ON THE "HEAT COAGULATION" OF PROTEINS.

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On heating solutions of many proteins, as temperature rises, an irreversible change of state occurs—so-called "heat-coagulation." Similarly, in the case of most proteins endowed with active physiological properties (*e.g.* ferments, toxins, lysins, opsonins, complements, etc.), at or about a particular temperature these properties are destroyed.

Up to the present it has been the almost universal practice to regard the temperature at which change of state or loss of activity occurs, as if it were a physical constant characteristic of the particular protein, this temperature being subject to small variations according to the conditions of experiment.

This may however be an entirely misleading way of regarding the matter and the well-known fact that the destruction of active properties, or precipitation as the case may be, does not occur instantaneously suggests a time process in which heat merely plays the subsidiary part of accelerator. This view of the coagulation of proteins was indeed advanced by Duclaux (1893) and has since been established by Famulener and Madsen (1908) for the destruction of the active properties in solutions of three antigens, vibriolysin, tetanolysin and goats'-serum-hæmolysin, and by Madsen and Streng (1909) for agglutinins.

We have occupied ourselves in the first instance with an investigation of the laws governing the precipitation of solutions of pure proteins because such a process lends itself more easily to accurate quantitative methods.

The fact that various proteins are more or less easily coagulated by heating their solutions has proved useful for their identification and

differentiation, see Hewson (1772), Kühne (1864), Fredericq (1877), Weyl (1877), Halliburton (1884).

It was apparent to Weyl that the temperature of heat coagulation, even if it were of the nature of a physical constant, was altered by a variety of conditions and Hammarsten (1879) found that serum globulin, the coagulation temperature of which was determined by Hoppe-Seyler to be 72° to 75° C., coagulated at 68° to 80° C. according to the rate of heating up and the concentration of protein and salt. Osborne and Campbell (1900) found a similar difference in the coagulation temperature of egg albumen by varying the concentration of protein and NaCl. A fourth factor which exerts a profound influence is the addition of even minute amounts of acid or alkali. Halliburton (1884) found in the case of serum albumen that neutralizing (to litmus) an alkaline solution lowered the coagulation temperature from 80° to 78° C. and, by the successive addition of small quantities of acid, it fell as low as 53° C.

The value of the determination of the temperature of heat coagulation as a means of differentiating proteins has been severely criticised by Haycraft and Duggan (1890), who point out that the act of coagulation takes a certain time, and that the temperature of coagulation, as usually studied, rises as the concentration of protein diminishes; it was also criticised by Duclaux (1893). On the other hand, the usefulness of the method when performed under standard conditions was defended by Hewlett (1892), and, provided the four conditions mentioned above be kept uniform, the observation of the temperature at which precipitation commences undoubtedly affords useful information. It will, however, be apparent from the experiments below, that, as insisted upon by Duclaux (1893), heating in solution is not a method which lends itself to the fractional separation of proteins unless the difference in the readiness with which they are thrown out of solution be very considerable. Were the process a reversible one it would be different and the method would compare in usefulness with fractional distillation.

Nature of heat coagulation.

The coagulation of proteins by heating their solutions is not a pure temperature effect. Water as such or in the form of steam is essential. Proteins in the dry or nearly dry condition can be heated to much higher temperatures without change. Cohnheim (1900, p. 141)

attributes to Michel and Wichmann the observation that albumen crystals can be heated to 150° C. in the dry condition without change. Again, in the case of proteins soluble in 70 % alcohol, 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 (Osborne, 1909, p. 21).

The contrast between the effect of heat upon proteins in the dry and moist state is a well-recognised fact, but, with the exception quoted above, we have been unable to discover in the literature any precise experimental data upon the point.

We therefore heated crystalline egg-albumen and methæmoglobin in a hot air-bath jacketted with oil. The proteins were freed as much as possible from moisture by squeezing in a press between filter papers, but still contained 20 % of water. Weighed portions of a few decigrams were placed in muslin bags and hung in the air bath through which a current of air passed. If only small masses are employed, it is not necessary to previously dry the protein, because as long as the residual water is being dissipated the material does not acquire the temperature of the bath. Samples were removed at intervals and estimation made of the protein still soluble in water. We found that crystallised egg-albumen was completely soluble after five hours' heating to 120° C. At 120° C. it was slowly changed and after four hours at this temperature 22 % was rendered insoluble in water. A similar sample in a sealed tube (*i.e.* in the presence of steam at the temperature of the bath) was rendered completely insoluble in a few moments. Methæmoglobin was unchanged by subjection for four hours to a temperature of 110° C. Observations were not made at higher temperatures with this protein.

So-called "heat coagulation" appears to be a reaction taking place between protein and water leading under suitable conditions to the precipitation of the former. If such is the case, it is difficult to imagine that it should occur only at or above any particular temperature, and an interpretation more in accordance with modern chemistry is that heat coagulation is a reaction with a high temperature coefficient, the reaction velocity of which varies considerably with different proteins and according to the acidity and saline content of the solution.

In order to test this interpretation and at the same time to determine the progress of the reaction we kept solutions of two pure proteins, oxyhæmoglobin and crystallised egg-albumen, at constant but different temperatures, removed samples at intervals and determined the amount of protein remaining unchanged. We are alive to the fact that by

so doing we are measuring the rate of change in a reaction which involves two distinct processes: (1) the reaction between water and protein, and (2) the separation of the product from a colloidal solution.

Our experiments were performed in the presence of ammonium sulphate which facilitates coherence of the precipitates and, as the filtrates from the partially coagulated solutions displayed no sign whatever of being altered in character and showed no further change on standing at a lower temperature, we believe that, under these conditions, the time occupied in the physical change involved in No. 2 is insignificant and that we are indeed measuring the slower interaction between water and protein.

Materials used. The *hæmoglobin* was prepared from horse's blood and crystallised from solutions of Am_2SO_4, as described by Schulz (1908). The crystals were separated from mother liquor by pressing between filter paper and were dissolved in distilled water. A 3 % solution of hæmoglobin in a weak solution of Am_2SO_4 was used for the experiments.

Crystalline egg-albumen was prepared from the whites of fresh eggs according to the method of Hopkins and Pinkus (1898). The crystals were pressed as dry as possible between filter paper. A strong solution in distilled water, containing 20·8 % albumen and 8·4 % Am_2SO_4 formed the stock solution; it was diluted with the necessary amount of water as required. The stock solution was kept in the cold room as it was not permissible to add a preservative. Even the presence of toluol we found to greatly increase the rate of the reaction.

Method. The protein solutions were placed in a test-tube of 200 c.c. capacity fitted with a stirrer of bent glass tube. The upper end of the stirrer passed through a glass bearing in the cork. Near this upper end a side piece was blown through which the samples were withdrawn. The lower end of this tubular stirrer (Fig. 1) was open, the upper closed and attached to a revolving spindle. The test-tube was immersed in a bath of water with half an inch of mineral oil upon the surface to minimise evaporation. The bath was continuously stirred

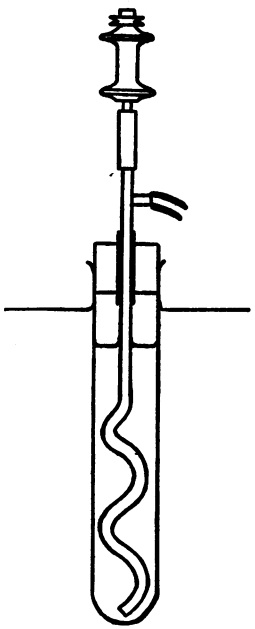

Fig. 1.

and maintained at a constant temperature by means of a gas flame controlled by a toluol regulator. The variations in the temperature of the bath seldom exceeded 0·1° C. during the experiment.

According to size and contents of the tube, 8 to 10 minutes were necessary for the solution to reach the temperature of the bath. A sufficient period having been allowed for warming up, samples of the tube's contents were withdrawn after successive intervals of time, cooled and filtered and the residual proteid determined.

Determination of the proteid content in the samples.

In the case of hæmoglobin, the residual proteid was determined colorimetrically by comparison with the first sample as standard (= 100). This comparison was carried out in a home-made apparatus, which was found to be much more convenient and accurate than any of the more elaborate ones which we tried.

Three test tubes of the same diameter and made of glass of the same tint were carefully selected. The three tubes were observed in a narrow cardboard box, sufficiently long to take them in a row, and deep enough to hold them comfortably. Two square openings exactly opposite one another were cut out in the longer sides of the box, through which the colours could be seen when the box was held up to the light. A cigarette paper was pasted over the opening on the illuminated side.

Two tubes contained the standard solution, diluted as necessary, while a third contained a known volume of the solution to be determined. This was diluted with successive small quantities of distilled water from a burette until its tint could not be distinguished from that of the two standard tubes between which it was placed. A further quantity of water was added until a difference in tint was again noticed. The mean between the readings when a difference could be detected was taken for end-point.

In the case of egg-albumen the concentration of residual unchanged proteid in the filtered samples was determined by boiling a given volume of the filtrate, made just acid with acetic acid, collecting the coagulum on a weighed filter paper, drying at 110° C., and weighing.

The reaction velocity of coagulation.

Hæmoglobin. The hæmoglobin solutions were experimented with as prepared and contained ammonium sulphate. Table I gives the results

of five experiments made with a solution of hæmoglobin containing about 3 % proteid (determined by weighing the coagulum obtained on boiling) at temperatures between 60° C. and 70° C. The residual concentrations of hæmoglobin are expressed in proportion to that of the first sample which is represented by 100. The first sample was taken after sufficient time had elapsed for the tube and contents to take the temperature of the bath, and time is reckoned from the taking of the first sample.

TABLE I. *Coagulation of hæmoglobin, 3 % solution.*

Exp.	Temp. of bath, ° C.	Time*, minutes $=t$	Concentration of hæmoglobin (first sample=100) $=C$	$\text{Log}_{10} C$	$K=\frac{\log C_0-\log C_n}{t_n-t_0}$
1	60	$0=t_0$	$100=C_0$	2·000	—
		30	54	1·732	·0090
		90	13·5	1·000	·0097
					mean ·0093
2	62·6	$0=t_0$	100	2·000	—
		20	42	1·623	·019
		45	12	1·079	·020
		70	4·8	·681	·019
					mean ·019
3	65·6	$0=t_0$	100	2·000	—
		10	35·5	1·550	·045
		20	11·0	1·041	·048
		30	5·0	·699	·043
					mean ·044
4	67·6	$0=t_0$	100	2·000	—
		3	61·4	1·788	·071
		6	34·8	1·542	·076
		9	24·9	1·396	·067
					mean ·074
5	70·4	$0=t_0$	100	2·000	—
		2	52·5	1·720	·16
		4	25·3	1·404	·15
		6	14·1	1·150	·15
		7·5	7·6	·886	·15
					mean ·15

* Time was reckoned from the taking of the first sample, *i.e.* from a time when the temperature of tube and contents was known to have reached that of the bath.

In the right-hand column of the table are given values for the velocity constant, calculated on the assumption that the process proceeds logarithmically. These are in good agreement, indicating that the reaction velocity at any moment is proportional to the concentration of unchanged hæmoglobin. This is graphically shown in Fig. 2, where logarithms of hæmoglobin-concentration are plotted against time, and five straight lines are obtained corresponding to the five experiments in Table I. As the temperature is raised, these lines become rapidly steeper, and the velocity constants in Table I become greater in value.

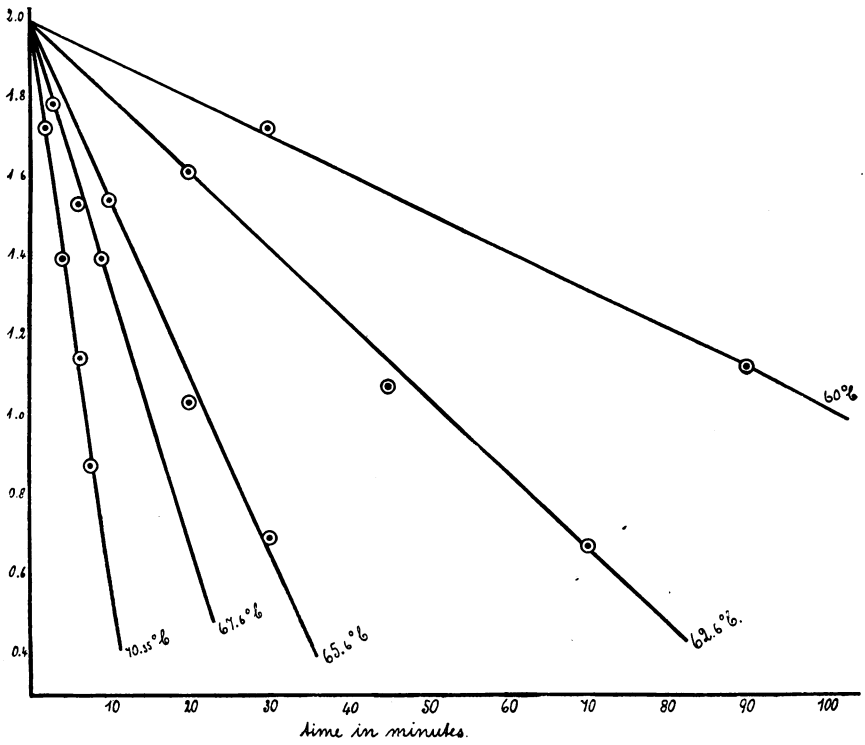

Fig. 2. Coagulation of hæmoglobin in 3 % solution at temperatures from 60° C. to 70° C. (original concentration = 100). Ordinates = logarithms of concentration of hæmoglobin.

From the results of these experiments it is evident that the change occurring on heating a solution of hæmoglobin is a reaction of the first order.

Our interpretation of the facts is as follows: Water reacts with hæmoglobin, and this reaction results in the splitting off of hæmatin and

the formation of an insoluble protein. As the water is in infinite excess, at any one temperature the rate of reaction is determined only by the residual concentration of the hæmoglobin. At ordinary temperatures this reaction between hæmoglobin and water is inappreciable, but is greatly enhanced by raising the temperature, in other words the reaction has a high temperature coefficient. This has been determined for coagulation of hæmoglobin and egg-albumen and will be discussed later.

What the nature of the cleavage may be we are ignorant but the phenomenon presents some analogy with the hydrolysis of cane sugar.

Crystallised egg-albumen. A 1 % solution of egg-albumen was used and the experiments were similar to those with hæmoglobin detailed above, except that the concentration of albumen in the samples removed at intervals was determined by boiling and weighing the precipitated protein. The temperatures employed varied between 69° and 77° C.

The crystals of egg-albumen were twice recrystallised from solutions of ammonium sulphate. The solutions contained free acid and required the addition of considerable quantities of alkali before the concentration of H^+ ions was reduced to that of water. The observations were made by determining the electromotive force of hydrogen concentration-cells and will be referred to again later. The fact is mentioned here to indicate that egg-albumen crystals are salts of the protein with the acid present at the time of their precipitation as was found by Osborne (1902) to be the case with Edestin crystals. Such salt formation by proteins has been abundantly shown among others by Skojvist (1895), Spiro and Pemsel (1898), Osborne (1899 I and II and 1902), Erb (1901), Pauli (1907), Moore and Bigland (1910). In water these salts hydrolyse to some extent and a solution containing salts of albumen, albumen and acid is produced. Under these circumstances, it might be anticipated that the progress of the reaction with hot water would be more complicated than was the case with hæmoglobin.

In Table II are set forth the results of four experiments showing the progress of coagulation in a 1 % solution of egg-albumen crystals at temperatures of 69° to 76·3° C. The solution was rendered alkaline to lacmoid solution by the addition of 5 c.c. $\frac{N}{10}$ AmOH to 100 c.c.[1] and

[1] The original albumen solution contained 1·039 % protein, so that the amount of ammonia added is equivalent to 4·81 c.c. $\frac{N}{10}$ AmOH per gram protein; after this dilution the solution contained 0·99 % albumen.

subsequent examination of the concentration of H^+ ions in this solution with a gas-chain battery by the hydrogen concentration-cell method indicated that the solution was only slightly more alkaline than distilled water. Nevertheless, a distinct formation of alkali albumen was detected after the solution had been heated for some hours. The residual concentration of protein in the solutions is given in milligrams per c.c.

TABLE II. *Coagulation of* 0·99 % *crystalline egg-albumen solution.*

Exp.	Temp. of bath, °C.	Time, minutes	Amt. of filtrate analysed, c.c.	Weight of coagulum, grams	Residual albumen, mgrs. per c.c.
1	69	10	15	0·1462	9·747
		85	15	0·1104	7·360
		180	14·5	0·0838	5·779
		360	14·3	0·0598	4·184
		741	21	0·0585	2·786
		1443	30	0·0604	2·013
2	71·1	11	13·5	0·1326	9·823
		37	17·2	0·1162	6·756
		62	11·7	0·0607	5·187
		90	18	0·0850	4·722
		146	19·5	0·0634	3·251
		280	23	0·0487	2·117
3	73	10	14·5	0·1379	9·512
		20	14·5	0·0818	5·642
		30	12·5	0·0545	4·360
		50	16	0·0470	2·938
		129	17·5	0·0270	1·543
		275	34	0·0393	1·156
4	76·3	10·5	12	0·0442	3·683
		13	12	0·0312	2·600
		17	20	0·0407	2·035
		22	18	0·0243	1·350
		30	40	0·0480	1·200

In Fig. 3, the concentration after definite intervals is plotted against time. The points appear to lie upon smooth curves, indicating that the process proceeds in an orderly manner. An examination of the figures showed that the relation of the concentration to time did not follow a simple logarithmic law, but decreased with time more quickly than would be accounted for if the rate were merely proportional to the concentration of protein.

We have made a large number of experiments with egg-albumen solution, the details of which we have not included in this paper. The results of these experiments showed general agreement with those

described above and afforded curves similar to those drawn in Fig. 3. For some time we were not aware how extremely sensitive this process is to a variety of influences, and that it is essential to work with the same solution if the results are to be compared. No preservative is permissible and the solutions must be frozen during the time between the observations. In the early portion of the work sufficient precautions were not taken, and these experiments cannot therefore be used for comparative purposes.

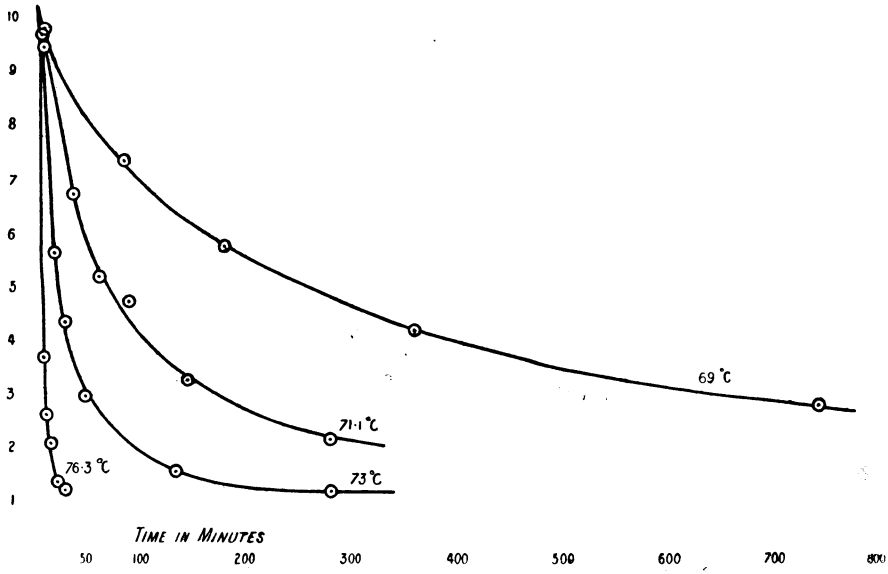

Fig. 3. Influence of temperature upon coagulation rate of egg-albumen. Coagulation of 1 % solution at temperatures from 69° C. to 76·3° C. Ordinates = concentration of albumen in milligrams per c.c.

TABLE III. *Temperature coefficient of heat coagulation of hæmoglobin.*

Material	Temperature of bath $=t$	Velocity constant $=K$	Log_{10} $(K\times10^3)$ $=\log k$	Mean logarithmic difference in velocity constant per 1° C.	Temperature coefficient, mean relative change in velocity constant for 1° C.	$\mu=\frac{2T_0T_n}{T_0-T_n}(\log_e K_0-\log_e K_n)$
Hæmoglobin	70·4 $=t_0$	0·15 $=K_0$	2·176 $=\log k_0$	—	—	—
	67·6	0·074	1·869	0·109	—	58,700
	65·6	0·044	1·643	0·111	—	59,410
	62·6	0·019	1·278	0·115	—	61,030
	60	0·0093	0·968	0·116	—	61,070
				mean 0·113	1·30	mean 60,050

Temperature coefficient of the reaction.

Hæmoglobin. The effect of temperature upon the reaction velocity of coagulation was studied by comparing the velocity constants at the various temperatures. The available data obtained from Table I are set forth in Table III, and it will be seen that between the temperatures of 60° C. and 70° C., rise of temperature has a consistent effect upon the rate of coagulation. This is in approximate obedience to a logarithmic

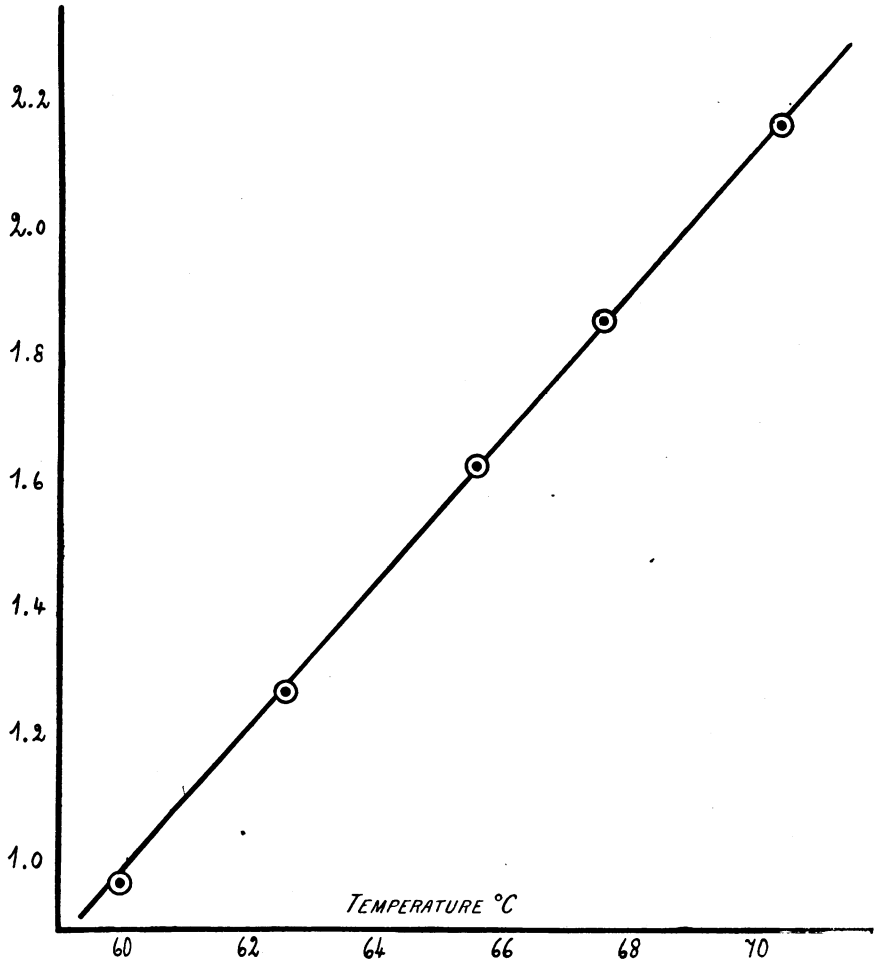

Fig. 4. Influence of temperature upon the reaction velocity of hæmoglobin coagulation (3 % solution), logarithms of mean values of the velocity constant (ordinates), K, plotted against temperature (abscissæ).

law, the mean logarithmic difference in velocity constant for a rise of temperature of 1° C., calculated from the various different experiments, remaining nearly constant in value throughout the range observed. This is expressed graphically in Fig. 4, where logarithms of velocity constants, plotted against temperature, fall upon a straight line.

The formula of Arrhenius, in which an additional factor having reference to the absolute temperatures is introduced, has, in many cases, been found to express the effect of temperature upon reactions, and would appear to be applicable here equally with the simpler law discussed in the preceding paragraph. The values of $\mu \left(= \frac{2T_o T_n}{T_o - T_n} \log_e \frac{K_o}{K_n}\right)$ are given in the last column of Table III, and a fair approximation to constancy is displayed. Over the small range of temperature of the experiments, it is difficult to say which expression is the better applicable.

From Table III the coagulation rate of hæmoglobin is seen to be increased 1·3 times for a rise in temperature of 1° C., *i.e.* 13·8 times for a rise in temperature of 10° C.

Egg-albumen. It is not possible, as was done in the case of hæmoglobin, to ascertain the temperature coefficient by a direct comparison of velocity constants at different temperatures, because the equation to the curve is unknown. It is legitimate, however, to compare the time taken for a similar reduction in concentration of unaltered albumen at various temperatures. In Fig. 3 concentrations of residual albumen are plotted against time, at four different temperatures; from the smoothed curves the times taken at each temperature for a concentration of albumen (A) to be reduced to concentration (B) can be determined. The reciprocals of these times are proportional to the average velocities of similar portions of the total coagulation process at the various temperatures and may be compared. In this way the figures in Table IV are obtained, giving the times taken for reduction of concentration of uncoagulated albumen from 9 mgr. per c.c. to 3 mgr. per c.c. in each case.

Rise of temperature within the limits employed has a consistent influence upon the average velocity of coagulation of egg-albumen and this influence is even greater than was the case with hæmoglobin. The average velocity of coagulation of egg-albumen is increased 1·91 times for a rise in temperature of 1° C., *i.e.* at the rate of about 635 times for 10° C.

TABLE IV. *Influence of temperature upon the coagulation of crystalline egg-albumen.*

Temp., ° C. $= t$	Time taken for reduction, 9 mgrs. per c.c. to 3 mgrs. per c.c., minutes	Average velocity during this period, mgr. per c.c. coagulated per minute $= V$	Log_{10} $(V \times 10^3)$	Mean logarithmic difference in average velocity per 1° C.	Temperature coefficient, mean relative change in average velocity per 1° C.	$\mu =$ $\frac{2T_0T_n}{T_n - T_0}(\log_e V_n - \log_e V_0)$
$69{\cdot}0 = t_0$	617·0	$0{\cdot}00973 = V_0$	0·988	—	—	—
71·1	155·0	0·03871	1·587	0·285	1·93	136,500
73·0	41·5	0·1446	2·160	0·294	1·97	141,600
76·3	7·2*	0·833	2·921	0·265	1·84	128,800
				Mean	1·91	

* Figure by extrapolation.

In the last column of Table IV are given the value of μ, if the formula of Arrhenius be supposed to hold in this instance. The value obtained from the various experiments shows a rough constancy and is nearly three times that obtaining in the case of hæmoglobin.

The temperature coefficient which we find for the coagulation of these two proteins by hot water, 1·30 and 1·91 times respectively for 1° C. rise in temperature, is an exceedingly high one compared with the effect of temperature upon most chemical reactions. In the majority of instances the reaction velocity is increased about 1·1 times for 1° C., that is 2 to 3 times for a rise of temperature of 10° C., and even the biological processes of germination of seeds, respiration of plants and growth of bacteria fall within this range. On the other hand, many reactions in which complex protein bodies are concerned have been shown to possess high temperature coefficients which are comparable with those obtained for heat coagulation. The destruction of emulsin by heat has, according to Tamman (1895), a temperature coefficient of 7·14 for 10° C. rise of temperature between the temperatures of 60° C. and 70° C. Bayliss (1908, p. 52) found the action of trypsin to be hastened 5·3 times for 10° C. rise of temperature. Meyer (1906) found the disinfection of spores of B. subtilis and B. robur by heat to be hastened from 4 to 5 times for 10° C. rise of temperature, in accordance with a logarithmic law. Ballner (1902) found disinfection of anthrax spores by steam to take place from 9 to 11 times more quickly by raising the temperature 10° C.; a simple logarithmic law and the law of Arrhenius are equally applicable to his results (H. Chick, 1908, p. 153). Disinfection of

vegetative forms of bacteria (B. paratyphosus) with phenol and other coal tar derivatives has a temperature coefficient of 8 to 10 for 10° C. rise in temperature[1].

The high temperature coefficient for coagulation of egg-albumen has a counterpart in that for the velocity of destruction by hot water of the hæmolysins in vibriolysin, tetanolysin and goat-serum. Famulener and Madsen (1908) found the influence of temperatue to be in accordance with the law of Arrhenius, and the velocity of this reaction to be doubled if the temperature were raised 1° C. Madsen and Streng (1909) showed the action of hot water upon some agglutinins to be similarly influenced by temperature.

All the high temperature coefficients mentioned above have been observed with reactions taking place in colloidal solutions which suggests that under these conditions the effect of temperature is produced in some other way than by merely enhancing the intrinsic energy of the molecules. In what other way temperature may operate, we have however no proof. Mr W. B. Hardy has suggested to us that alteration in the mean size of the protein aggregates, whereby the total surface was increased, might explain a rise in reaction velocity. The heat coefficient of egg-albumen may be in part so explained, but, since from the osmotic pressure determinations of Hüfner and Gansser (1907) and Roaf (1909) hæmoglobin would seem to exist in solution in single molecules, some other interpretation must be looked for in this case.

It must not be overlooked that in all these observations the range over which the influence of temperature has been studied is a very small one—in our own experiments 10° C.—and there is no experimental proof that the effect of temperature remains the same over a wider range. We have, however, observed the formation of an insoluble precipitate in faintly acid solutions of egg white after one week at 37° C., and it is a matter of common observation that protein solutions slowly deposit an insoluble precipitate of protein on long standing.

From the results set forth in this paper it is clearly inaccurate to consider that a protein coagulates at any particular temperature. It is true that proteins heated with water under exactly similar circumstances usually commence to precipitate at or about some definite temperatures, but it is misleading to look upon the "coagulation temperature" as if

[1] On the other hand disinfection by mercuric chloride or silver nitrate has a much lower coefficient, viz. 2—4, (H. Chick (1908), B. paratyphosus; Madsen and Nyman (1907) anthraxspores).

it were of the nature of a physical constant of the particular protein. It is due to the high temperature coefficient of this reaction that observations of the so-called coagulation temperature have been of service. A real distinction is, however, afforded by the *rate* at which a protein is coagulated at any given temperature and under similar conditions as to reaction and salt content.

Effects of acid and alkali upon rate of coagulation of egg-albumen.

It has long been known that the addition of acid or alkali influences the readiness with which protein is thrown out of solution by hot water. Halliburton (1884) found that with solutions alkaline to litmus, the temperature at which precipitation occurred, if at all, was not much influenced by the degree of alkalinity. On the other hand, acid lowered the temperature of coagulation; if acid were added in equal quantity to a solution of albumen the first additions produced a small fall in coagulation temperature, and this effect became progressively more marked with each successive addition. Halliburton's results as to the general effect of acid in increasing the readiness with which proteins were precipitated from their solutions were confirmed by Haycraft and Duggan (1890).

A certain amount of confusion upon this subject is due to the fact that in the past the criterion of neutrality has been arrived at with the use of indicators, substances which in many cases act as stronger acids or bases than the proteins themselves. Our observations with solutions of egg-albumen have led us to the conclusion that directly the concentration of OH^- ions in a solution rises above a minimum (about twice the concentration in distilled water) some alkali-albumen is formed on heating. The question therefore resolves itself into the effect of acid.

Having ascertained that it is improper to speak of a protein as having any particular coagulation temperature, we have studied the effect of addition of acid upon the velocity of coagulation at constant temperature. For this purpose hæmoglobin could not be used owing to its extreme sensitiveness to acid and the experiments were therefore confined to crystalline egg-albumen.

In Table V are given the results of six experiments made with the same material and at the same temperature, 69° C., and differing only in the reaction of the albumen solution employed, which varied from the material as prepared, with markedly acid reaction, to the limit

TABLE V. *Coagulation of crystalline egg-albumen, 0·99 % solution, with original acidity, and after addition of various amounts of ammonia, at 69° C.*

Exp.	No. of c.c. $\frac{N}{10}$ AmOH added per gram protein	Time, minutes	Amount of filtrate analysed, c.c.	Weight of coagulum, grams	Residual albumen, mgrs. per c.c.
1	0	0	control	—	9·901
		8	22	0·1262	5·737
		10	23	0·1022	4·444
		11	24·5	0·0989	4·037
		12·5	22·5	0·0800	3·555
		15	34·5	0·0999	2·896
2	1·60	10	16	0·1260	7·876
		15	16·5	0·1105	6·697
		22	22·5	0·1257	5·587
		30	23	0·1111	4·831
		50	32	0·1172	3·663
		80	18	0·0506	2·811
3	2·40	10	17	0·1461	8·594
		20	16	0·1168	7·301
		40	20	0·1180	5·900
		81	18	0·0795	4·416
		150	22·5	0·0806	3·582
		337	28·5	0·0716	2·512
4	3·20	10	17	0·1458	8·576
		20	15·5	0·1246	8·040
		40	8·5	0·0636	7·482
		86	30	0·1809	6·030
		165	21	0·1025	4·881
5	3·53	10	12·7	0·1201	9·458
		30	15·5	0·1279	8·251
		60	16	0·1144	7·151
		120	18·5	0·1058	5·719
		241	18·7	0·0802	4·288
		420	14·8	0·0428	2·892
		600	36	0·0709	1·969
6	4·01	10	13	0·1230	9·461
		40	12·5	0·1000	8·001
		90	16	0·1073	6·707
		215	17·5	0·0836	4·777
		300	20	0·0810	4·050
		645	24	0·0589	2·454
7	4·81	See Table III, Experiment at 69° C.			

of alkalinity possible without formation of alkali albumen[1] (Exp. 7). This is after all a very small range, but was the utmost within the compass of our experimental method for any one temperature. As it was, the reaction rate in the most acid condition (Exp. 1) was so rapid as to make the observations only just possible, whereas the experiment with neutral solution occupied 12 hours.

The results are shown graphically in Fig. 5 where the concentration of residual albumen in solution for seven different degrees of acidity are plotted against time. The observations fall upon smooth curves. As in

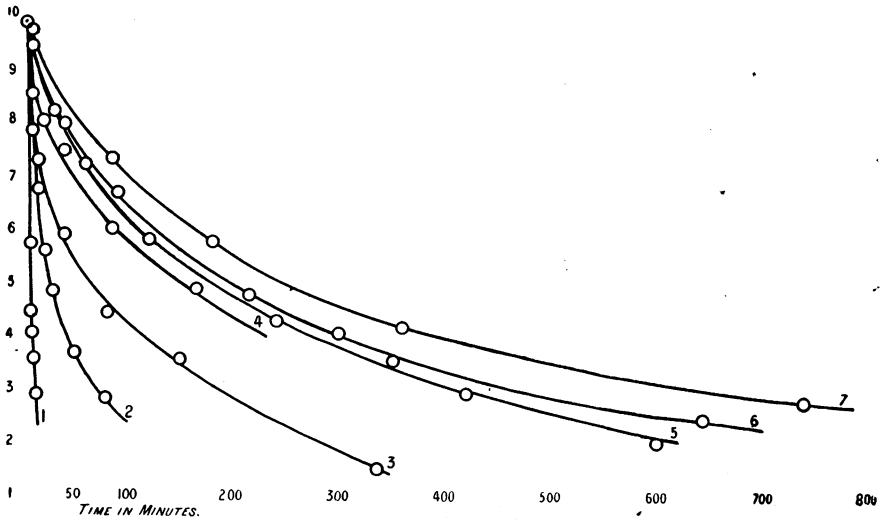

Fig. 5. Influence of acid upon coagulation rate at 69° C. of egg-albumen in 1 % solutions to which varying amounts of alkali had been added. Ordinates = concentration of albumen in milligrams per c.c. Abscissæ = Time minutes.

1. Original solution, reaction acid, *i.e.* H^+ ion concentration greater than distilled water.
2. 1·6 c.c. $\frac{N}{10}$ AmOH added per gram protein, reaction acid, ,, ,,
3. 2·4 ,, ,, ,, ,, ,, ,,
4. 3·2 ,, ,, ,, ,, ,, ,,
5. 3·53 ,, ,, ,, ,, ,, ,,
6. 4·01 ,, ,, ,, reaction alkaline, *i.e.* H^+ ion concentration less than distilled water.
7. 4·81 ,, ,, ,, ,, ,, ,,

[1] It must be remembered that the original solution of twice re-crystallised egg-albumen crystals is decidedly acid and that we have studied the effect of varying the acidity by adding different amounts of alkali. This method was employed owing to the fact that at that time we had no means of ascertaining the true neutral point of the solution. It has no disadvantage other than rendering the description involved.

the previous series the reaction rate falls off more rapidly than can be accounted for by the decrease in concentration, but the nature of the curve has not been ascertained.

Therefore, for a quantitative comparison of the rates of coagulation with different degrees of acidity the same expedient had to be adopted as before and the average velocities derived from the times taken for the concentration to be reduced from 6 mgrs. per c.c. to 3 mgrs. per c.c. in each case. The figures were obtained from the smoothed curves of Fig. 5, and are set forth in Table VI.

Examination of Table VI or a glance at Fig. 6, in which the mean velocity of coagulation is plotted against no. of c.c. $\frac{N}{10}$ alkali added per gram protein, reveals the fact that as the acid is removed the rate of coagulation falls, at first very rapidly, afterwards more slowly, the effect being in no simple proportion to the alkali added.

TABLE VI. *Effect of acid upon rate of coagulation of a* 0·99 % *solution of crystalline egg-albumen. Total volume of solution constant* = 157·5 *c.c.*

Composition solution no. of c.c. $\frac{N}{10}$ AmOH added per gram protein	Reaction of solution	Time taken for reduction in albumen concentration of 6 mgrs. per c.c. to 3 mgrs. per c.c., mins. (from Fig. 5)	Average velocity during this period mgrs. per c.c. coagulated per minute
0	Original acidity	7·1	·4225
1·60	Acid	57·5	·0522
2·40	,,	150	·0200
3·20	,,	240	·0125
3·53	,,	300	·0100
4·01	alkaline	370	·00811
4·81	,,	480	·00625

These results confirm in the main those of Halliburton (1884) with serum proteins already referred to (p. 405). In our experiments the effect of acid is more striking, but that is to be explained as follows. The influence of acid is here measured by comparing coagulation rates at constant temperature, whereas Halliburton compared the "coagulation temperatures," that is to say temperatures at which coagulation takes place at approximately the same rate. For a process with a high temperature coefficient the former method is the more accurate. An amount of acid which would double the coagulation rate at a given temperature would lower the apparent "coagulation temperature" only about 1° C.

Little further could be made of the facts until we had found out the true neutral point of the solution, *i.e.* the point where the concentration

of hydrogen ions equalled that in distilled water, and had also determined what influence the withdrawal of acid was producing upon the real acidity of the solution (concentration of hydrogen ions).

This we have been enabled to carry out owing to the kindness of Dr N. T. M. Wilsmore of University College, who not only placed the resources of his laboratory at our disposal but with infinite pains instructed us in the use of the gas-chain battery and generally looked after us while we made the observations. Subsequently we installed the apparatus in our own laboratory and repeated the determinations.

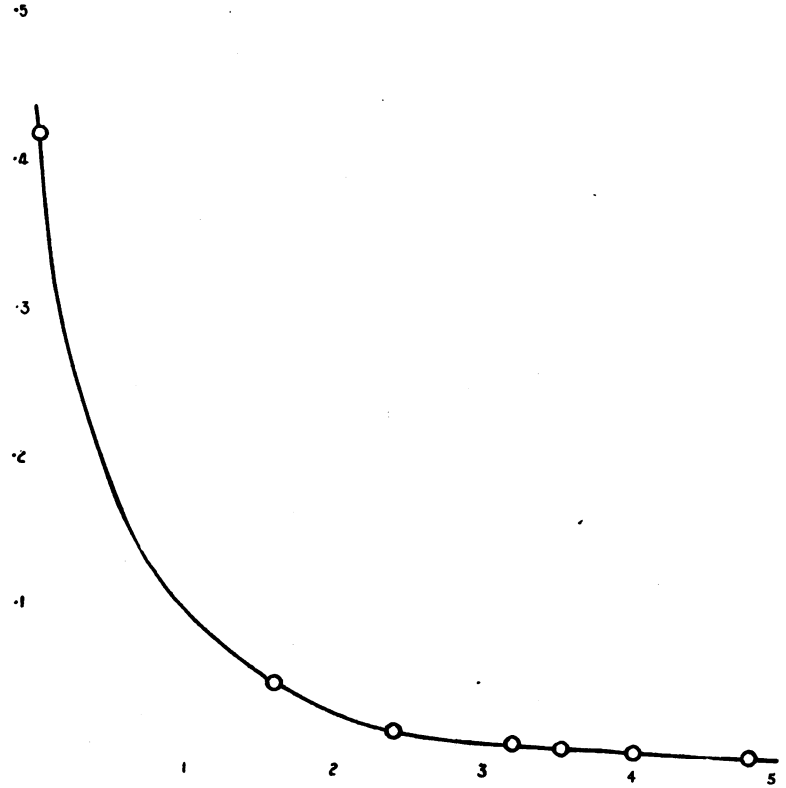

Fig. 6. Influence of acid upon coagulation rate of egg-albumen. Ordinates = milligrams per c.c. coagulated per minute. Abscissæ = number of c.c. $\frac{N}{10}$ AmOH added to the original acid solution per gram protein.

The particular deposition of the apparatus we used was that described by Wilsmore (1900). Two hydrogen electrodes were employed; one dipped into $\frac{N}{10}$ HCl, and the other into the solution whose

E.M.F. was to be measured. The two cells were connected by a vessel containing a saturated solution of ammonium nitrate, in order to minimise the contact potential between the two solutions. The temperature was maintained at 18° C. The solutions under investigation contained 1 % egg-albumen, 0·4 % ammonium sulphate and varying quantities of $\frac{N}{10}$ AmOH. After adding the alkali the solutions stood overnight in order that equilibrium might be attained.

We first ascertained that the E.M.F. given when one cell contained $\frac{N}{10}$ HCl and the other $\frac{N}{1000}$ HCl was nearly the theoretical value, viz. ·1154 volt. In the series of observations detailed in the table the actual value obtained under these conditions was 0·1150 volt. The small error due to the presence of 0·4 % Am_2SO_4 in one cell only was subsequently ascertained to be + ·004 volt and in the table the observed values for E.M.F. have been corrected by this amount.

TABLE VII. *Concentration of* H^+ *ions in* 0·99 % *solution after addition of various quantities of* $\frac{N}{10}$ AmOH. *Total volume of solution constant.*

Solution in contact with 1st H. electrode,		Solution in contact with 2nd H. electrode, c.c. $\frac{N}{10}$ AmOH added per grm. protein	E.M.F. volts (corrected)	Calculated concentration of H^+ ions
$\frac{N}{10}$ HCl		0·00	—	251×10^{-7} N.
		0·20	0·211	$222 \times ^{-7}$ N.
		1·20	0·238	75·0 ,,
		2·20	0·263	27·7 ,,
	Acid	2·84	0·294	7·96 ,,
	↑	3·17	0·316	3·33 ,,
		3·53	0·334	1·62 ,,
		3·60	0·344	1·09 ,,
	Neutral	4·35	0·360	0·58 ,,
	↓ alkaline	4·80	0·369	0·41 ,,

The results of these observations are set out in Table VII, where the number of c.c. $\frac{N}{10}$ AmOH added per gram of proteid, the E.M.F. observed and the concentration of hydrogen ions corresponding thereto appear in parallel columns. The calculation of the concentration of the hydrogen ions is made by means of the general formula of Nernst for the case of similar electrodes and solutions containing univalent ions, viz;

$$\text{E.M.F.} = 0{\cdot}0577 \log_{\cdot 10} \frac{\text{concentration } H_1^+}{\text{concentration } H_2^+}.$$

The results permit of important conclusions regarding the composition of our egg-albumen solution. The 1 % solution of egg-albumen crystals, as originally made up, contained a concentration of free acid of 251×10^{-7} normal, and required the addition of 4·0 c.c. $\frac{N}{10}$ AmOH to reduce that concentration to that of pure water, so that the total acid combined with 1 gram of protein as crystals was 0·0004 equivalents and of this amount 0·7 % was free in the solution. Egg-albumen crystals, crystallised from acetic acid ammonium sulphate solutions were shown by Osborne (1899) to consist to some considerable part of protein combined with acid to form a salt or series of salts. What in our solutions was the nature of the acid we have not determined, but, as they were prepared in the first instance from a solution of egg-white (containing excess of ammonium sulphate) and subsequently twice recrystallised from strong ammonium sulphate solutions, it is probable that the acid radicle was SO_4[1]. The amount of hydrolysis is surprisingly small, when we consider that the solution cannot be stronger than about $\frac{1}{300}$th normal and is probably less, but, as we are at present ignorant how far the protein is saturated with acid, it is impossible to draw any conclusions as to the strength of egg-albumen as a base.

Our observations do not afford any simple explanation of the effect of acid upon the coagulation rate of a protein solution. Having ascertained that "heat coagulation" was a reaction between the protein and water in which heat and acid play the subsidiary part of accelerators we thought that, as is the case with the hydrolysis of sugar, the velocity of the reaction might be conditioned by the concentration of hydrogen ions present. At constant temperature the rate is, however, not entirely dependent upon their concentration, as $\frac{\text{velocity}}{H^+ \text{ concentration}}$ is not a constant. In Fig. 7 the logarithms of the mean coagulation rate at 69° C. (Table VI) and the logarithms of the hydrogen ion concentration are both plotted against the alkali added per gram of protein, but, although indicating a certain parallelism in the mediate portion of the curve, there is clearly another factor involved. At first (*i.e.* near the neutral point) the hydrogen ion concentration rises much more quickly than the coagulation rate, then becomes nearly proportional and finally the rate of coagulation increases more rapidly than the concentration of the hydrogen ions.

[1] Osborne (1899, II) has shown that crystals of Edestin prepared in an analogous manner in the presence of NaCl contain the chloride.

It may well be that the apparent relation, such as it is, of coagulation rate to concentration of hydrogen ions is a spurious one and that the gradual conversion of the protein to protein salt, or to a series of protein salts of increasing acidity and different velocity coefficients accounts for the influence of acid upon the rate of coagulation.

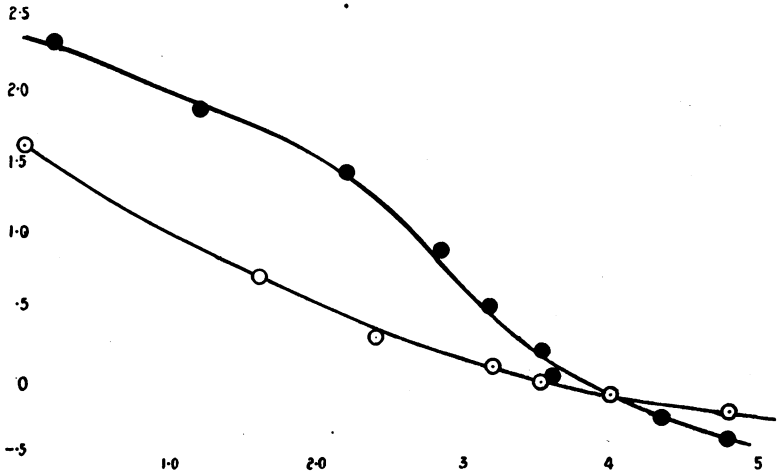

Fig. 7. Relation between average velocity of egg-albumen coagulation and the concentration of free acid (H^+ ions) in the solution.

● = hydrogen ion concentration, ordinates = $\log_{10}$ (concentration of H^+ ions $\times 10^7$).

○ = average velocity of coagulation, ordinates = $\log_{10}$ (average velocity of coagulation $\times 10^2$).

Abscissæ = number of c.c. $\frac{N}{10}$ AmOH added per gram protein to the original acid solution.

TABLE VIII. *Effect of acid upon coagulation rate of a* 1·003 % *solution*[1] *of egg-albumen, comparison of sulphuric and acetic acids.*

Temp. = 70° C., time = 55 minutes.

Composition of solution	Amount of filtrate after coagulation taken for analysis, c.c.	Weight of coagulum, grams	Uncoagulated protein, mgrs. per c.c. filtrate	% coagulated
Control, 10 c.c. albumen solution + 1 c.c. H_2O	—	—	91·2	—
10 c.c. albumen solution + 0·33 c.c. $\frac{N}{10}$ H_2SO_4 + 0·6 c.c. H_2O	4·40	0·0224	50·9	44·2
10 c.c. albumen solution + 0·16 $\frac{N}{10}$ H_2SO_4 + 0·16 $\frac{N}{10}$ HA + 0·66 c.c. H_2O	4·81	0·0243	50·5	44·6

[1] This solution has been made neutral to litmus with ammonia.

We thought to throw light upon this question by comparing the effect of equivalent quantities of sulphuric acid and acetic acid upon coagulation rate. From Table VIII it will be seen that the effect of the weaker and stronger acids was almost the same. At first this appeared to settle the matter but on calculating the concentration of free acid (about $N \times 10^{-5}$) it was of such a dilution that even acetic acid may be considered to be completely ionised.

We cannot therefore decide to what extent the influence of acid upon the reaction rate is to be attributed to variation of hydrogen ion concentration, and to alteration in the nature and proportion of salts of protein in the solution respectively.

Diminution of free acid on coagulation of egg-albumen.

It is common experience that on heating a protein solution, previously rendered neutral or faintly acid to litmus paper, it becomes progressively less acid or even alkaline *to litmus paper* as coagulation proceeds. Due allowance for this phenomenon was made by Halliburton (1884, p. 155) when using the fractional coagulation method for separating the proteins of serum. Corin and Bérard (1888) were also aware of the phenomenon and sought to compensate[1] for it in their endeavour to differentiate the proteins in egg-white. Haycraft and Duggan (1890) suggested that the phenomenon might account for the continual "raising of coagulation temperature" during the progress of coagulation.

The observations detailed in a previous part of this paper confirming those of Spiro and Pemsel (1898), Osborne (1899), and Erb (1901), show that, when acid in not too great an amount is added to solutions of crystalline egg-albumen, the greater proportion is immediately combined, but a small amount remains free owing to the hydrolysis of the salt.

The fate on coagulation of this free acid was quantitatively investigated as follows: A series of albumen solutions were prepared, all containing approximately 1 % crystalline egg albumen, but possessed of different degrees of acidity. The concentration of H^+ ions in these

[1] They attempted to adjust the reaction of the filtrate from each successive coagulation to the same point by first adding NaOH until it just showed pink with phenol-phthalein and then adding a constant amount of acid per unit volume. This procedure would be successful were it not for the fact that the protein content varied in each successive filtrate. Corin and Bérard were unaware of the salt formation of proteins, and did not realise that in consequence solutions in which their successive fractions were coagulated were progressively more and more acid. This no doubt explains partly the difference in the conclusions arrived at by these authors and other observers.

solutions was determined both before and after the solutions had been heated to 90° C. for 5 minutes. The results are given in Table IX., which shows that for small concentration of acid, up to $\frac{1}{10{,}000}$ normal, the amount withdrawn from the solution is nearly proportional to the initial concentration of free acid. Above this strength the acid removed becomes proportionally less.

The relation has not been further investigated, but is not unlike that found in absorption phenomena. We found that egg-albumen precipitated by heating in nearly neutral solution, washed and added to $\frac{1}{3000}$ normal HCl removes acid from the solution.

TABLE IX. *Showing the amount of acid fixed during coagulation of 0·99 % albumen solutions of differing acidity.*

Solution in contact with 1st H electrode	Solution in contact with 2nd H electrode	E.M.F., volts		Calculated concentration of H+ ions in albumen solution		Calculated equivalents of acid fixed per gm. protein
		As made up	After heating	As made up	After heating	
$\frac{N}{10}$ HCl	No. 1 alb. sol.	·3010	·3451	$6{\cdot}07 \times 10^{-7}$ N.	$1{\cdot}04 \times 10^{-7}$ N.*	$0{\cdot}51 \times 10^{-7}$
	No. 2 ,,	·2422	·3100	63·4 ,,	4·24 ,,	5·98 ,,
	No. 3† ,,	·1634	·2051	1470 ,,	279 ,,	131 ,,
	No. 4† ,,	·1097	·1192	12600 ,,	8590 ,,	459 ,,

* Neutrality is equivalent to $\cdot 8 \times 10^{-7}$ N.

† Solutions 3 and 4 contained ·91 % and ·87 % albumen respectively.

Summary and Conclusions.

The process of "heat coagulation" has been studied with solutions of crystallised hæmoglobin and crystallised egg-albumen. The complete solubility of both proteids after exposure to dry heat at high temperatures (110° C. – 130° C.) indicates that "heat coagulation" of protein solutions is not a pure temperature effect, but a reaction between water and protein.

In the case of solutions of hæmoglobin the rate of coagulation at any moment is proportional to the concentration of residual hæmoglobin, *i.e.* it is a reaction of the first order.

Coagulation of solutions of egg-albumen crystals is also an orderly time process, but the rate decreases more rapidly as coagulation proceeds than can be accounted for by the decrease in concentration of uncoagulated protein. The explanation of this increased complexity is attributed to want of homogeneity in the composition of egg-albumen crystals, and

to the changing conditions due to the absorption of free acid by the coagulum as formed, see below.

The conclusion of Osborne that egg-albumen crystals as prepared by Hopkins' method consist of salts of protein with the acids used in their preparation is confirmed.

In the case of 1 % solutions of egg-albumen, twice recrystallised from ammonium sulphate solution, the combined acid was equal to 0·0004 equivalents per gram of protein and the proportion of acid free was 0·7 % of the total (0·0000251 N).

On addition of alkali to the original solution most was employed in decomposition of the acid protein salts, and diminution of the free acid was very gradual.

The effect of acid upon coagulation rate was studied in a series of respectively less and less acid, neutral, and faintly alkaline solutions prepared by addition of different amounts of alkali to the original acid solution.

The effect of acid upon coagulation rate is considerable. The addition to a solution of egg albumen crystals of 4 c.c. $\frac{N}{10}$ alkali per gram protein (*i.e.* the amount necessary to neutralise) reduced the reaction rate to $\frac{1}{60}$th.

The influence of acid in accelerating the coagulation rate of a neutral solution of egg-albumen is at first relatively small; with each successive addition of acid its influence becomes disproportionately greater.

The mean coagulation rate of egg-albumen is not directly proportional to the hydrogen ion concentration. The velocity of the reaction increases at first more slowly and subsequently more quickly than the hydrogen ion content. It is possible however that hydrogen ion concentration may be a factor in determining reaction rate.

As, however, most of the acid added, combines to form salts it may be that the whole or part of its effect upon reaction rate is due to such salts reacting with water more rapidly than protein itself and the more acid salts more rapidly than the less acid salts.

The free acid in a solution of egg-albumen crystals diminishes and even disappears as coagulation proceeds. The quantity of free acid fixed by the coagulation of a definite quantity of protein is at first nearly proportional to the concentration of free acid; as this concentration increases, the amount fixed falls more and more short of proportionality. The curve suggests that the phenomenon is one of adsorption

by the coagulated particles, as protein already coagulated and washed absorbs free acid from solutions in which it is suspended.

Coagulation of both proteins is influenced by temperature in accordance with the Law of Arrhenius or some similar logarithmic law. The temperature coefficient is exceedingly high, viz. 1·91 per degree centigrade for egg-albumen and 1·3 per degree for hæmoglobin.

"Heat coagulation" is a reaction between protein and water and the effect of temperature is merely to accelerate it. The reaction has an extraordinarily high temperature coefficient and the velocity is influenced to a marked degree by a variety of conditions, most important among which is the presence of acid. It is improper to speak of proteins as having any particular "coagulation temperature" at which they are thrown out of solution. Notwithstanding this, the temperature, at which under precisely similar conditions a precipitate is first visible, may be useful in differentiating proteins, and can even be utilised to separate to some extent two proteins which at the same temperature are possessed of greatly different reaction rates.

The existence of an optimum temperature of the reaction, which is characteristic of many processes in which proteins in solution are concerned, *e.g.* various enzyme actions, and which has even been regarded as evidence of "vital action," distinguishing these reactions from ordinary chemical ones, (Duclaux, 1910) is capable of a simple interpretation. In the solution two processes are at work: (1) the operations under the influence of the enzyme, and (2) the destruction of the enzyme by water. If the influence of temperature upon the velocity of the enzyme destruction is greater than upon the velocity of the enzyme action, as the temperature rises, the effect of the former, negligible at first, counteracts the latter to a greater and greater extent, and a point must sooner or later be reached when the rate of destruction is so great that the enzyme action is rapidly arrested.

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