ON THE "HEAT COAGULATION" OF PROTEINS. PART III. THE INFLUENCE OF ALKALI UPON REACTION VELOCITY.

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We have pointed out in the papers we have previously published upon the subject ((1) and (2)) that the "heat coagulation" of proteins as ordinarily observed consists of two distinct processes (1) "denaturation[1]," or the reaction between protein and hot water, and (2) agglutination, or the separation of the altered protein in a particulate form.

In our previous work we investigated the first process using solutions of pure hæmoglobin and egg-albumen containing a small proportion of ammonium sulphate (0·3 % to 0·4 %) and possessing different slight degrees of acidity. In these cases agglutination occurred at a rate very greatly in excess of denaturation; the latter process thus became the limiting factor in the complete reaction and the reaction-rate could therefore be studied without complication. The reaction velocity of denaturation was found to be increased with the amount of free acid present. Since the latter was decreased continuously with the gradual precipitation of the protein, the course of the reaction departed from that followed by a "reaction of the first order" to which type it conformed if means were taken to maintain constant acidity during the process ((2), p. 5).

In the present investigation we have studied heat coagulation in the presence of free alkali.

The material used was again recrystallised egg-albumen from which the ammonium sulphate had been removed by dialysis. Solutions

[1] We have adopted the word "denaturation" as an English equivalent of "Denaturierung" which is in general use in German to denote the first process in the precipitation of proteins by hot water.

were made, all containing 1 % protein and various concentrations of free alkali, by addition of a standard solution of sodium hydroxide. A control experiment was made with the solution in its normal reaction, which was slightly acid (hydrogen-ion concentration equal to 10^{-5} normal). The method of experiment was similar to that described in our first paper ((1), p. 407), viz.:—estimation of the residual protein at successive intervals of time. In this case, however, the conditions obtaining in the solution were such as prevented the agglutination of the denaturated protein. It was therefore necessary to adjust the reaction and salt-content of the cooled samples (see below) so that the altered protein should be precipitated. The temperature selected was 64·5° C.; all the solutions were warmed to 20° C. before placing in the thermostat and eight minutes' interval was allowed for the tube and contents to attain the required temperature, before the first sample was removed.

The samples were quite unchanged in appearance but, on acidifying, more or less precipitation occurred according to the time during which the solution had been heated.

It has been shown that for satisfactory precipitation of heated proteins a definite, slightly acid, reaction is necessary. The optimum acidity for agglutination of denaturated egg-white was found by Sörensen and Jürgensen(3) to be 2×10^{-5} normal and that of denaturated serum-proteins was previously found by Michaelis and Rona(4) to be $0·3 \times 10^{-5}$ normal, and to coincide with the iso-electric point of the proteins. In the presence of electrolytes these conditions may become very much modified. We have found, for example, that at 69° C. denaturated, pure egg-albumen in the presence of a small concentration of ammonium sulphate agglutinated over a range of hydrogen-ion concentration extending from $10^{-4·6}$ ($2·5 \times 10^{-5}$) normal to $10^{-7·4}$ ($·004 \times 10^{-5}$) normal ((1), pp. 421 and 423).

In order to bring about agglutination the following procedure was adopted: the samples were acidified by the addition of 1/10th normal acetic acid in slight excess of the amount of alkali originally added, and enough pure sodium chloride was then added to saturate[1] the solution. After standing for 24 hours, the coagulum on boiling was determined in a measured volume of the filtrate. In calculating the concentration of unaltered albumen in the original sample, due allowance was made for dilution of the protein-content by addition of acetic acid and saturation

[1] This was found to be a useful method of securing complete precipitation of denaturated protein in cases where the reaction was not correctly adjusted to be at the best point for agglutination.

with sodium chloride. In the control (Exp. 1, Table I) agglutination occurred naturally, but the samples were nevertheless saturated with sodium chloride for the sake of uniformity.

The "alkalinity" of the various solutions was also investigated. Direct determinations of the hydrogen-ion concentration were made electrically, using the apparatus recommended by Michaelis and Rona(5), which we have found to be specially suitable for small quantities of liquid. In their method the hydrogen electrodes are maintained in a

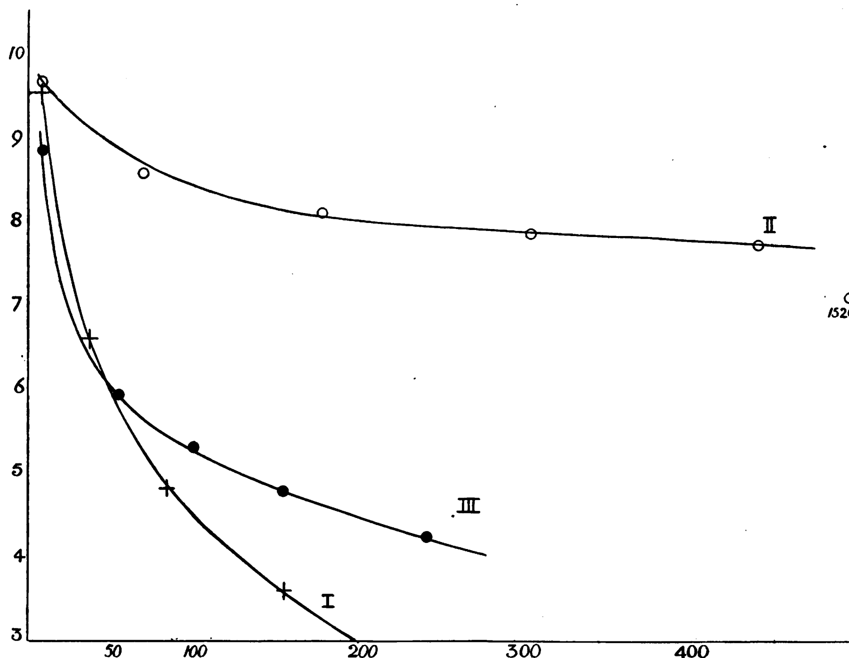

Fig. 1. Denaturation of crystalline egg-albumen, 1 % solution, at 64·5° C.
(I) In original (faintly acid) reaction.
(II) After addition of 5·0 c.c. N/10 NaOH per gram protein.
(III) ,, ,, 10·0 ,, ,, ,, ,,
Ordinates = concentration of residual albumen in milligrams per c.c.
Abscissæ = time in minutes.

still atmosphere of hydrogen, and equilibrium is attained almost instantaneously[1] if the electrodes are dipped into their respective solutions to a depth of 1 to 2 mm. only. Contact between the standard cell

[1] It is necessary to make a reading as soon as possible after completing the circuit, because with the use of concentrated potassium chloride in the contact thread there is a tendency for fluid to soak up into the thread from either cell and dilute the salt; a contact potential is thereby developed and the electromotive force in the circuit falls.

containing 1/10th normal HCl and that containing the solution under investigation was made by means of a concentrated solution of potassium chloride (3·5 normal). The contact potential was found to be nearly obliterated by this means and as the electromotive force measured was comparatively large (usually more than 0·5 volt) no correction was applied. Concentration of hydroxyl-ions was calculated from the hydrogen-ion concentration thus determined, taking the dissociation constant of water to be equal to $10^{-14.14}$ (Sörensen (6), p. 161).

The results of the experiments are given in Table I, and graphically expressed in Fig. 1. In Exp. 2, where the reaction was on the alkaline

TABLE I. *Coagulation of dialysed crystalline egg-albumen,* 1·03 % *solution, at* 64·5° C., *in natural reaction (acid) and after addition of sufficient amount of sodium hydroxide to make the solution alkaline in reaction.*

Exp.	N/10 NaOH added per grm. protein, c.c.	Time, minutes	Concentration* of residual albumen, mgrs. per c.c.	Concentration of H+ in terms of normality	Calculated concentration of OH− in terms of normality†	Time taken for reduction of concentration of residual albumen from 9 mgs. per c.c. to 7·12 mgs. per c.c., minutes
1	0	0 = control	10·30	$10^{-4.91}$ normal (124×10^{-7} normal)	—	18·5
		8	9·61	—	—	
		37	6·64	—	—	
		83	4·85	—	—	
		154	3·61	$10^{-5.48}$ normal (33×10^{-7} normal)	—	
2	5·0	0 = control	10·30	$10^{-10.36}$ normal	$10^{-3.78}$ normal ($1·67 \times 10^{-4}$ normal)	14·72
		8	9·73	—	—	
		69	8·63	—	—	
		177	8·15	—	—	
		303	7·89	—	—	
		440	7·75	$10^{-9.43}$ normal	$10^{-4.71}$ normal ($0·19 \times 10^{-4}$ normal)	
		1520	7·12	$10^{-8.90}$ normal	$10^{-5.24}$ normal ($0·058 \times 10^{-4}$ normal)	
3	10·0	0 = control	10·30	$10^{-11.40}$ normal	$10^{-2.74}$ normal (18×10^{-4} normal)	13·5
		8	8·91	—	—	
		54	5·96	—	—	
		99	5·33	—	—	
		153	4·80	—	—	
		240	4·25	$10^{-11.12}$ normal	$10^{-3.02}$ normal ($9·6 \times 10^{-4}$ normal)	

* Corrected for dilution by saturation with NaCl and also by addition of N/10 acetic acid in case of experiments 2 and 3.

† Dissociation constant of water being taken as $10^{-14.14}$.

side of the neutral point (hydroxyl-ion concentration equal to $10^{-3.8}$ normal), denaturation proceeded so slowly at 64·5° C. that after more than 24 hours had elapsed, only 30 % of the total protein was altered by heat. On addition of more alkali (Exp. 3, hydroxyl-ion concentration equal to $10^{-2.7}$ normal) the denaturation-rate had increased so as to be comparable with that in the original, slightly acid, solution (Exp. 1).

The progress of the reaction in alkaline solutions conforms to no simple law. The reaction-velocity does not remain proportional to the concentration of residual protein, but departs more and more from the theoretical value as the experiment proceeds. This is due to the progressive diminution in alkalinity (see Exp. 2) which is taking place in the solution at the same time. This change in reaction is exactly comparable with the progressive diminution of acidity during coagulation in acid solutions (see Exp. 1, and our previous paper, (2), Tables III and IV, p. 8).

The denaturation process when alkalinity is kept constant. An attempt was made to keep "alkalinity" constant during the process of denaturation by means of the presence of excess of solid magnesium oxide, a saturated solution of which possesses a hydroxyl-ion concentration of about $10^{-4.0}$ normal. A solution of pure egg-albumen, containing about 0·5 % ammonium sulphate, was shaken up with excess of magnesium oxide and left for 24 hours. The solution was gradually warmed up to 60° C., so that loss of equilibrium owing to changed solubility of magnesium oxide at the higher temperature might be avoided, and then placed in the thermostat at 65° C., at which temperature denaturation proceeded at a convenient rate. The initial concentration of egg-albumen was about 0·9 % and the residual protein after the various intervals of time was determined in the usual way.

TABLE II. *Coagulation of pure egg-albumen solution in presence of excess of magnesium oxide at* 64·8° C.

Time, minutes $=t$	Residual albumen, mgs. per c.c. $=C$	$\text{Log}_{10} C$	$K=\frac{1}{t_n-t_0}(\log C_0-\log C_n)$	Concentration of OH^- ions, in terms of normality
10	—	—	—	$10^{-4.14}$ normal ($7·3\times10^{-5}$ normal)
$41=t_0$	$8·676=C_0$	·938	—	—
102	—	—	—	$10^{-4.16}$ normal ($6·8\times10^{-5}$ normal)
162	8·032	·905	·00028	—
312	7·100	·851	·00032	$10^{-4.32}$ normal ($4·8\times10^{-5}$ normal)
1380	3·880	·589	·00026	—

Even in the presence of excess of magnesium oxide, the concentration of hydroxyl-ions was not maintained absolutely constant and was

reduced during the experiment to 70 % of its original value (see Table II). This reduction is small compared with what occurred in the other experiments in which no such precautions were taken (Table I). Having nearly maintained constancy in hydroxyl-ion concentration denaturation proceeded in fair agreement with the unimolecular law, and the velocity constant, K (column IV), remained reasonably constant in value.

It was found that in the presence of magnesium oxide the denaturated protein was almost completely agglutinated in the alkaline solution and little further was precipitated on acidification This is to be ascribed to the influence of the strongly electro-positive Mg^{++} upon the negatively charged protein particles.

Influence of hydroxyl-ion concentration upon denaturation velocity. Since no direct comparison of velocity constants was possible in the case of Exps. 1, 2 and 3 (Table I), the rates of denaturation were compared by considering the times taken in the different instances for denaturation of exactly similar proportions of the original egg-albumen present. This was done by means of the curves in Fig. 1, and the numbers obtained are given in the last column of Table I. Unfortunately it was only possible to institute a comparison over a small portion of the curves, because of the slow rate in Exp. 2, but the figures obtained are very striking. Increase of original alkalinity from a concentration of hydroxyl-ions equal to $10^{-3.8}$ to $10^{-2.7}$ was accompanied by an increase in average denaturation-rate of over a hundredfold.

In a previous paper we showed ((1), Tables V and VI, pp. 419 and 421) that at 69° C. coagulation-rate progressively decreased as the original acidity (hydrogen-ion concentration equal to $10^{-4.60}$ normal) was neutralised by addition of ammonia. These experiments were carried to the neighbourhood of the neutral point (hydrogen-ion concentration equal to 10^{-7} normal), but before this was reached the changes in reaction-velocity became less marked, the absolute differences in hydrogen-ion concentration being also inconsiderable when compared with those existing between the more acid solutions. It would therefore appear that in the neighbourhood of the neutral point change in hydrogen- or hydroxyl-ion concentration is less effective in modifying denaturation-rate than in regions further removed.

There are other instances of the influence of acid and alkali on the effect of hot water upon protein bodies which offer an analogy with what

we have found to obtain in the case of egg-albumen. Famulener and Madsen(7) showed that the destruction by hot water of the activity of the antigens, vibriolysin, tetanolysin, and goat-serum hæmolysin, proceeded as a reaction of the "first order," that the reaction-velocity was at a minimum when the solution was neutral in reaction and was greatly and progressively increased on addition of either acid or alkali. Streng(8) showed that the rate of destruction by hot water of "coli-agglutinin" was greatly increased by addition of alkali. He also showed that while in some cases the reaction conformed to the "monomolecular type," in many others, including those solutions to which alkali had been added (p. 96), the reaction-velocity during the progress of the reaction became progressively lower than the value it should have maintained if it had remained proportional to the concentration of unchanged agglutinin. We have shown ((1), p. 408) that the coagulation of crystalline oxyhæmoglobin by hot water proceeds as a reaction of the first order, and Hartridge(9), who has confirmed our results with oxyhæmoglobin and has shown the same to be true for alkaline methæmoglobin, finds that in the latter case the reaction-rate greatly exceeds that of hæmoglobin at the same temperature. One of us (H. C.(10))[1] showed that the death-rate of a suspension "of bacteria in" hot water, which also proceeds as a reaction of the unimolecular type, was accelerated to a surprising extent by the addition of small quantities of either acid or alkali to the distilled water in which they were heated.

Famulener and Madsen regarded the above accelerating effect of acids and alkalies as an example of catalysis. We should prefer to explain it as due to a difference in properties exhibited by protein according to whether it exists alone or in combination with acid or alkali as a salt. We are of opinion that the protein salts are denaturated at a greater rate than the amphoteric protein at the same temperature, and that the rate of denaturation depends upon the degree of salt formation which has taken place.

There are a number of observations suggesting that protein salts have a greater attraction for water than electrically neutral proteins. Pauli and Handovsky(11) and Schorr(12) have shown that proteins are less readily precipitated by alcohol from their solutions after addition of acids or alkalies. Solutions of protein salts have also a greater viscosity than unionised protein. The same observation was made by Laqueur and Sackur(13) in case of casein. Fischer(14) has

[1] See Table XXVI, p. 276. In Exp. 1, $K=1{\cdot}65$ is a misprint for $K=0{\cdot}65$.

demonstrated the increased imbibition of water by gelatine, muscle and other protein bodies if acid or alkali be added to the solution in which the protein fragments are placed. How far this is due to the electrical charge borne by the protein particles in this condition is difficult to say. Chiari(15) has recently come to the conclusion, from experiments with gelatine, that the minimum imbibition of water takes place in fluids whose reaction (hydrogen-ion concentration equal to 2×10^{-5} normal) coincides with the iso-electric point of the protein.

The increased reaction-velocity of protein denaturation on addition of acids and alkalies falls into line with these observations. We may suppose the protein salt to be in more intimate association with the water which is known to be necessary for the denaturation-change.

The addition of neutral salts to acid or alkaline solutions of proteins has been shown to produce an analogous antagonistic effect in all the above instances. Precipitability by alcohol is restored, viscosity is lowered (Pauli and Handovsky and Schorr, *loc. cit.*), imbibition of water is reduced (Fischer, Chiari), and the reaction-velocity of denaturation in acid solution has also been shown by us to be greatly reduced in presence of neutral salts ((2) p. 17).

SUMMARY AND CONCLUSIONS.

1. Denaturation-rate of egg-albumen in alkaline solutions is shown to be increased with increasing concentration of hydroxyl-ions exactly as was previously shown to be the case with increase of hydrogen-ion concentration in acid solution.

2. Continuous removal of hydroxyl-ions as denaturation proceeds takes place in alkaline solution, just as in acid solution we found hydrogen-ion concentration to diminish.

3. If precautions are taken to keep the concentration of hydroxyl-ions constant during the process, denaturation proceeds as a reaction of the first order (as was also shown in the case of acid). If no such device is employed, denaturation-rate does not remain proportional to the concentration of unchanged albumen, but departs farther and farther from this value owing to the progressive fall in "alkalinity."

4. The influence of acids and alkalies on denaturation-rate is compared with their effect on the viscosity and precipitability by alcohol of protein solutions and upon the imbibition of water by protein; it is suggested that protein in the form of salts is in more intimate association with water.

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