Chick and Martin (1) have shown that the heat coagulation of proteins consists of two distinct processes; viz., (a) denaturation, the alteration of the protein under the influence of heat, and (b) agglutination, the separation of the altered protein in a particulate form. Agglutination, in which hydrogen ion concentration and electrolyte contents play important rôles (2), is now a fairly well understood phenomenon of colloidal chemistry, while the significance of denaturation is still obscure.

Although denaturation is made evident by agglutination, the latter is not a necessary consequence of the former. In this paper we are concerned only with the alteration which heat-coagulable proteins undergo under the influence of heat, and we will designate it as heat denaturation to distinguish it on the one hand from denaturation by other agencies and on the other from heat coagulation, which would include the physical process of agglutination. No little confusion in the literature has resulted from the failure to distinguish the two processes comprised in heat coagulation of proteins.

The word denaturation is used loosely to designate the change of proteins from a soluble to an insoluble form brought about by a large variety of chemical and physical agents, including acids, alkalies, alcohol, acetone, salts of heavy metals, alkaloidal reagents and dyes (3), and heat, light, and pressure (4). Chick and Martin (5) consider heat denaturation as a reaction between protein and water which implies in all probability a hydrolysis. Robertson,¹

¹ Robertson (4), pp. 304-311.
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on the contrary, supports the old view of Hofmeister that heat denaturation is a phenomenon of dehydration. Excluding the possibility of intramolecular rearrangement for which there is no experimental evidence, the heat denaturation must then be either a hydrolysis or a dehydration.

In a previous paper (6) we have shown that when a protein is denatured by dilute acids and alkalies at ordinary temperatures, an increase occurs in the acid- and base-binding powers as well as in the reactivity toward the phenol reagent of Folin and Denis. Since both these changes are known to accompany the hydrolysis of proteins, we concluded that the denaturation of proteins by dilute acids and alkalies is a hydrolytic process. We have reason to believe that the denaturation of proteins in the presence of H₂O and under the influence of most agents is a hydrolytic process. In this paper we will show that the heat denaturation of proteins is exactly similar to the denaturation of proteins by acids and alkalies and that the two apparently different processes are one, that of hydrolysis. Before presenting our experiments, however, we will review briefly the evidences bearing upon the dehydration theory.

Robertson⁵ has found that casein dissolved in anhydrous formic acid is not precipitated by cupric chloride until sufficient water has been added to cause a change of the color of the solution from green to blue. If alcohol is added instead of water, no precipitate is formed until the color of the solution has changed from green to brown. Since water and alcohol are obviously hydrating and dehydrating agents, respectively, and since the changes of the color of cupric chloride indicate changes in the degree of hydration as generally believed, Robertson concluded “that protein may be thrown out of solution by electrolytes in two grades of hydration, the one of high, and the other of very low hydration.” (The former process he called precipitation, and the latter, coagulation). From these experiments he concluded also that the process of heat coagulation is not one of hydration but of dehydration of the protein.

Robertson’s interpretation of the precipitation of casein by cupric chloride is probably correct, but there is little similarity between the heat denaturation of proteins in an aqueous solution and the precipitation of casein in anhydrous formic acid solution with cupric chloride to warrant the transfer of the conclusion from one process to the other. The precipitate in Robertson’s experiments is cupric caseinate, while the heat-coagulated protein is protein as such. The precipitation of casein from formic acid solution by cupric chloride and water or alcohol is a revers-

⁵ Robertson (4), pp. 125-129.
ible process, for the precipitate produced by water redissolves on adding more formic acid, and that produced by alcohol redissolves on adding water. Heat coagulation is, on the contrary, an irreversible process.

Robertson argues that the irreversibility of heat coagulation (denaturation) is only apparent, and he cites the observation of Corin and Ansiaux (7) that if a solution of protein be cooled and vigorously shaken just as the first traces of heat coagulation (flocculation) appear, the incipient coagula will pass again into solution.

We have repeated this experiment with only negative results, but it has occurred to us that if the denatured protein will revert to the original soluble form by cooling and shaking after it has flocculated to give a turbid solution, it should revert so much more readily, if the denatured protein is not allowed to flocculate at all. We have heated a solution of egg albumin to which a minimal amount of acid or alkali (0.5 cc. of 0.1 N HCl or NaOH for 50 cc. of 0.1 per cent egg albumin solution) has been added to prevent flocculation. On neutralizing a part of the heated solution the protein was precipitated, showing that denaturation has occurred. The remainder of the solution was cooled and left in an ice box under sterile conditions. After 2 months the solution was neutralized and the protein precipitated as before. The heat-denatured protein, therefore, cannot revert to its original form and the process is accordingly an irreversible one.

As another evidence of the reversible character of heat denaturation Robertson cites the observation of Berczeller (8) that the surface tension of a protein solution which is so salt-free as not to coagulate on heating, nevertheless diminishes on heating, and rises again on cooling and standing. The true explanation of this is found in Berczeller's paper. Although his protein solutions did not flocculate, they did become turbid, and the rise of the surface tension took place as the result of the protein particles becoming large enough to separate out from the solution. This is,

3 It cannot be urged that the denaturation of the protein which occurs in solutions containing such a minute amount of acid or alkali is essentially different from that which occurs in a neutral solution or at the isoelectric point of the protein. Since proteins are denatured in acid as well as in alkaline solutions, it follows that the rate of denaturation must pass through a minimum as we go from the acid to the alkaline side. There is no reason to believe, however, that the character of the reaction should change abruptly at any point in the pH range. Furthermore, heat coagulation as ordinarily carried out in practice certainly does not represent a reaction of the isoelectric protein.
Heat Denaturation of Proteins

Robertson (9) found that the solubility of casein in alkaline solutions was considerably augmented by carrying out the process of solution at temperatures above 40°C. Osborne (10) observed an increase in alkalinity and electrical conductivity of caseinate solutions upon heating. In explaining these facts Robertson assumes that there is an equilibrium between protein and water of the type

\[ HXOH + HXOH \rightleftharpoons HXXOH + H_2O \]

and that the influence of heat on protein consists in shifting the equilibrium from left to right, that is, in the direction of higher complexes, with consequent decrease in the base-binding power and increase in the amount of casein dissolved for a given amount of alkali. This explanation is plausible, but the fact that in none of the solutions in which more casein was dissolved at higher temperature than would be dissolved at room temperature did he observe any appreciable tendency towards precipitation of casein on cooling, seems to show that the casein has undergone some decomposition. Even granting that his explanation is correct as applied to casein, the conclusion is still unwarranted that heat denaturation is the result of repeated condensation of the protein molecule, since free casein itself is insoluble (10) and is like a denatured protein.

Chick and Martin (1) have shown that heat coagulation (denaturation) is a monomolecular reaction. This would exclude the possibility of polymerization which is a reaction of higher order.

That water is essential for the heat denaturation of proteins has been known for some time. According to Wichmann (11) albumin crystals can be heated to 150°C in the dry condition without change. Osborne (12) found in the case of proteins soluble in alcohol that such alcoholic solutions may be boiled without precipitating the protein, but, if diluted with water, some coagulation occurs when the solution is heated to the same temperature. Confirmatory to this observation, Chick and Martin (5) found that crystallized egg albumin was completely soluble after 5 hours heating at 120°C, although in the presence of steam the albumin became completely insoluble in a few moments. Robertson tried to harmonize this observation of Chick and Martin with his dehydration theory by assuming that dry protein has been deprived of the elements of water in its end -NH₂ and -COOH groups, and that these, consequently, cannot react to form polymers of the protein. We have already pointed out that heat denaturation, being monomolecular, cannot be a process of polymerization. If heat denaturation is a process of dehydration at all, it can only be one by internal neutralization of the NH₂ and COOH groups. The protein should then be denatured as it loses water on drying which is contrary to fact.

Nor can the failure of the dry protein to be denatured be accounted for by the reaction occurring in a solid system which is extremely slow.
as Robertson contends further. Chick and Martin found that egg albumin was completely denatured in the presence of steam. Here the albumin was not dissolved in water, and the reaction occurred not in a liquid system, but in a solid system, or between gas (water vapor) and solid according to our interpretation.

There is another line of evidence which Robertson mentioned in support of his theory. From the observations of some investigators that heat is evolved when a protein is hydrolyzed, Robertson concludes that the hydrolysis of protein is an exothermic reaction and that, therefore, according to van't Hoff's principle of mobile equilibrium, the effect of heat must be to shift the equilibrium point of the system protein-amino acids in the direction of condensation. Since the heat of reaction of protein hydrolysis is extremely small, the reliability of the few available data is open to question, especially as some other observers have failed to detect any evolution of heat. Even if the evolution of heat in protein hydrolysis is real, we still have to know the secondary reactions which occur and the amount of substances entering into these reactions, before the heat of the main reaction—hydrolysis—can be calculated. In the experiment on trypic digestion of casein reported by Henderson and Ryder (13) for instance, some heat would be produced from the neutralization of sodium carbonate by the phosphoric acid and dicarboxylic amino acids liberated from casein, and the slight evolution of heat which they observed is certainly no convincing proof of the exothermic character of protein hydrolysis.

The fact that proteins are hydrolyzed far more readily at 100° than at ordinary temperature shows that heat favors hydrolysis. Whether this is due to the acceleration of the reaction toward equilibrium or to a shift of its equilibrium point in favor of hydrolysis is a matter of no moment in the present discussion.

It is seen from the above review that all the available evidence is in favor of the hydrolysis theory of heat denaturation of proteins.

We have observed many points of similarity between the denaturation of proteins by heat and that by dilute acids and alkalis at ordinary temperatures. The denatured protein in either case is insoluble in neutral water or salt solution or, more accurately, at the isoelectric point of the protein, but redissolves in the presence of slight excess of acid or alkali. The velocity of denaturation in both processes is increased by increasing the hydroxyl ion concentration in alkaline solution, and the hydrogen ion concentration in acid solution (1, 6, 14). In both processes a decrease of hydrogen ion concentration occurs in acid solution and a decrease of hydroxyl ion concentration in alkaline solution (1, 6, 14). We have found further that the chromogenic value of
proteins (when allowed to react with the phenol reagent of Folin and Denis) increases when denatured by heat as well as when denatured by acids and alkalies, and that the increase in the case of heat-denatured protein does not disappear on cooling, showing the irreversible character of the reaction. Non-protein substances are liberated when the protein is denatured by either process.

The similarity between heat denaturation and denaturation by acids and alkalies has been observed not only in the general features mentioned above, but also in certain anomalous cases. We have reported previously that horse serum albumin remains unchanged in 0.05 N HCl solution as far as solubility is concerned (also 1 per cent in Na₂SO₄, which, however, has no bearing upon the present discussion), even after the solution has stood 2 months, although in 0.02 N HCl the albumin is denatured and precipitates out, with or without neutralization of the acid. Exactly the same relation holds for the heat denaturation of horse serum albumin. The albumin is not denatured by heat in 0.05 N HCl solution, although in 0.02 N HCl it is denatured and the denatured protein flocculates with or without neutralization of the acid. Pigeon's egg albumin undergoes chemical change in 0.05 N NaOH solution as shown by a marked production of ammonia and a decrease of titrable alkalinity, but the solution gives no precipitate or turbidity on neutralization. The behavior of pigeon's egg albumin on heating is exactly the same. Neutral or acid solutions become turbid on heating, but when an alkaline solution is heated, no opalescence is observed even after neutralization. We cannot at present explain these anomalies, but whatever be the underlying reason, they go to show that the denaturation of proteins by heat and that by dilute acids and alkalies are the same kind of process.

Chick and Martin have shown that heat denaturation is not purely a temperature effect which begins suddenly at a particular temperature, the so called coagulation temperature, but that it is a chemical reaction with a large temperature coefficient. The reaction which underlies the heat denaturation of proteins, must, therefore, take place also at ordinary temperatures, though at an extremely slow rate. In the presence of sufficient hydrogen ion or hydroxyl ion concentration, however, the reaction is accelerated. Conversely, the denaturation of the protein in faintly acid or alkaline solution is very slow at ordinary temperatures, but the reaction is accelerated by a rise in temperature.
EXPERIMENTAL.

Effect of Heat Denaturation on Chromogenic Value of Protein.—Three tubes of hen’s egg albumin solution were prepared as follows: (1) 1 cc. of 1 per cent albumin plus 15 cc. of H₂O; (2) 1 cc. of 1 per cent albumin plus 15 cc. of H₂O plus 0.1 cc. of NH₄Ac; and (3) 1 cc. of 1 per cent albumin plus 15 cc. of H₂O plus 0.1 cc. of Na₂CO₃.

Tubes 2 and 3 were placed in boiling water for 5 minutes. After cooling 0.5 cc. of phenol reagent and 3 cc. of 20 per cent Na₂CO₃ were added to all three tubes, made up to 25 cc., and mixed. Using Solution 1 as a standard set at 20 mm., Solutions 2 and 3 read 16.1 and 13.0 mm., respectively, in a Duboscq colorimeter. The HAc and Na₂CO₃ were used to prevent the flocculation of the denatured protein which would otherwise interfere with the calorimetric determination. Heating the solution for 10 minutes gave practically the same results, showing that the denaturation has long been completed. In another experiment the solutions were cooled, and the protein was precipitated with 2 cc. each of 10 per cent sodium tungstate and 2/3 N sulfuric acid, and the precipitates were redissolved in 2 cc. of the tungstate for the determination of the chromogenic value. Practically the same results were obtained. These experiments show (1) that heat denaturation increases the chromogenic value of the protein, (2) that the protein denatured in acid solution differs from that denatured in alkaline solution, and (3) that the increased chromogenicity lies mainly in the protein itself, although some non-protein chromogenic substances are liberated, as the following experiment shows.

Liberation of Non-Protein Chromogenic Substances from Protein on Heating.—20 cc. of 1 per cent hen’s egg albumin solution plus 25 cc. of H₂O in a 100 cc. Erlenmeyer flask were heated under cover for 10 minutes in boiling water. After cooling, 2.5 cc. of 2/3 N sulfuric acid were added, followed by 2.5 cc. of 10 per cent sodium tungstate. The resulting precipitate was filtered. A non-protein filtrate was prepared similarly from an unheated albumin solution. To 35 cc. of each filtrate were added 0.25 cc. of phenol reagent and 3 cc. of 20 per cent sodium carbonate solution. Compared with suitable standards the color intensity of the filtrate from the unheated albumin was found to correspond to 0.14 mg. of tyrosine while that from the heated albumin corresponded to 0.20 mg. of tyrosine. Calculation shows about 0.43 mg. of “tyrosine” was split off from 1 gm. of albumin.
Increase in Acid- and Base-Binding Powers of Protein Attending Heat Denaturation.—Chick and Martin have shown that a decrease of hydrogen ion concentration takes place when a protein is coagulated in acid solution, and a decrease of hydroxyl ion in alkaline solution. This can be simply demonstrated in the following way. Place in each of four test-tubes 5 cc. of 0.2 per cent egg albumin solution. Add to two tubes 0.1 cc. of 0.1 N HCl and 1 drop of methyl red. To the other two tubes add 0.1 cc. of 0.1 N NaOH and 1 drop of phenolphthalein. Place one tube of each pair in boiling water for a few minutes and cool. Compared with the unheated solutions the change in reaction of the heated solutions is striking.

Anomalous Behavior of Horse Serum Albumin on Heating in Acid Solution.—In the following set of experiments it will be seen that horse serum albumin behaves normally in alkaline or faintly acid solutions, but in 0.05 N HCl solution denaturation does not occur.

(1). 5 cc. of 1 per cent horse serum albumin (faintly acid), heated, flocculated without neutralization.
(2). 5 cc. of 1 per cent horse serum albumin + 0.5 cc. of 0.1 N HCl, heated, gave the same result as (1).
(3). 5 cc. of 1 per cent horse serum albumin + 1 cc. of 0.1 N HCl, heated, gave the same result as (1).
(4). 5 cc. of 1 per cent horse serum albumin + 2 cc. of 0.1 N HCl, heated, flocculated poorly without neutralization.
(5). 5 cc. of 1 per cent horse serum albumin + 5 cc. of 0.1 N HCl, heated, did not flocculate with or without neutralization. But the neutralized solution did flocculate on further heating, showing that the albumin was not denatured by the first heating.
(6). 5 cc. of 1 per cent horse serum albumin + 0.5 cc. of 0.1 N NaOH, heated, flocculated on neutralization.
(7). 5 cc. of 1 per cent horse serum albumin + 5 cc. of 0.1 N NaOH, heated, gave the same result as (6).

Anomalous Behavior of Pigeon’s Egg Albumin on Heating in Alkaline Solution.—The following set of experiments shows that pigeon’s egg albumin becomes insoluble when heated in acid or faintly alkaline solution, but in solutions containing moderate amounts of alkali the denatured product is soluble.

(1). 5 cc. of 1 per cent pigeon’s egg albumin (faintly acid), heated, solution became opalescent without neutralization.
2. 5 cc. of 1 per cent pigeon's egg albumin + 0.5 cc. of 0.1 N HCl, heated, solution became opalescent after neutralization.

3. 5 cc. of 1 per cent pigeon's egg albumin + 5 cc. of 0.1 N HCl, heated, gave the same result as (2).

4. 5 cc. of 1 per cent pigeon's egg albumin + 0.5 cc. of 0.1 N NaOH, heated, became opalescent after neutralization.

5. 5 cc. of 1 per cent pigeon's egg albumin + 1 cc. of 0.1 N NaOH, heated, no opalescence with or without neutralization. The neutralized solution gave no opalescence on further heating, showing that the albumin had been changed by the first heating.

6. 5 cc. of 1 per cent pigeon's egg albumin + 5 cc. of 0.1 N NaOH, heated, gave the same result as (5).

DISCUSSION.

In our previous paper on the denaturation of proteins by dilute acids and alkalies we advanced the hypothesis that the fundamental reaction underlying denaturation is one of hydrolysis involving probably some especially labile linkages. Proteins which contain these labile linkages are subject to denaturation by dilute acids and alkalies, while those lacking these labile linkages are not subject to such denaturation.

If this hypothesis is true and if heat denaturation is the same process as denaturation by dilute acids and alkalies, it follows that those proteins which are subject to denaturation by one agent should be also subject to denaturation by the other. This is actually the case. Albumins, globulins, and hemoglobins are denatured by heat as well as by dilute acids and alkalies, while gelatin is denatured by neither. Albumoses and peptones which have lost the labile linkages through peptic digestion are not coagulable by heat nor are they denatured by dilute acids and alkalies.

In our previous paper we cautioned the use of acids and alkalies in isolating proteins on account of the danger of denaturation. The use of heat should be even more restricted, since heat causes denaturation so much more rapidly, and the protein is almost always denatured when heated for any length of time. The denatured protein differs, to be sure, only slightly from the original protein in the contents of most amino acids. But some amino acids which react with the phenol reagent—probably tyrosine and tryptophane—have been shown to be split off very readily. It is probable that some other amino acids which do not react with phenol reagent are also liberated when the protein is denatured.
Heat Denaturation of Proteins

It is well known that the non-protein blood filtrate prepared by heat coagulation contains more nitrogen than those prepared in the cold by means of protein precipitants in faintly acid solution. We have found the same to be true of non-protein chromogenic substances, and this is easily explained by the findings reported in this paper.

SUMMARY.

Evidence is adduced to show that the heat denaturation of proteins is the same process as the denaturation of proteins by dilute acids and alkalies at ordinary temperatures, and that the underlying reaction is one of hydrolysis.

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