proteins and in this way find out for each protein how many denatured molecules polymerize to form a native protein. We are now doing this by osmotic pressure measurements.

## VI.

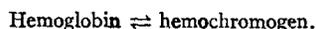
*The Globins and Histones.*

The group of proteins known as the histones seems to form an exception to this view of the proteins. But the exception is more apparent than real. These proteins are not coagulated by heat (12). They are soluble in acids and rather strong bases. They are flocculated at their isoelectric points and the flocculation is greatly increased by small concentrations of salts. It would seem that these proteins have the properties of denatured proteins. As a matter of fact they probably are denatured, and there is nothing surprising about this, for 10 per cent  $H_2SO_4$  is used in preparing them (13) and such treatment would denature any protein. Their properties are similar to those of the globins (14), and the latter too must be regarded as denatured proteins. Here again this is what would be expected, for in the preparation of globin from hemoglobin hematin is first formed, and hematin is a denatured protein. The globins and histones should not form a separate group of proteins. They are merely denatured proteins containing large amounts of the more alkaline amino acids

## VII.

*The Equilibrium between Genuine and Denatured Protein.*

It has been shown (1) that in a hemoglobin system there is an equilibrium of this nature:



When the system is heated, or when acid, alkali, or alcohol is added to it the equilibrium is shifted to the right; the hemoglobin is denatured. It is only at a moderately low temperature and near the neutral point that much hemoglobin exists. If an alkaline solution of hemochromogen is neutralized, most of the hemochromogen is precipitated. A small amount, however, goes back to form hemoglobin, which can readily be detected spectroscopically. This experiment shows that the denaturation of hemoglobin is a reversible process. The reversibility is, however, masked by the flocculation of denatured hemoglobin—hemochromogen. The denaturation of proteins is regarded as an irreversible process, but it is the flocculation of denatured protein that obscures the reversibility of the process.

The ease with which the equilibrium is shifted varies from protein to protein. Reduced hemoglobin is much more easily denatured, it is known, than is oxyhemoglobin, edestin and euglobulin more easily than egg albumin (15); and in biological reactions other substances may influence the ease of the shift. In the agglutination of bacteria Bordet found that after an agglutinin reacts with the bacteria they can be flocculated only if a small concentration of salt is present. This process (as has often been noted) is in many ways similar to the coagulation of proteins. We merely want to point out that an agglutinin seems to be concerned with the equilibrium between genuine and denatured protein. The equilibrium between genuine and denatured protein will be affected if the denatured protein decreases the surface tension of the solvent. This is the case in the egg albumin system where the denatured protein which is but slightly soluble goes to the surface and is precipitated so that a film of denatured egg albumin appears at the interface (water-gas, or water-collodion). If the film is continually removed by shaking the equilibrium between genuine and denatured egg albumin will be shifted entirely in the direction of the latter, so that practically no more egg albumin remains in solution (16).

#### VIII.

It would be very interesting to know what group is uncovered when a protein is denatured. The presence of this group on the surface of the molecule converts the protein into an entirely dif-

ferent substance. It would appear to be a non-polar group, for when it is uncovered by denaturation the resulting molecules have much less affinity for water than before; they become less soluble, concentrate at interfaces, and lose water (5). The uncovering of this group also seems to prevent the molecules from manifesting their specificity. The differences between genuine and native proteins as regards solubility, reaction with salts, etc., have often been pointed out. The difference in specificity which, however, is very important from the biological point of view, has scarcely been noticed.

## IX.

*The Specificity of Proteins.*

Perhaps the two most delicate manifestations of protein specificity are the absorption spectra of the hemoglobins and the precipitin reactions of proteins in general. It has recently (17) been shown that the positions of the absorption bands of oxy- and carboxy-hemoglobin vary from species to species and even within the individuals of the same species. These differences have been correlated with the affinities of the hemoglobins for oxygen and carbon monoxide. If these hemoglobins are denatured it is found (1) that the resulting hemochromogens have their absorption bands in the same position—to 1 Å. u.—and that they have the same affinities for carbon monoxide. Denaturing hemoglobin—*i.e.* converting it into hemochromogen—renders it non-specific. The chemical basis of specificity has, however, not been destroyed, for when the denaturation is reversed by converting the hemochromogen into hemoglobin the latter is as highly specific as it was originally. If, for instance, we compare the hemoglobins of rabbit and man, we find that their HbCO absorption bands are in different positions and that their affinities for carbon monoxide are different. The hemochromogens made by treating these hemoglobins with alkali are globin compounds containing *different* globins, and yet their absorption bands are in the same position (within the experimental error of 1 Å. u.) and they have the same affinities for carbon monoxide. When the solutions of these hemochromogens are neutralized the hemoglobins reappear (*i.e.* the denaturation is reversed), and these are found to be exactly as they

had been before. Here, then, we have proteins that are highly specific when native, but not detectably specific when denatured. The same phenomenon has been noticed with the precipitin reaction (18). Whereas genuine egg albumins of different species give rise to highly specific precipitins, the precipitins produced by the injection of denatured egg albumin have only a group specificity. Apparently, then, a protein must be in its native form if it is to be able to manifest its specificity.

If, as was stated above, the globins are really denatured proteins, they should not have any species specificity. Browning and Wilson (19) found that in the complement fixation reaction globins do not show species specificity.

The difference in specificity between genuine and denatured proteins brings us to the highly interesting work of Obermeyer and Pick (20) and Landsteiner (21). They showed that the introduction of chemical groups into proteins endowed those proteins with antigenic properties characteristic of the groups introduced. The species specificity of the protein was lost; the specificity of the group introduced was the only specificity detectable. Landsteiner found "dass man die Azoproteine in gleicher Weise durch Serumreaktionen scharf unterscheiden kann, wie artverschiedene natürliche Eiweisskörper, und sie beweisen, dass die serologische Spezifität der untersuchten Substanzen wirklich von der chemischen Beschaffenheit der bei der Kupplung eingetretenen Gruppen abhängig ist." Now it is interesting to note that in preparing his protein solutions Landsteiner treated them with an equal volume of 1 N NaOH. Since much less alkali would have sufficed to denature the proteins, there can be little doubt that Landsteiner's proteins were denatured. Since denatured proteins do not show species specificity,<sup>4</sup> it would be expected that if Landsteiner's proteins manifested any specificity at all, within any one group of proteins, *viz.*, the albumins, it would be the specificity of the groups introduced. It was probably the denaturation rather than the introduction of new groups that obliterated the species specificity of the native proteins. There is

<sup>4</sup> Just how much specificity a denatured protein possesses is not known. We are discussing species specificity and not the specificity of groups of proteins.

no evidence that the introduction of a group into a *native* protein would wipe out the species specificity of that protein. Hemoglobin is just such a protein if we consider hem to be the group. Hemoglobin, however, has marked species specificity as shown by direct chemical methods and by serological ones (22).

## X.

## CONCLUSIONS.

1. The processes of denaturation and coagulation of hemoglobin are like those of other proteins.
2. When hemoglobin is denatured it is probably depolymerized into hemochromogen.
3. When other proteins are denatured they, too, are probably depolymerized. Conversely, native proteins can be regarded as aggregates of denatured proteins.
4. The globins and histones are to be regarded as denatured proteins rather than as a distinct group of proteins.
5. The factors affecting the equilibrium between native and denatured proteins have been considered.
6. A non-polar group is uncovered when a protein is denatured.
7. It has been shown that judged by the two most sensitive tests for the specificity of proteins, it is only when proteins are in the native form that they are highly specific.

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