ON THE "HEAT COAGULATION" OF PROTEINS. PART II. THE ACTION OF HOT WATER UPON EGG-ALBUMEN AND THE INFLUENCE OF ACID AND SALTS UPON REACTION VELOCITY.

By HARRIETTE CHICK, D.Sc., *Assistant, Lister Institute of Preventive Medicine,* AND C. J. MARTIN, M.B., D.Sc., F.R.S., *Director of the Lister Institute of Preventive Medicine.*

In our first communication on this subject(1), we showed that "heat coagulation" was a reaction between proteins and water which progressed in an orderly manner, the reaction velocity being accelerated to an extraordinary degree by raising the temperature or by increasing the concentration of hydrogen-ions in the solution. In the case of hæmoglobin the reaction was found to be of the first order, but with pure crystallized egg-albumen the rate of coagulation diminished more quickly as the reaction progressed than could be accounted for by the diminishing concentration of protein. How far this falling off in rate was due to the observed simultaneous *progressive* diminution in acidity as the protein left the solution, we were unable to ascertain; we contented ourselves with the surmise that it might be so explained, but were unable to express the progress of the reaction by any simple formula.

From an analysis of our results Sutherland(2) arrived at the opinion that the data justify the conclusion that the heat coagulation of egg-albumen is a reaction of the second order, the rate of the reaction at any moment being proportional to the square of the concentration of residual albumen $\left(\frac{-dc}{dt} = kc^2\right.$, or the velocity constant $\left. k = \frac{1}{t}\left(\frac{1}{C_t} - \frac{1}{C_0}\right)\right.$, where C_0 and C_t represent the concentration of unchanged albumen at the beginning and end of the time t). We could not understand how Sutherland had obtained so constant a value for this expression from our experimental results. We had at the time tested our experimental results to see how they might accord with this interpretation,

but with two exceptions (*loc. cit.* Exps. 5 and 6, Table V, p. 419) the constant fell continuously to two-thirds or one-half of its initial value during the progress of the reaction. We have again calculated the constant for the various experiments, and obtained values different from Sutherland's, and on carefully going over his paper find that he has assumed that the reaction was proceeding at constant temperature from the time when the albumen solution was placed in the thermostat. This assumption is unjustified, because by so doing the time, five to ten minutes, taken for the solution to warm up is neglected. Only after this has taken place can the reaction be studied. When this mistake is rectified the figures lend no support to the interpretation that in the case of albumen we have to deal with a reaction of the second order. Further, Sutherland has not considered change in reaction of the solution which is taking place all the time; this is a disturbing factor of sufficient magnitude to invalidate the argument.

Sutherland's second deduction from our observations, viz.: that, over the range studied, the coagulation rate of egg-albumen is also directly proportional to the concentration of hydrogen-ions (as determined in the solution before heating), is subject to the same error, and when this is rectified the proportionality is approximate only over a small interval.

The average velocity, as determined from the time occupied in reducing concentration of albumen from 6 to 3 mgs. per c.c. (*loc. cit.* Tables VI and VII and Fig. 7), when plotted against concentration of hydrogen-ions, gives a curve (Fig. 1) showing that the influence of initial acidity on rate becomes progressively more marked as the former increases.

For example, change in the average velocity, on altering concentration of hydrogen-ions from 25 to 50×10^{-7} normal, was in the proportion of 5·5 to 10·5 or nearly 1 to 2. In more acid solutions a greater proportional change took place, and on increasing concentration of hydrogen-ions from 125 to 250×10^{-7} normal, the average velocities were increased from 28 to 84 or 1 to 3 (see Fig. 1)[1].

We do not think, however, that the exact relation of reaction velocity to hydrogen-ion concentration can be arrived at by such experiments as those published in our previous paper. The conditions are too

[1] We also have evidence that in more acid solution still (hydrogen-ion concentration equal to 4000×10^{-7} normal) an increase of only 15 % in concentration of hydrogen-ions was sufficient to double the average velocity of coagulation.

complicated. Both the concentration of protein and the concentration of acid are changing all the time as the separation of protein from the solution takes with it acid or what amounts to the same thing possibly sets free abase. All we can claim for these experiments is that they show changes in acidity, in the region where concentration of hydrogen-ions is equal to 10^{-5} normal to 10^{-7} normal, to exert a very potent influence upon the reaction rate, which accounts for the old established observation that increase of acidity lowers the "coagulation temperature" of protein solutions.

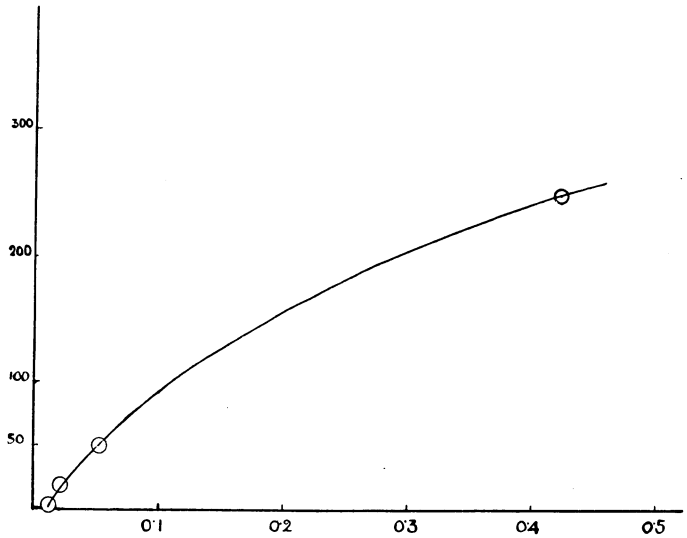

Fig. 1. Relation between average velocity of egg-albumen coagulation (in 1 % solution) and concentration of hydrogen-ions in the solution.
Ordinates = concentration of hydrogen-ions in terms of normality ($\times 10^7$).
Abscissæ = average velocity of coagulation in mgs. per c.c.

Heat coagulation of egg-albumen a reaction of the first order when precautions are taken to maintain concentration of hydrogen-ions constant.

Enough has been said to show that it is hopeless to attempt to ascertain the nature of the reaction between hot water and egg-albumen unless the concentration of hydrogen-ions can be maintained constant during the observations.

Our first endeavour to arrive at such constancy was to introduce as much as possible of a weakly ionised acid, such as butyric acid, in the

hope that the amount of acid withdrawn during coagulation would not materially diminish the concentration of hydrogen-ions, in other words that the large un-ionised fraction would act as a reservoir of hydrogen-ions. Accordingly an experiment was arranged in which coagulation took place in a concentration of butyric acid equal to $\frac{1}{10}$th normal.

The method of experiment in this and all the subsequent experiments in this paper was exactly similar to that described in our previous paper(1), p. 408. The material used was a solution of pure crystallized egg-albumen prepared according to the method of Hopkins and Pinkus(3) and recrystallized. The 1 % solution of albumen here employed contained 0·26 % of ammonium sulphate. The details of the experiment are given in Table I.

TABLE I. *Coagulation of a* 1 % *solution of crystalline egg-albumen at* 56·2° C. *in presence of excess of butyric acid; initial hydrogen-ion concentration* $= 10^{-3.39}$ *normal* (4070×10^{-7} N), *concentration at* 20 *minutes* $= 10^{-3.47}$ (3370×10^{-7} N) *normal.*

Time, minutes, $= t$	Amount of filtrate analysed, c.c.	Weight of coagulum, gms.	Residual albumen, mgs. per c.c. $= C$	$\frac{1}{t - t_0}(\log C_0 - \log C)$*
0	Control	—	10·100	—
$8 = t_0$	11·2	·0623	$5·563 = C_0$	—
10·1	14·6	·0554	3·795	·0790
12	18·3	·0513	2·803	·0742
15	19·2	·0363	1·891	·0669
20	50	·0634	1·268	·0535

* The values of this expression are in this and other cases calculated with Briggs' logarithms in place of natural logarithms.

The experiment was only partially successful for the concentration of hydrogen-ions was reduced by about 15 % during the progress of the reaction, falling from 4070×10^{-7} normal at the moment of commencing turbidity, to 3370×10^{-7} normal after twenty minutes had elapsed and most of the protein had been precipitated. We therefore tried to ensure constancy of acidity by working in a saturated solution of a very weak acid in presence of undissolved acid. For this purpose we chose boracic acid, which, even at a temperature of 50° C., at which temperature about a 9 % solution is obtained, does not give rise to a concentration of hydrogen-ions too high for our purpose. In a saturated solution at 51° C., the temperature at which the experiment was made, the concentration of hydrogen-ions is rather less than N/1000, ($10^{-3.1}$ normal or $8·0 \times 10^{-4}$ normal).

Precautions were taken to purify the boracic acid used by recrystallization. Excess of the crystals was added to a 1·5 % solution of albumen, warmed up slowly to about 49° C. When the tube containing the solution was placed in the bath at 51° C. a small further excess was added, with the object of supplying a reservoir as the acid was removed. Under these circumstances five minutes was found adequate for the tube and contents to take the temperature of the bath. From time to time samples were removed, cooled, filtered and the residual concentration of albumen determined in the ordinary way.

TABLE II. *Coagulation of a* 1·5 % *solution of crystalline egg-albumen at* 51·1° C. *in presence of saturated boracic acid; concentration of hydrogen-ions constant throughout,* $= 10^{-3.1}$ *normal* $(8000 \times 10^{-7}\,\mathrm{N})$[1].

Time, minutes, $=t$	Amount of filtrate analysed, c.c.	Weight of coagulum, gms.	Residual albumen, mgs. per c.c. $=C$	$\frac{1}{t-t_0}(\log C_0 - \log C)$
$5=t_0$	12	·1243	$10{\cdot}358=C_0$	—
15	15	·1306	8·707	·0075
30	19·5	·1332	6·831	·0072
62	28·8	·1098	3·813	·0076
101	30	·0650	2·166	·0071

The details are given in Table II. In the fifth column are given values of the velocity constant, calculated on the assumption that coagulation-rate is proportional only to the protein concentration. A very constant value is obtained, showing that this assumption is true and can be demonstrated if precautions are taken to prevent alteration in concentration of acid. The same result is shown in Fig. 2, where logarithms of concentration of protein as ordinates are plotted against time as abscissae, and the experimental points are seen to lie on a straight line.

The value of this velocity constant in other experiments where no effort was made to keep acidity constant (*loc. cit.* Tables II and V), decreased during the course of the reaction to from 1/2 to 1/7 of its original value. In the experiment with excess of un-ionised butyric acid the decrease in the value of the constant (calculated in the same way) fell to 0·67 of its original value (last column of Table I). The relative change in concentration of hydrogen-ions during this experiment

[1] By matching the tint with methyl-orange given by a saturated solution of boracic acid at 51° C. with that given by a citrate mixture, according to the method of Sörensen[4].

was small, but it occurred at an acidity where small changes in reaction have a very large effect upon coagulation rate.

We therefore conclude that, when freed from disturbance consequent on changing acidity, coagulation of egg-albumen, as we had found to be the case with hæmoglobin, proceeds as a reaction of the first order, coagulation rate at any moment being proportional to concentration of protein.

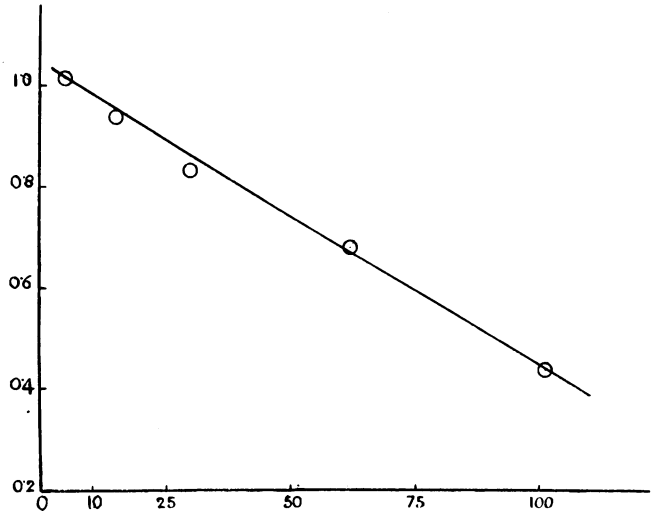

Fig. 2. Coagulation of egg-albumen in presence of saturated boracic acid at 51·1° C.
Ordinates = $\log_{10}$ (concentration of residual albumen in mgs. per c.c.).
Abscissae = time in minutes.

On the progressive change of acidity which occurs during coagulation.

The following experiments were undertaken with the view of elucidating the more usual and complex case of egg-albumen coagulation where no precautions are taken to maintain a constant reaction throughout the process. The diminution in concentration of hydrogen-ions which takes place during coagulation was studied by measurements made after successive intervals of time during the process, and the corresponding concentration of residual albumen was also ascertained.

The details of two such experiments, 1 and 2, are given in Tables III and IV, Exp. 1 made at 68·7° C. with an initial acidity of 135×10^{-7} normal, Exp. 2 at 71° C. in the presence of a concentration of sodium

chloride equal to normal and an initial acidity of 45×10^{-7} normal. Both solutions contained 0·26 % ammonium sulphate[1].

In both experiments free acid was progressively removed from the solution together with the protein. The coagulation of unit weight of protein does not remove quite the same amount of free acid throughout the experiment, but a less amount as coagulation proceeds. This might have been anticipated from observations in our previous paper(1), (p. 427) in which the amount of acid fixed by coagulation of one gram of protein varied from $0{\cdot}5 \times 10^{-7}$ to 459×10^{-7} equivalents, when the initial free acid in the solution was changed from 1×10^{-7} to $8590 \times 10^{-7} \times$ normal.

A chance relationship between residual protein and hydrogen-ion concentration emerges from an analysis of the data given in Tables III and IV. If these be plotted one against the other, the points fall upon a straight line or very shallow curve, so that over the small range of acidity of these two experiments the two are nearly proportional.

Further, within a portion of the range of acidity of most of our experiments on reaction-rate, an approximate proportionality also exists between velocity of reaction and concentration of hydrogen-ions, as may be seen from Fig. 1 above, in which the portion of the curve between the ordinates 10^{-5} and $10^{-7} \times$ normal is, within the error of experiment, linear.

These two results explain the fact that coagulation of egg-albumen, without compensation for varying acidity, may occasionally approximate to a reaction of the second order, see column 6, Tables III and IV. This is, however, merely coincidental for, as we have shown above, the velocity of reaction uncomplicated by changes in acidity is simply proportional to protein concentration.

This spurious relation of rate to square of protein-concentration is due to the facts to which attention has just been drawn, that, over a certain range of acidity, rate of reaction is nearly proportional to hydrogen-ion concentration, which in its turn is roughly proportional

[1] When the very slight acidity (10^{-5} to $10^{-7} \times$ N) obtaining in these experiments is taken into account, an error is apparent owing to neutralisation of an appreciable amount of the acid by the soluble alkali contained in the glass vessel in which the coagulation was carried out. A control experiment was made at 71° C. with a similar glass tube containing a solution of corresponding low acidity ($33 \times 10^{-7} \times$ N), but containing no protein, and this was shown to be the case, the acidity being reduced to $11{\cdot}2 \times 10^{-7}$ N. Most of the decrease, however, (to $14{\cdot}5 \times 10^{-7}$ N) took place during the first 10 minutes, and since in experiments 1 and 2, Tables III and IV, the determinations of concentration of acidity and protein began only after 10 minutes had elapsed, the error is not very great. It would, however, have been better if the experiments had been carried out in quartz vessels.

to the concentration of protein remaining. Under these conditions, a reaction of the first order simulates one in which the reaction velocity at any moment is proportional to the square of the protein concentration, because owing to the nearly linear relationships happening to obtain between concentration of protein and hydrogen-ions on the one hand, and hydrogen-ions and reaction-velocity on the other, it is as if the concentration of protein affected the rate of reaction twice over.

In the two experiments under consideration the value of the constant $k = \frac{1}{t}\left(\frac{1}{C} - \frac{1}{C_0}\right)$, where C_0 = the initial concentration of protein, and C the concentration after time t, falls off only slightly as the reaction proceeds. In most of the experiments set out in our previous

TABLE III. EXP. I. *Progressive change in acidity during coagulation of a* 1 % *solution of crystalline egg-albumen at* 68·7° C.

Time, minutes, $= t$	Amount of filtrate analysed, c.c.	Weight of coagulum, gms.	Residual albumen, mgs. per c.c. $= C$	$\frac{1}{t - t_0}(\log C_0 - \log C)$	$\frac{1}{t - t_0}\left(\frac{1}{C} - \frac{1}{C_0}\right)$	Concentration of H^+ ions in terms of normality $\times 10^7$
0	Control	—	9·750	—	—	135 N
$10 = t_0$	20	·1560	$7·800 = C_0$	—	—	79·3
14	28	·1744	6·228	·024	·00807	57·4
20	41·9	·2046	4·887	·020	·00764	42·4
37	44·4	·1360	3·066	·015	·00740	19·6

TABLE IV. EXP. II. *Progressive change in acidity during coagulation of a* 1 % *solution of crystalline egg-albumen at* 71° C., *in presence of a concentration of* NaCl = *normal solution.*

Time, minutes, $= t$	Amount of filtrate analysed, c.c.	Weight of coagulum, gms.	Residual albumen, mgs. per c.c. $= C$	$\frac{1}{t - t_0}(\log C_0 - \log C)$	$\frac{1}{t - t_0}\left(\frac{1}{C} - \frac{1}{C_0}\right)$	Concentration of H^+ ions in terms of normality $\times 10^7$
0	Control	—	9·750	—	—	45·0
$10 = t_0$	19	·1785	$9·395 = C_0$	—	—	33·5
30	21·1	·1511	7·161	·0059	·00166	18·2
100	36·3	·1493	4·113	·0039	·00152	9·53
310	38	·0716	1·884	·0023	·00145	2·07

paper(1), (Tables II and V), the constant decreased in value to a much greater extent.

In some cases in presence of more concentrated salts, (see Table VIII, p. 19), a very perfect agreement happened to be maintained and coagulation proceeded in extraordinary agreement with the progress of a reaction of the second order.

The union of egg-albumen with acid (1) *in the cold, and* (2) *on coagulation by hot water.*

It is an old observation that when protein is added to acid a diminution of acidity and of conductivity takes place, and that the solutions become still less acid after coagulation by heat has occurred. Details of some observations upon this subject and some fresh experiments made by ourselves were given in our previous paper (*loc. cit.* Tables VII and IX). These experiments we have endeavoured to complete in the present instance.

(1) *Fixation of acid by egg-albumen in the cold.* That proteins form compounds with acids and bases was pointed out as long ago as 1866 by Platner(5), since when the reaction between proteins and acids has been studied by a variety of methods. As this only indirectly concerns us, and as the literature has recently been collected by Brailsford Robertson(6), it is unnecessary to refer to it in detail.

The observations of Bugarszky and Liebermann(7) and Moore and Bigland(8) on the equilibrium of proteins with acid, however, call for mention as they bear directly upon the point under consideration. Bugarszky and Liebermann determined, by means of the concentration cell, the fall in acidity when varying quantities of protein were added to 0·05 N. HCl. Moore and Bigland placed a definite amount of protein solution and acid in a dialyser surrounded by a known volume of water and titrated the acid in the water outside the membrane after a sufficient time had elapsed for equilibrium. Both sets of observers found that the amount of acid taken up by protein varies with the concentration of free acid, but it is to be regretted that in neither case were the experiments made with pure proteins. These experiments show that the acid combined reaches a maximum above which practically no more is taken up.

The amount of acid fixed in the cold by a constant weight of pure egg-albumen in concentration of hydrogen-ions from 1 to 250×10^{-7} normal was incidentally determined by us in our previous experiments(1)

(p. 423). These observations have now been extended nearly to the point where the protein begins to suffer attack by the acid. Measured amounts of standard H_2SO_4 were added to a fixed quantity of a 1 % solution of pure crystallized egg-albumen; water was added to make each solution up to a constant volume, and about two hours allowed for equilibrium to be reached.

Following the method of Bugarszky and Liebermann, the actual free acid was deduced from the observed concentration of hydrogen-ions in the solution, as determined electrically by means of a hydrogen concentration cell. The method was exactly the same as that already described in detail(1) (p. 422). As H_2SO_4 is not completely ionised in the strengths with which we were working, we had to calculate the concentrations of free H_2SO_4 from our determinations of the hydrogen-ions present. For this purpose we took the values for the dissociation of this acid in different strengths arrived at by Kohlrausch[1] from conductivity experiments. We plotted dissociated acid obtained from Kohlrausch's determinations against total concentration of acid and drew a smoothed curve. From the curve we read off the concentration of $\frac{1}{2}$ H_2SO_4 corresponding to the particular hydrogen-ion concentration required.

The results of the experiments are given in Table V and graphically represented in Fig. 3, where equivalents of acid fixed per gram protein ($\times 10^3$) are plotted against the final concentration of acid ($\times 10^3$). The amount of acid combined rises rapidly at first, then more slowly, and from the curve, is apparently approaching its maximum in a concentration of 0·03 normal $\frac{1}{2}$ H_2SO_4.

From the tables given by Bugarszky and Liebermann the HCl fixed per gram of mixed egg-white proteins in equilibrium with different concentrations of free acid can be obtained. These values do not coincide with our own for equivalents of H_2SO_4 and pure egg-albumen but are considerably less[2]. The general form of the curve obtained by plotting these values is, however, the same.

The reversibility of this action between protein and acid was demonstrated by Moore and Bigland(8) (p. 40), and confirmed in the present instance. Two experiments were made (Nos. 9 and 10) to correspond to Experiments 5 and 6 in Table V; the solutions were made up to contain twice the concentration of acid and twice the concentration of protein that was present in the earlier experiments.

[1] Landolt, Börnstein and Meyerhoffer. *Physikal.-chem. Tabellen*, No. 229 *b* Berlin, 1905.

[2] H_2SO_4 probably combines to some extent as $R\text{-}NH_2HSO_4$.

After contact for two hours, the solutions were both diluted twice with distilled water, so that they then became identical in every respect with the solutions of Experiments 5 and 6. The final concentration of hydrogen-ions was then measured in solutions 9*a* and 10*a*, and agreed very nearly in each case with that of the corresponding previous Experiments 5 and 6. This showed that the absorption of acid in the 2 % solution was reversible and that readjustment took place on dilution.

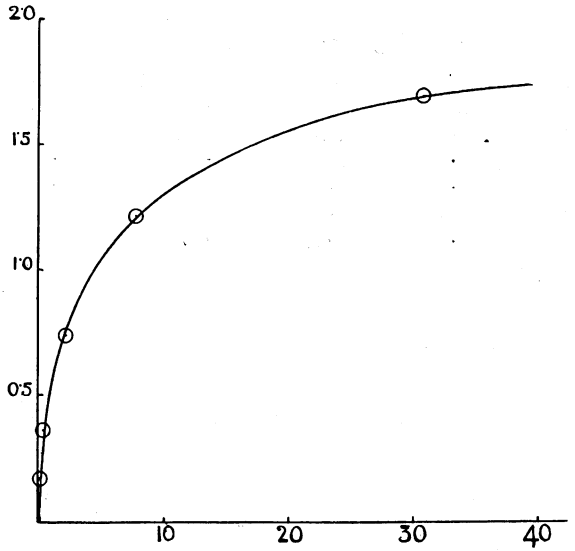

Fig. 3. Fixation of acid (H_2SO_4) by 0·95 % solution of egg-albumen at room temperature.
Ordinates = equivalents of acid fixed per 0·95 gram protein ($\times 10^3$).
Abscissæ = final concentration of acid in terms of normality ($\times 10^3$).

(2) *Further fixation of acid on coagulation.* Earlier observations on the diminution of the acidity of protein solutions on coagulation were referred to in our previous paper, where (p. 427) we also recorded the amount of free acid fixed in our own experiments by one gram of egg-albumen in solutions containing varying concentration of hydrogen-ions. Recently Sörensen and Jürgensen(9) have also made similar measurements, and further shown that starting with amounts of acid giving the same hydrogen-ion concentration, the fall in concentration of hydrogen-ions is less with a weak than with a strong acid. The smaller fall in hydrogen-ion concentration in the case of the weaker acid might have been expected, as the un-ionised fraction serves as a reservoir.

TABLE V. *Fixation of acid by crystallised egg-albumen* (0·95 % *solution*) *at room temperature.*

Total volume in each case = 52·5 c.c.

Exp.	Amount of 1 % albumen solution taken, c.c.	Amt. of N. $\frac{1}{2}(H_2SO_4)$ (or equivalent) added, c.c.	Concentration of free acid ($\frac{1}{2}H_2SO_4$) which would have resulted in absence of protein, in terms of normality, $\times 10^3$	Actual final concentration of H^+, in terms of normality, $\times 10^3$	Calculated[1] final concentration of $\frac{1}{2}H_2SO_4$, in terms of normality, $\times 10^3$	Equivalents of acid fixed per 0·95 gm. protein, $\times 10^3$
1 *a*	50	(0·1 c.c. N/1 AmOH)	—	·00094 N	—	—
1	50	0	—	·0154	—	—
2	50	0·1	1·905 N	·071	·071	·183
3	50	0·2	3·81	·195	·195	·361
4	50	0·5	9·52	2·04	2·13	·739
5	50	1·0	19·05	6·78	7·86	1·119
6	50	2·5	47·62	22·50	30·66	1·696
	2 % solution					
9	50	2·0	38·10	11·12	13·55	—
9 *a*	Exp. 9, diluted twice with distilled water		19·05	6·55	7·55	1·150
10	50	5·0	95·25	38·91	5·80	—
10 *a*	Exp. 10, diluted twice with distilled water		47·62	22·81	31·16	1·646

In their single experiment bearing on this point, which appears to have been made with undialysed egg-white solution, they found the total acid neutralised per gram of protein was approximately the same when they started with such amounts of hydrochloric, lactic and acetic acids as to afford the same hydrogen-ion concentration (*loc. cit.* Table XII, p. 427). We have not obtained this result with pure egg-albumen, but as was found to be the case in our earlier experiments, the amount of acid which disappears per gram of egg-albumen coagulated is primarily a function of the hydrogen-ion concentration.

A comparison of Exps. 1 and 2, and 3 and 4 in Table VI below shows that the amount removed is dependent upon hydrogen-ion concentration whether the particular acid be a strong or a weak one. With a concentration of hydrogen-ions equal to about $10^{-4.1}$ normal the amount withdrawn was greater with acetic than with hydrochloric acid, with a concentration equal to about $10^{-2.6}$ normal the reverse was true, but

[1] From Kohlrausch's determinations of conductivity, Landolt, Börnstein and Meyerhoffer. *Physikal.-chem. Tabellen*, No. 229 *b*. Berlin, 1905.

it will be seen that whereas the removal of the acid in the case of hydrochloric produced a considerable fall in acidity, in the case of acetic this effect was much less.

TABLE VI. *Disappearance of acid added and alteration in hydrogen-ion concentration on coagulation of a* 1·25 % *solution of egg-albumen containing* 0·36 % Na_2SO_4 *and previously acidified with hydrochloric and acetic acid respectively.*

Exp.	Acid added	Amount of acid added in 100 c.c., no. of c.c. N/10 (or equivalent)	Amount of acid remaining in 100 c.c. filtrate after coagulation in terms of no. of c.c. N/10 NaOH (or equiv.) required to neutralise to phenolphthalein	Total acid fixed in terms of equivalents acid fixed per gm. protein × 10^3.	Concentration of H^+ ions in terms of normality		Additional[1] acid fixed on coagulation in terms of equivs. per gm protein × 10^3
					In original solution after addition of acid in the cold	In filtrate after coagulation by heating	
1	HCl	3	0·2	0·22	$10^{-4.19}$ normal (650×10^{-7} N)	$10^{-4.71}$ normal (195×10^{-7} N)	·0036
2	,,	13·65	2·78	0·87	$10^{-2.61}$,, (24600×10^{-7} N)	$10^{-2.72}$,, (19500×10^{-7} N)	·041
3	$C_2H_4O_2$	13·65	9·12	0·36	$10^{-4.05}$,, (892×10^{-7} N)	$10^{-4.23}$,, (590×10^{-7} N)	—
4	,,	997·1	990·6	0·52	$10^{-2.63}$,, (23400×10^{-7} N)	$10^{-2.65}$,, (22500×10^{-7} N)	—

Interpretation of the fixation of acid on coagulation.

The amphoteric character of proteins was explained by Emil Fischer by their polypeptide constitution. As complex amino-acids they form salts with acids and alkalies, the acid joining on to the NH_2 group with change of the valency of nitrogen from triad to pentad, and the base displacing the H in the carboxyl. The salts with acids hydrolyse in water so that they are only stable in the presence of a small excess of acid. A further source of acidity is the dissociation of the hydrogen of the carboxyl group.

When a protein salt with an acid, *e.g.* HCl, is dissolved in water a certain amount of the acid is split off by hydrolysis until equilibrium is attained. If such solution be dialysed the free acid passes through the membrane and a further hydrolysis occurs. In time the whole of

[1] These amounts were calculated from the determinations of H^+ ion concentration, which in the case of HCl are proportional to the concentration of acid.

the HCl is removed and a chlorine-free solution is obtained which possesses a faint acidity, approximately 10^{-5} normal which apparently represents the electrolytic dissociation of the protein.

If such a solution be heated to a sufficient extent to precipitate the egg-albumen, *pari passu* with the separation of the protein, the acidity, as we have shown above, diminishes.

The disappearance of this small amount of acidity could be adequately explained on the supposition of electrolytic dissociation of the protein, for in this case the removal of the protein would also remove the hydrogen-ions. This explanation is the one adopted by Sörensen and Jürgensen(9) to explain their observation that if to the originally alkaline egg white solution HCl be added until the acidity equals that of a concentration of hydrogen-ions of about 10^{-5} N and then coagulation takes place, the acidity diminishes, but, nevertheless, the whole of the chlorine is found in the filtrate. They conclude from this that the HCl added to arrive at the iso-electric point was entirely occupied in the neutralisation of some base contained in the originally alkaline egg white solution, and none was available to form hydrochloride with the amino-group of the albumen. If their supposition were correct the solution would contain only the hydrogen salt of the protein, and chlorine combined with some base originally attached to the protein. In such a solution the whole acidity might, as they point out, be due to the electrolytic dissociation of the protein which is behaving as a weak acid.

We tried to repeat these experiments of Sörensen and Jürgensen and to extend them so as to include the case of more acid solutions where salt formation between protein and acid undoubtedly exists. This seemed to us important because from their results it might be inferred that in the coagulation of solutions containing salts of proteids with acids the latter did not leave the solution with the precipitated protein. We found, however, that a solution of purified crystals of egg-albumen which has been dialysed until it is free of Am_2SO_4 does not form a precipitate on heating in the presence of HCl. Precipitation occurs, however, if neutral salts are added, but the amount of a chloride which is necessary to effect agglutination makes the total chlorides to be estimated in the filtrate so great that the determination of any loss of chlorine by the fixation of HCl is rendered uncertain. A very small addition of a sulphate of sodium, potassium or ammonium is able to bring about complete separation of the heated protein in a particulate form. In the presence of 0·014 N. HCl and 0·36 % Na_2SO_4 we found that although acid disappeared on coagulation the whole or

nearly the whole of the chlorine remained in the filtrate. On subsequent experimentation we ascertained, however, that a corresponding quantity of SO_4 had combined with the coagulum. We therefore had recourse to H_2SO_4 for acidifying the solution of egg-albumen crystals and a small quantity of Na_2SO_4 was added to facilitate separation of the coagulum. Only a small amount of this salt is required because SO_4 assists the agglutination of denaturated egg-albumen much more powerfully than Cl.

In the following experiment, the precise details of which are set out in Table VII, 500 c.c. of 1 % egg-albumen was made up, containing an amount of acid which, in the absence of protein, would have made the solution equal to 0·05 N ($\frac{1}{2}$ H_2SO_4). It also contained about 0·1 % of Na_2SO_4. The whole was placed in a Jena flask and heated with a reflux condenser in a bath of boiling water for 30 minutes. At the end of that time it was cooled down and filtered with precautions to avoid evaporation. Portions of the filtrate were taken for the estimation of total acid by titration with $\frac{1}{50}$th N. NaOH and phenolphthalein as indicator, and for the gravimetric determination of total sulphates.

TABLE VII. *Showing that the protein-acid-salt formed on addition of* H_2SO_4 *to a solution of pure crystalline egg-albumen is precipitated as such on coagulation by hot water.*

Material	Amount of acid, in terms of N/10, $\frac{1}{2}H_2SO_4$, added in 100 c.c. of the solution, c.c.	Amount of Na_2SO_4 in terms of N/10, Na_2SO_4, added in 100 c.c. of the solution, c.c.	Total amount of SO_4, in terms of N/10, $\frac{1}{2}SO_4$, added in 100 c.c. of the solution, c.c.	Amount of acid, in terms of c.c. N/10 NaOH, added for neutralisation to phenolphthalein, remaining in 100 c.c. filtrate after coagulation, c.c.	Total amount of SO_4, in terms of c.c. N/10, $\frac{1}{2}SO_4$, present in 100 c.c. filtrate, c.c.	Equivalents of acid fixed per gram protein, $\times 10^3$	Equivalents of $\frac{1}{2}SO_4$ fixed per gram protein, $\times 10^3$
1 % sol. egg-alb.	20	14·26	34·26	9·64	25·36	1·04	0·89

From Table VII it will be seen that 1 gram of egg-albumen on coagulation in 1 % solution had removed 0·00105 equivalent of acid and about 10 % less of SO_4.

It is clear, therefore, that on coagulation of egg-albumen in more acid solutions the bulk of the acid leaves the solution with the protein. In other words the salt is precipitated as such.

The want of agreement between disappearance of acid and sulphate indicates that some base other than protein has combined with a

portion of the acid. The possibility of a trace of ammonia or di-amino-acid being split off during coagulation was considered, but excluded as the filtrate was found to be nitrogen-free. The only other sources from which a base could be derived are the ash of the protein and alkali from the glass of the vessel in which the protein solution was heated. The ash in our solution of protein was only 0·0016 gr. per gram of egg-albumen; this, even if all composed of MgO or CaO, is a little short of the amount required for combination with the small excess of SO_4 found in the filtrate. On the other hand it is unlikely that such an amount of alkali could be dissolved out of the glass of a Jena flask during 30 minutes' heating at 100° C.

We propose to ascertain for certain whether the small amount of ash contained in our egg-albumen is indeed removed by boiling in this concentration of acid.

Another explanation of the loss of acidity on coagulation is that CO_2 is driven off by the heating. This view, which was formulated by Michaelis and Rona[10] is quite inadequate to explain the large amount of acid disappearing from the more acid solutions of pure egg-albumen we employed, and the progressive nature of the disappearance, for it must be remembered that we were working with crystallized egg-albumen and not with diluted serum or egg-white. Moreover, in determining the hydrogen-ion concentration hydrogen was passed through the cell until a constant reading was obtained whereby CO_2 would be displaced. It is not possible to work with serum proteins in such acid solution as we employed with egg-albumen, owing to failure of agglutination, but there is little doubt that our results could be applied to serum proteids.

Sörensen and Jürgensen[9] (p. 424) have also replied to this theory of Michaelis and Rona by showing that a diminution in hydrogen-ion concentration took place in solutions of egg-white and blood-serum where all carbon-dioxide had been previously driven off by passing through a continuous stream of hydrogen in strongly acid solutions. They also showed that no extra carbon-dioxide was produced during coagulation.

Our conclusions regarding the relation of acid and protein during heat coagulation of pure crystalline egg-albumen may be summarised as follows.

(1) The amount of acid combined as protein-acid-salts, when acid is added to a solution of protein, depends on the concentration of free acid. Conversely, these salts undergo hydrolysis which accounts for the free acid always present in such solution: *e.g.* with HCl.

$$\text{Prot.} - \text{HCl} \rightleftharpoons \text{Prot.} + \text{H}^+ + \text{Cl}^-.$$

(2) On treatment by hot water the protein-acid-salts are precipitated as such; the percentage of acid combined with them being dependent on the acidity of the solution at the time of coagulation.

(3) The interaction between egg-albumen and hot water, if acidity be kept constant, proceeds as a reaction of the first order.

(4) The velocity of the reaction is conditioned by the acidity (hydrogen-ion concentration) of the solution.

(5) Unless precautions be taken to maintain the acidity of the solution at a constant level, the concentration of free acid progressively diminishes as the protein is precipitated.

(6) The conclusions embodied in (3), (4) and (5) explain why under ordinary circumstances the reaction between egg-albumen and hot water appears to be of a more complicated nature.

(7) From (2) combined with (4), it appears that the original protein is acted upon by hot water less readily than protein-acid-salts, and further that these salts suffer attack at a rate which is dependent upon the amount of acid they contain[1]. If this is so, a satisfactory explanation of the progressive diminution of acidity during coagulation is forthcoming, as the separation from the solution of protein-acid-salt will disturb the equilibrium between hydrolysed and unhydrolysed salt. Some portion of the free acid and free protein will therefore combine. This phenomenon will be progressive.

Effect of salts upon the velocity of "heat coagulation" of proteins.

The effect of salts upon "coagulation temperature" has been studied by K. V. Starke(11), Haycraft and Duggan(12), Osborne and Campbell(13), Joh. Starke(14), Pauli and Handovsky(15) and others. All these workers found that the temperature at which precipitation was first observed was raised with increasing concentration of salts.

A higher "coagulation temperature" is, as was shown in our previous paper, an indication of the diminution of "coagulation-rate" at constant temperature.

In our experiments we studied coagulation-rate by the same method as before. Solutions containing 1 % of albumen were made by dilution from a stock solution of egg-albumen crystals. The stock albumen solution contained Am_2SO_4 so that the 1 % solution of egg-albumen had in addition to protein 0·26 % of the salt. It was not found necessary to get rid of this small amount of Am_2SO_4 by dialysis. The rate of coagulation at 70·9° C. was measured in the control solution and also in similar solutions containing in addition various quantities of sodium chloride and extra ammonium sulphate. In the case of the stronger salt solutions the time necessary for the experiments was so long that precautions had to be taken to avoid errors due to evaporation of the

[1] A possible catalytic influence of the hydrogen-ions cannot be excluded.

protein solution. This was successfully done by using a rubber cork and by filling a mixture of vaseline and paraffin wax into the glass bearing in the cork through which the stirrer passed. Under these conditions the loss of water was negligible.

The coagulation-rate was measured up to a concentration of three times normal (equivalents) in the case of ammonium sulphate and of sodium chloride. It was not possible to work with higher concentrations of salt as the rate of coagulation became too slow to study conveniently at

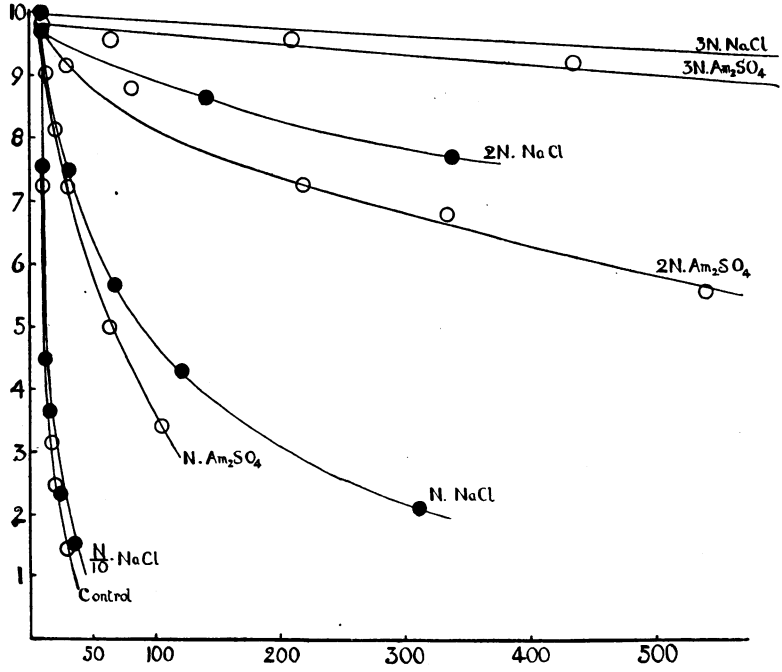

Fig. 4. Effect of addition of salt, NaCl and Am_2SO_4, in various concentrations, upon coagulation rate of 1 % solution of egg-albumen at 70·9° C.
—•— Experiments with NaCl. —o— Experiments with Am_2SO_4.
Ordinates = concentration of residual albumen in mgrs. per c.c.
Abscissæ = time in minutes.

70·9° C., the temperature of experiment. The details of our results are set forth in Table VIII below and are also graphically represented in Fig. 4, where the concentration of residual albumen in the different experiments has been plotted against time.

It will be seen that the presence of salts greatly diminishes coagulation-rate. From the smoothed curves it appears that in a concentration of NaCl equal to normal the rate is only $\frac{1}{16}$th, and in twice normal

TABLE VIII. *Coagulation of* 1 % *solution of pure crystalline egg-albumen, with original salt content* (= 0·26 % Am_2SO_4) *and after addition of* NaCl *and* Am_2SO_4 *in varying concentration at* 70·9° C.

Exp.	Salt added	Concentration of salt in terms of normality	Time, minutes = t	Amount of filtrate analysed, c.c.	Weight of coagulum, grs.	Concentration of residual albumen, mgrs. per c.c. = C	$\frac{1}{C}$	$\frac{1}{t_n - t}\left(\frac{1}{C} - \frac{1}{C_n}\right)$ *
1	Control	0	10	8·7	·0455	5·231	·191	·0242
			12	14	·0622	4·443	·225	·0250
			16·17	20	·0630	3·150	·318	·0258
			20	20	·0492	2·460	·407	·0268
			$30 = t_n$	50	·0742 = C_n	1·484	·675	—
							Mean	·0250
2	NaCl	0·1 N	10	10	·0556	5·560	·180	·0191
			12	15·4	·0690	4·480	·223	·0189
			15	35·3	·1290	3·655	·273	·0192
			23	26·4	·0613	2·322	·431	·0191
			$25 = t_n$	26·9	·0409 = C_n	1·520	·658	—
							Mean	·0191
3	NaCl	N	10	12·5	·1214	9·711	·103	·00129
			31	15·5	·1160	7·485	·134	·00127
			67	20	·1130	5·650	·177	·00128
			121	23·3	·0964	4·137	·242	·00130
			$311 = t_n$	32·5	·0664 = C_n	2·043	·490	—
							Mean	·00128
4	NaCl	2 N	10	11·1	·1074	9·676	·103	·0000948
			142	12	·1034	8·617	·116	·0000923
			$337 = t_n$	15	·1142 = C_n	7·614	·134	—
							Mean	·0000935
5	NaCl	3 N	10	Control	—	10·000	·100	·0000143
			940	15	·1258	8·388	·119	·0000115
			1420	18	·1495	8·306	·120	·0000143
			2393	20	·1501	7·505	·133	·0000158
			$3025 = t_n$	21	·1466 = C_n	6·982	·143	—
							Mean	·0000140
6	Am_2SO_4	1·03 N	13	11	·0992	9·018	·111	·00199
			20	20	·1628	8·140	·123	·00201
			30	19·5	·1407	7·215	·139	·00207
			63	22·3	·1113	4·992	·200	·00224
			$105 = t_n$	46	·1565 = C_n	3·403	·294	—
							Mean	·00208
7	Am_2SO_4	2·03 N	10	8·6	·0843	9·803	·100	·000154
			30	15·5	·1421	9·170	·109	·000143
			81	10	·0877	8·770	·114	·000148
			219	21·5	·1549	7·205	·139	·000134
			335	22·4	·1504	6·716	·149	·000160
			$541 = t_n$	26·4	·1450 = C_n	5·493	·182	—
							Mean	·000148
8	Am_2SO_4	3·03 N	10	10	·0966	9·660	·103	·00000412
			66	11·3	·1078	9·540	·105	·00000412
			210	16·2	·1542	9·519	·105	·00000438
			435	16·3	·1485	9·110	·110	·00000461
			1055	24·2	·1892	7·819	·128	·00000535
			$2495 = t_n$	35	·1694 = C_n	4·841	·205	—
							Mean	·00000452

* The values of the constant are calculated using the *last* determination of albumen concentration as standard, owing to the greater accuracy, as regards time, with which these samples can be taken in the quick experiments.

only $\frac{1}{34}$th of that in the control. The effect of Am_2SO_4 was somewhat less. This effect of salts is not due to any influence on the second phase of coagulation, viz. the separation of the altered protein in the particulate form, because in the samples drawn this was completed so that all filtered quickly and well, affording clear filtrates in which no further precipitation occurred.

In the last column of Table VIII are given the values of

$$\frac{1}{t_n - t}\left(\frac{1}{C_n} - \frac{1}{C_t}\right)^1,$$

where C_n the final concentration and C the concentration after time t has elapsed. Although the values show a satisfactory constancy, for reasons stated above, we consider this approximation to a reaction of the second order (the reaction velocity at any moment being proportional to the square of the concentration of residual protein at that moment) is a mere coincidence. This coincidence has, however, proved convenient, as it enables the effect of salts to be quantitatively expressed by comparing the velocity constants characteristic of each experiment.

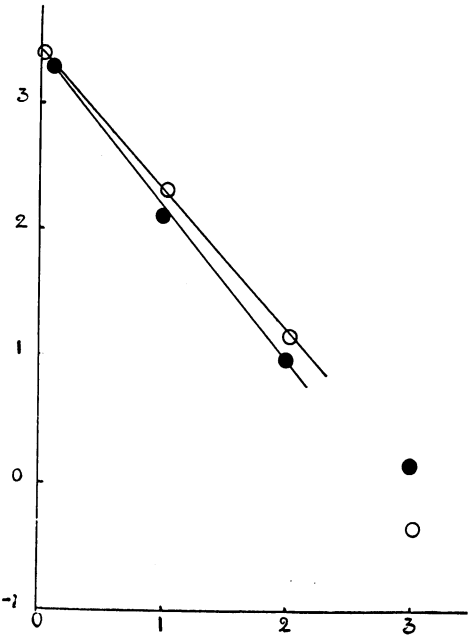

Fig. 5. Relation between the concentration of salt and the coagulation-rate as measured by the velocity constant.

Experiments with NaCl •; experiments with Am_2SO_4 o.
Ordinates = $\log_{10}$ (velocity constant $\times 10^5$).
Abscissæ = concentration of salt in terms of normality.

When we plotted the logarithms of the velocity constants obtained for different concentrations of the same salt against the reciprocal of the concentrations of salt, between the limits 0 and twice normal, the points were very nearly in a straight line (Fig. 5), showing that as

[1] See footnote to Table VIII.

the concentration of salt in the solution is increased arithmetically the velocity of coagulation diminished geometrically.

This relation, speculations as to the significance of which appear below, is maintained up to a concentration of twice normal NaCl and Am_2SO_4, but the observation with three times normal salt indicates that with stronger solution the effect of NaCl and Am_2SO_4 becomes slightly less and slightly more[1] potent respectively.

This great retardation of the rate of denaturation by neutral salts becomes of practical significance in the estimation of proteins by the method of Devoto(16). Two hours' heating on a water bath at 100° C. does not complete the process in the presence of saturated Am_2SO_4. The efficacy of Hopkins'(17) modification is due to the subsequent heating in dilute salt solution. The difficulty can also be got over by heating for 5 minutes at 110° C. in an autoclave.

The effect of the addition of neutral salts upon the hydrogen-ion concentration of protein solutions. It occurred to us that the effect of neutral salts in slowing reaction-rate might be, in whole or in part, due to change in the hydrogen-ion concentration of the protein solutions. We, therefore, determined by the electrical method the concentration of hydrogen-ions in our egg-albumen solution, and the same made up to contain a concentration of NaCl varying between $\frac{1}{10}$th normal and twice normal. The results are given in Table IX, and show that acidity is indeed progressively lowered with each increase in concentration of salt. The addition of an amount of salt to make the

TABLE IX. *Effect of addition of various amounts of* NaCl *upon hydrogen-ion concentration and coagulation velocity of* 1 % *egg-albumen solution at* 70·9° C.

Concentration of NaCl in terms of normality	H^+ concentration, in terms of normality	Coagulation velocity as measured by the velocity constants (from Table VIII)
0	$10^{-4.88}$ normal (132×10^{-7} N)	·0250
0·1 N	$10^{-4.88}$,, (132 ,,)	·0191
1 N	$10^{-5.30}$,, (50 ,,)	·00128
2 N	$10^{-5.73}$,, (19 ,,)	·0000935

[1] Pauli and Handovsky(15), working with thoroughly dialysed ox-serum, found that the effect of ammonium sulphate in hindering coagulation-rate (as measured by determining "coagulation temperature") was reversed at a concentration between 2× and 3× normal. In the above experiments we find that the rate of lessening coagulation velocity is on the contrary increased. Further experiments with ammonium sulphate and a solution of pure egg-albumen showed a progressive slowing of coagulation-rate up to the point where precipitation was imminent. We have not made experiments with serum.

solution = N/10 had no appreciable effect, but in normal solution of NaCl the hydrogen-ion concentration was less than 1/2, and in twice normal solution was 1/7th of that of the control solution to which no salt had been added. The most obvious explanation of this action is that in presence of sufficient amount of salt the hydrolysis of the protein-acid-salt is hindered.

This effect of salt in lessening the acidity of solutions containing protein is inconsistent with the conclusions of other observers, derived from indirect methods of measuring hydrogen-ion concentration. Pauli and Handovsky [15], using indicators, found that adding salts to an acidified solution of ox-serum resulted in an increased acidity. Hardy [19] found the same result with salt and "acid albumen."

These observers appear to have been misled by their indicators. Pauli and Handovsky's estimations of acidity were made by comparison of the colour given with indicators, such as methyl-orange and alizarine. The colour of indicators, particularly that of methyl-orange, has since been shown by Sörensen [4] (p. 240) to be modified by the presence of any considerable amount of protein. Michaelis and Rona [20] have found that the tint of an indicator in a solution having a particular hydrogen-ion concentration may also be influenced by the presence of salts, although the amount of hydrogen-ions as determined by the concentration cell remains constant. Different indicators are affected in different directions. With some, the addition of salt induced an apparently more alkaline reaction, *e.g.* litmus, with others a more acid reaction, *e.g.* methyl-orange and alizarine. The latter are the two indicators used by Pauli and Handovsky and Hardy in their experiments.

We have repeated the experiments with the protein materials employed by these observers, using methyl-orange as an indicator, and obtained the same results as they did, that is to say, the change of colour invariably indicated *increase in acidity*.

However, in every case where direct measurement of hydrogen-ions was made, the solutions were found to be made more alkaline by the addition of sodium chloride; this was true of experiments made over a wide range of acidity, viz. from $10^{-1.7}$ normal to $10^{-4.08}$ normal, or 189000×10^{-7} normal to 832×10^{-7} normal, roughly between N/100 and N/10,000. These results are set forth in Table X.

Having ascertained that the addition of neutral salts to a slightly acid solution of protein produced a quite considerable diminution in acidity, it occurred to us that the fall in reaction-rate produced by salts might not be a direct effect of salts *per se* but be indirectly brought about through the diminution in hydrogen-ion concentration occasioned by their presence. We were somewhat strengthened in this opinion by the fact that we had previously discovered that within certain limits there exists a similar logarithmic relation, but in the inverse sense, between acid added to a protein solution and velocity of coagulation. If the logarithms of the average velocity in three experiments detailed in our previous paper (1) Table VI, see also Table XI below), made with a protein solution after addition of different amounts of acid per gram protein, be plotted against acid added, they fall upon a line, indicating

that as the acid varies arithmetically the velocity of reaction varies geometrically.

From a comparison of Tables IX and XI, however, it is seen that the diminution in acidity caused by the addition of NaCl is insufficient to account for the whole effect of the salt in lessening coagulation velocity. We were able to arrive at this conclusion because in the experiments set forth in Table XI a decrease in hydrogen-ion concentration from $10^{-5.30}$ × normal to $10^{-5.75}$ × normal, caused by addition of alkali, the coagulation-rate (average velocity) was reduced to $\frac{2}{3}$ths. In one of our experiments with salt (Table IX) an almost precisely

TABLE X. *Effect of addition of* NaCl *to a concentration of* 1 % *upon the reaction of solutions containing various proteins, previously acidified with various acids.*

Material	Total volume, c.c.	Acid added	Amount of acid added, in c.c. N/10 (or equiv.)	NaCl present %	Concentration of H^+ in terms of normality
Egg-white, diluted 1 in 32, boiled; 5 c.c. diluted to 8 c.c. by addition of water, acid, etc.	8	0	0	0	$10^{-8.00}$ normal ($0{\cdot}1 \times 10^{-7}$ N)
	8	$C_2H_4O_2$	0·7	0	$10^{-4.08}$ normal (832×10^{-7} N)
	8	,,	0·7	1·2	$10^{-4.25}$ normal (563×10^{-7} N)
	8	H_2SO_4	0·7	0	$10^{-2.89}$ normal (12900×10^{-7} N)
	8	,,	0·7	1·2	$10^{-3.64}$ normal (2300×10^{-7} N)
Horse-serum, dialysed, diluted 1 in 25; 25 c.c. diluted to 50 c.c. by addition of water, etc.	50	HCl	12	0	$10^{-1.75}$ normal (189000×10^{-7} N)
	50	,,	12	1·0	$10^{-1.86}$ normal (178000×10^{-7} N)

TABLE XI. *Effect of change of acidity (induced by addition of different amounts of alkali to an originally acid solution) upon average coagulation velocity of* 1 % *egg-albumen solution at* 69° C. *(from previous paper, loc. cit., Table VI).*

No. of c.c. N/10 AmOH added per gm. protein	H^+ concentration, in terms of normality (from Fig. 7, *loc. cit.*)	Av. velocity of coagulation, during the period in which concentration of residual albumen was being reduced from 6 mgs. per c.c. to 3 mgs. per c.c., in mgs. per c.c.
0	$10^{-4.6}$ normal (251×10^{-7} N)	·4225
1·6	$10^{-5.3}$,, ($50{\cdot}1$,,)	·0522
2·4	$10^{-5.75}$,, ($17{\cdot}8$,,)	·0200

similar reduction in acidity (hydrogen-ion concentration reduced from $10^{-5.30} \times$ normal to $10^{-5.73} \times$ normal) was caused by increasing the concentration of sodium chloride from normal to twice normal; in this instance, however, the velocity constant was reduced to nearly $\frac{1}{14}$th of its value.

It follows, therefore, that a retardation of reaction by neutral salts is produced in two ways:

(1) To a small extent by lowering the hydrogen-ion concentration of the acid protein solution.

(2) To a large extent by some direct influence of strong salt solutions.

This effect is cumulative. If a particular addition of salt slows the rate by $\frac{1}{x}$, by a further addition of an equal amount the rate is reduced by $\frac{1}{x^2}$.

The tendency for the alteration of coagulation-rate of protein on addition of salt or acid to assume a logarithmic relationship to the salt or acid added is rather striking. It has been suggested to us by Mr W. B. Hardy that, assuming rate of reaction to be a function of total surface of particles of protein, such an effect would be produced if additions of salt and acid were to consistently increase and diminish respectively the mean size of the molecular aggregates, whereby the total reacting surface of the protein would be diminished logarithmically in the case of salt, and increased logarithmically in the case of acid. A similar explanation is suggested to explain the greatly enhanced effect of temperature upon the coagulation velocity of proteins (see below).

Effect of the presence of Am_2SO_4 *on the temperature coefficient of the reaction between egg-albumen and hot water.*

In our previous paper we showed that the temperature coefficient of the reaction called heat coagulation was, both in the case of hæmoglobin and egg-albumen, an extraordinarily high one, viz. 1·3 times per 1° C. with the former and 1·91 per 1° C. with the latter. In the presence of a concentration of Am_2SO_4 equal to twice normal the effect of temperature upon rate of reaction of egg-albumen appears from a comparison of the two experiments detailed in Table XII to be considerably less, viz. 1·57 per 1° C. At present it is not clear what the significance of these high temperature coefficients is, or how the presence of salt modifies them. It has been suggested that the phenomenon may be related to some

change in the state of aggregation of the particles with rise of temperature, whereby the extent of the reacting surfaces is greatly increased, in addition to the usual increase in molecule energy.

TABLE XII. *Effect of temperature upon coagulation velocity of egg-albumen in* 1 % *solution in presence of a concentration of* Am_2SO_4 *equal to twice* (2·03 ×) *normal.*

Temperature, °C.	Time, minutes, $=t$	Amount of filtrate analysed, c.c.	Weight of coagulum, grms.	Concentration of residual albumen, mgrs. per c.c. $=C$	$\frac{1}{C}$	$\frac{1}{t_n - t}\left(\frac{1}{C_n} - \frac{1}{C}\right)$
75·45	10	10	·0907	9·070	·110	·00114
	15	15	·1292	8·613	·116	·00114
	23	16·7	·1351	8·091	·124	·00115
	36	21·8	·1536	7·046	·142	·00112
	50	31·5	·1959	6·220	·161	·00108
	$130 = t_n$	23	·0932	$4·052 = C_n$	·247	—
					Mean	·00113
70·9	(From Table VIII)		...	...	...	·000148

It is necessary to again emphasise the fact that complete "heat coagulation" of proteins (as investigated by the method of estimating residual protein at successive intervals of time) consists of two processes:

(1) the union of the protein and hot water ("denaturation"),

(2) subsequent agglutination and separation of the product.

The first may take place without the second, as when a 10 % solution of serum or egg-white is boiled without adjusting the reaction. We have lately made a separate study of the agglutination process, the results of which will form another communication. We are convinced that in the above and in our previous experiments we have been investigating the nature of the first process, uncomplicated by the second, for the following reasons:

(1) The samples as they were taken from the partly coagulated solutions filtered easily and clearly, and the filtrates showed no further change on standing.

(2) The temperature coefficient of this first process in coagulation we have found to be extremely high, coagulation-rate being increased 1·3 and 1·9 times for rise of 1° C. in temperature in the case of hæmoglobin and egg-albumen respectively, whereas we have ascertained that the temperature coefficient of the second process—agglutination—under

circumstances in which this phenomenon is complete, is very much lower: about 2 to 2·5 for 10° C. rise in temperature or 1·10 for 1° C.

(3) The effect of salt supports the above conclusion, for whereas we find that the addition of sodium chloride and other salts facilitates the separation of the "denaturated" egg-albumen in a particulate form, it has been shown above to greatly delay the rate of coagulation as studied by us.

We therefore conclude that agglutination-rate is here much in excess of denaturation-rate, and that the velocity of the latter is the limiting factor of the complete process in all our experiments.

Summary and Conclusions.

1. "Heat coagulation" of egg-albumen consists of two processes, viz.

(1) the reaction between the protein and hot water ("denaturation"), and

(2) the separation of the altered protein in a particulate form (agglutination).

In the experiments which form the basis of the present and previous communications, it was always arranged that the rate at which (2) occurred was greatly in excess of that of (1). Accordingly, in our study of "heat coagulation" and the influence of various factors upon the rate of the reaction, we are merely concerned with (1), as this is the limiting factor in the process.

2. The action of hot water on egg-albumen, which constitutes the first stage in "heat coagulation," is, if means be taken to prevent change in acidity during the process, a reaction of the first order. The same was previously found to be true for hæmoglobin.

3. During the process, as the protein is precipitated, free acid is progressively removed from the solution. The consequent progressive diminution in the acidity of the solution accounts for the fact that the reaction appeared to be of a more complicated character.

4. Further experiments on the fixation of acid by pure egg-albumen in the cold, showing the relation of amount of acid fixed to the acidity of the solution and the reversibility of the process are presented.

5. The extra amount of acid fixed by egg-albumen on coagulation and its dependence (1) upon the total concentration of acid and (2) upon acidity (hydrogen-ion concentration) has been determined.

6. The disappearance of the small additional amount of acid on coagulation is explained as follows: coagulation disturbs the equilibrium between hydrolysed and unhydrolysed protein-salt since the unhydrolysed salt is acted upon by hot water and precipitated much more rapidly than the protein itself. This disturbance of equilibrium is adjusted by a corresponding diminution in the concentration of the products of hydrolysis, *i.e.* a combination of the protein with free acid. In this way the free acid progressively disappears.

7. The effect on coagulation-rate of the presence of salts (NaCl and Am_2SO_4) up to a concentration of three times normal has been studied and shown to greatly lower the rate of reaction.

8. Up to a concentration of salt equal to twice normal the effect on coagulation-rate varies geometrically as the additions of salt are varied arithmetically.

9. The addition of neutral salts to an acid protein solution disturbs the equilibrium between protein and acid so that less free acid is present. The influence of salts in lowering coagulation-rate may be to some extent thus explained, but the major part of the effect must be due to some direct influence of the salts upon the system.

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