CXX. THE KINETICS OF PROTEIN DENATURATION.

PART III. THE INFLUENCE OF NEUTRAL SALTS ON THE VELOCITY OF THE HEAT DENATURATION OF OXYHAEMOGLOBIN.

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Aronstein [1874] and Heinsius [1874] first pointed out that if a protein solution be dialysed until free from electrolytes then no coagulation occurs on boiling. If, however, a solution so treated is cooled and a little salt added in the cold, flocculation immediately takes place. From this it is inferred that although the process of denaturation can take place in the absence of salt, yet the salt, presumably as electrolyte, is necessary for the final stage in coagulation, viz. flocculation.

Other early investigations on the effect of neutral salt on proteins are those of Virchow [1854] and of Hofmeister and his pupils [1888, etc.]. These however were mainly concerned with flocculation by salts in the cold, and from the results obtained these authors concluded that inorganic salts threw proteins out of solution by dehydrating them.

Later, Ostwald [1907] showed that certain alkali salts lowered the "coagulation temperature" of egg-albumin. From the temperatures at which he worked, namely 20–40°, it is probable that he was dealing here with flocculation as distinct from denaturation.

Pauli and Handowski [1910] showed that the addition of neutral salts to alkali protein increased the coagulability by heat, the salts of the alkaline earths being more effective than those of the alkali metals. This effect they explained as being due to the depression of the ionisation of the protein owing to the formation of salts of the type $Cl.KH_2N.R.COONa$, thus bringing the properties of the solute nearer to those of the original protein.

The first to distinguish between the action of neutral salts upon flocculation in the cold and upon heat denaturation were Michaelis and Rona [1910] who state that while neutral salts diminish the rate of flocculation in the cold they do not delay the rate of heat coagulation.

Variation of the ammonium sulphate concentration of the solution was then studied by Micko [1911] who found that while the "coagulation temperature of *egg*-albumin was unaffected by the addition of ammonium sulphate that of serum-albumin and of milk-albumin was raised by the addition of ammonium sulphate."

In contrast to this, Chick and Martin [1912] state that both ammonium sulphate and sodium chloride greatly reduce the rate of denaturation of *egg*-albumin and also the critical increment of the process.

Further, Lepeschkin [1922] states that increase of neutral salt concentration increases the rate of denaturation when the salt concentration is low and diminishes it when the salt concentration is high, leaving it unaltered at intermediate concentrations. He is of the opinion that the effects produced by the salts are to some extent specific.

It will be apparent from the above that no little confusion exists in the literature on this point, on account of the lack of distinction between the effect of salts in the cold, an action which they exert *per se* and their influence as external factors on the heat denaturation of proteins, which latter seems to be a reaction between the protein, the ions of water, and the water of hydration of the protein.

In the present work the effect of varying concentrations of ammonium sulphate, sodium chloride and sodium sulphate on the velocity of the heat denaturation of oxyhaemoglobin has been studied.

Experimental.

The solutions used were "neutral," that is to say the p_H as determined by use of the quinhydrone electrode at 37° was 6·76, at which point the concentration of hydrogen ion is equal to that of hydroxyl ion. The solutions contained various amounts of neutral salts.

Addition of neutral salt to the solution in general increases the hydrogen ion activity. It was decided to work at a constant hydrogen ion activity as this is the factor which it is desired to eliminate in the present work. As Sørensen, Heyrup and Lang [1921] have shown, the effect of salts on the p_H as measured by the hydrogen gas electrode and the quinhydrone electrode respectively is not the same. It was therefore decided to take as a standard of "neutrality" that solution which, at 37°, possesses a p_H value of 6·76 as measured by the hydrogen gas electrode, when correction was made to normal pressure of hydrogen over the solution[1]. In each case a small amount of acid or alkali was added to bring the p_H to 6·76.

[1] In order that the p_H of a solution might be determined *rapidly* the correction to be applied to a reading made by means of the quinhydrone electrode at any given salt concentration was determined.

To effect this the E.M.F. of a series of such cells as

Gold foil in presence of saturated quinhydrone	Buffer solution containing x% neutral salt	Platinum black saturated with hydrogen gas

The oxyhaemoglobin was prepared in the manner described in Part I. The same analytical technique was employed. As before the salts used were Merck's "puriss" thrice crystallised.

For the sake of brevity only the mean velocity constants are quoted. Each value recorded is the average of the mean value obtained from at least two concordant duplicate experiments.

SECTION 1. *The influence of varying ammonium sulphate concentrations on the velocity of the heat denaturation of oxyhaemoglobin in a solution of* p_H 6·76.

Table I. *Summarising table.*

70°

Concentration of ammonium sulphate (g. per 100 cc.)	(equivalents per litre)	$k_{uni} \times 10^4$
0·0067	0·00102	25·7
0·1	0·0152	27·0
1·0	0·152	28·0
5·0	0·758	45·5
6·0	0·910	49·0
7·5	1·137	54·0
10·0	1·52	36·7
15·0	2·274	14·0
20·0	3·032	5·7

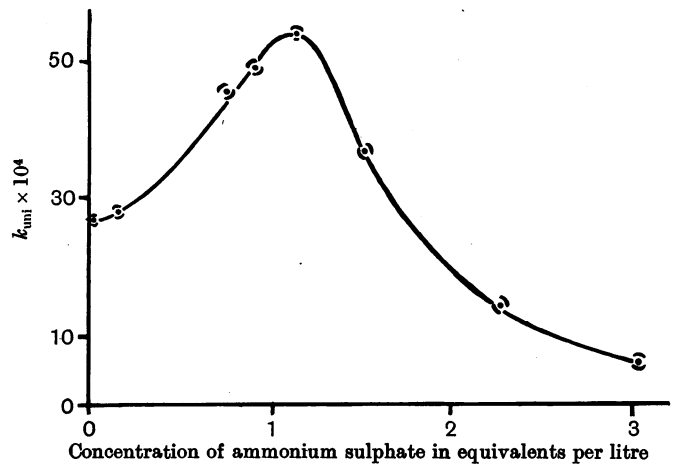

Fig. 1. Heat denaturation of oxyhaemoglobin at 70°. Variation of k_{uni} with concentration of ammonium sulphate.

was determined at 37°. The same solution was used throughout the cell, thus eliminating liquid/liquid potential difference.

The results of these experiments are as follows:

Neutral salt	$(NH_4)_2SO_4$	Na_2SO_4	NaCl
Concentration in normality	positive correction in millivolts		
0·0	0 0	0·0	0·0
0·5	1·9	1·8	1·6
1·0	3·6	3·6	3·2
2·0	7·2	7·1	6·4
3·0	—	10·5	—
4·0	14·1	—	12·1

From this table correction curves were drawn and values interpolated from the graphs were used in determining p_H by means of the quinhydrone electrode.

The data of Table I are plotted in Fig. 1. The effect shown here is noteworthy. Up to a concentration of 7·25 % of ammonium sulphate the velocity of denaturation is increased by increasing the amount of ammonium sulphate in the solution. From this point up to a concentration of 20 %, the velocity is steadily diminished, eventually falling below the initial value. This accounts for the contradictory statements referred to in the Introduction.

The ammonium sulphate seems to exert two opposing influences. One causes the velocity of denaturation to increase and the other causes it to decrease. The theoretical significance of this will be discussed later.

In the following table are given the results of a series of experiments analogous to those just cited.

Table II. *Summarising table.*

64°

Concentration of ammonium sulphate		
(g. per 100 cc.)	(equivalents per litre)	$k_{uni} \times 10^5$
0·0067	0·00102	34·1
0·1	0·0152	34·4
1·0	0·152	36·0
5·0	0·758	51·0
6·0	0·910	56·0
7·5	1·137	54·5
10·0	1·52	31·5
15·0	2·27	9·1
20·0	3·03	2·44

The data of Table II are plotted in Fig. 2.

These data again show the same effect as was noted in the case of the same reaction at 70° with the exception that, whereas the maximum occurred at a concentration of 7·25 % ammonium sulphate at 70°, it appears at a concentration of 6·7 % ammonium sulphate at 64°. This indicates that the action of the salt which tends to diminish the reaction rate is stronger at lower temperatures, so coming into play at lower concentrations.

SECTION 2. *The influence of varying concentrations of sodium chloride on the velocity of the heat denaturation of* 1 % *oxyhaemoglobin in a solution of* p_H 6·76 *at* 65°.

Table III. *Summarising table.*

Concentration of sodium chloride		
(g. per 100 cc.)	(equivalents per litre)	$k_{uni} \times 10^4$
0·054	0·00924	5·05
2·0	0·342	6·2
5·0	0·855	8·5
10·0	1·71	12·0
15·0	2·57	14·5
20·0	3·42	16·4

The data of Table III are plotted in Fig. 2. The results of this section show that with increasing concentration of sodium chloride up to 3·42 *M* (20·0 %) the velocity of denaturation rises steadily. There is a slight slowing off in the reaction rate with concentration but no actual fall of the velocity of denaturation. This means that the effect which caused an increase in the rate of reaction in the case of ammonium sulphate is now much stronger than the

other effect, namely that of reducing the reaction rate, which, it is evident, in this case does not predominate.

SECTION 3. *The influence of varying concentrations of sodium sulphate on the velocity of the heat denaturation at 65° of oxyhaemoglobin in solution of* p_H 6·76.

Table IV. *Summarising table.*

Concentration of sodium sulphate		$k_{uni} \times 10^4$
(g. per 100 cc.)	(equivalents per litre)	
0·015	0·000211	5·2
2·0	0·282	15·8
3·0	0·422	19·5
4·0	0·563	20·2
5·0	0·704	19·1
6 0	0·845	16·1
10·0	1·408	11·2
15·0	2·11	6·4
20·0	2·82	2·54

The data of this table are plotted in Fig. 2.

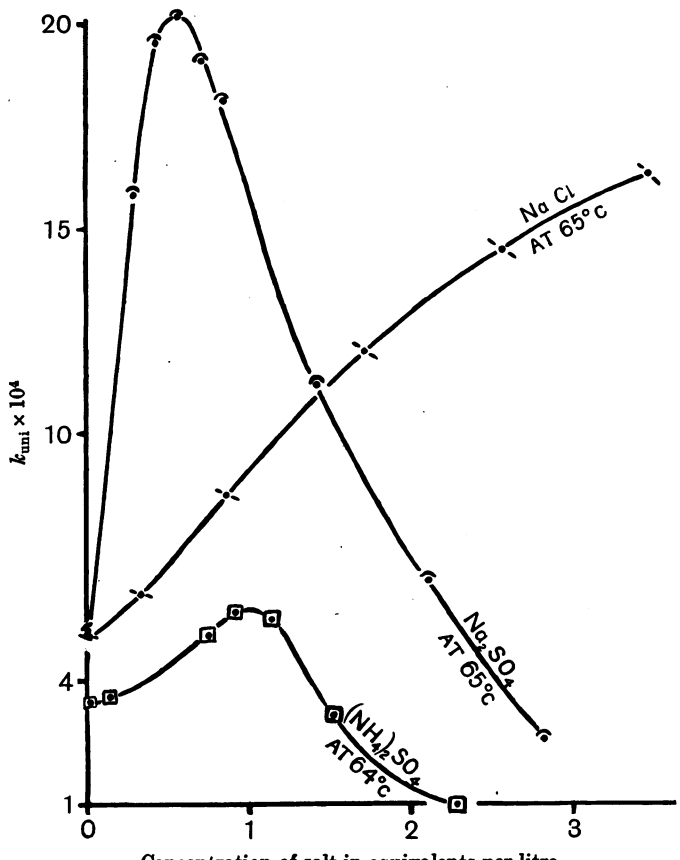

Fig. 2. Heat denaturation of oxyhaemoglobin in neutral solution. Variation of k_{uni} with salt concentration.

Here it is apparent that of the two opposing influences exerted by the sodium sulphate that which causes a reduction in the rate of denaturation sets in at a much lower concentration of this salt than in the case of ammonium sulphate. The relative effects of all the three salts studied are brought out in Fig. 2.

SECTION 4. *Heat denaturation of* 1 % *oxyhaemoglobin containing* 10 % *ammonium sulphate at* 68°.

In view of the theoretical considerations to follow it is convenient at this point to interpolate the following table which shows the variation of k_{uni} with p_H at 68° in presence of 10 % ammonium sulphate, although strictly this type of result belongs to Part I.

Table V. *Summarising table.*

p_H	$k_{uni} \times 10^3$
5·76	3·8
6·22	1·56
6·52	1·16
6·90	1·75
7·40	3·7

The data of Table V are plotted in Fig. 3, in which are also given the data of Table III, Part I, which shows the variation with p_H of the velocity of denaturation of 1 % oxyhaemoglobin containing 0·5 % ammonium sulphate. It will be seen that the two curves are similar save that the addition of the ammonium sulphate has displaced the curve p_H/k_{uni} downwards and to the acid side. The significance of this will be considered later.

DISCUSSION.

It is suggested that the action of the neutral salts depends on two distinct effects. It has been shown in Part I that with changing p_H the velocity of denaturation of oxyhaemoglobin in solutions containing relatively little salt (0·5 %) passes through a minimum when the activity of hydrogen ion is the same as that of hydroxyl ion, *i.e.* at the same p_H as that employed in the present instance. It is suggested that the first effect of the neutral salt (that which predominates in the lower concentrations of the sulphates and over the whole range in the case of sodium chloride) is to displace the minimum of the p_H/k_{uni} curve to one side. Thus the p_H 6·76 will no longer correspond to the minimum velocity but to some other, higher rate of reaction (cp. Fig. 3), thus causing the initial rise of velocity when the p_H is kept at 6·76 and the concentration of salt is increased.

The second action of the neutral salts is to depress the actual velocity of denaturation. This effect, predominating as it does in the higher concentrations of salt, is attributed to the dehydrating action of the salts. It is thought that this effect will only become apparent when the dehydrating action of the salts is sufficiently great to interfere with the hydration of the protein. This is

borne out by the relative magnitude of the influence of the various salts. In order of increasing power to depress the velocity they are

$$NaCl < (NH_4)_2SO_4 < Na_2SO_4.$$

This is also the order of increasing dehydrating power. Further, it will be noted in the case of the experiments with ammonium sulphate that this effect comes into play at lower concentrations of salt the lower the temperature. This is also in accord with the present view. In Part I it has been suggested that heat denaturation is a hydrolysis brought about by hydrogen and hydroxyl ions and likewise involving the water of hydration of the protein.

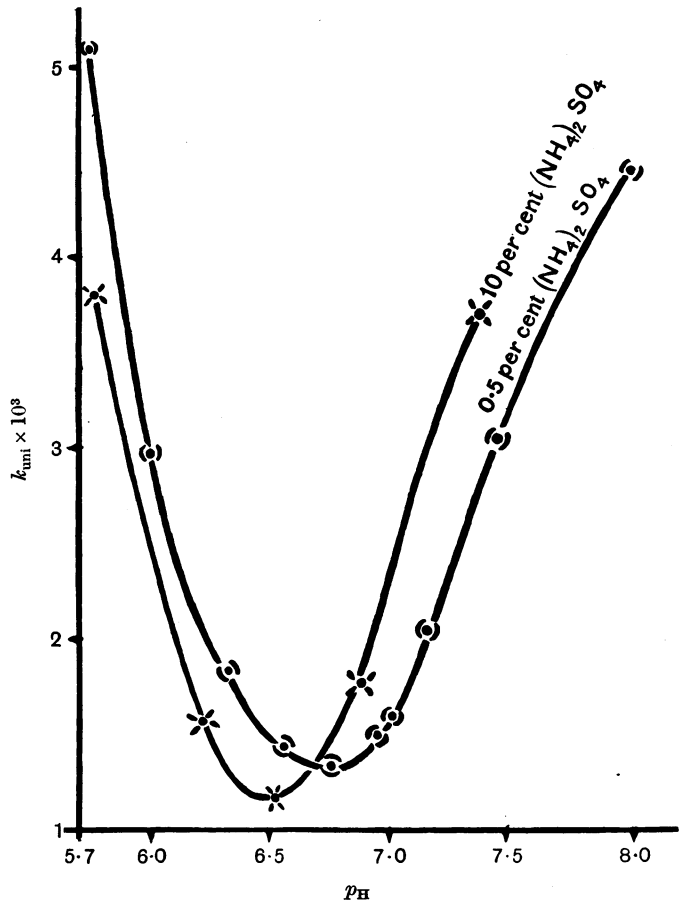

Fig. 3. Heat denaturation of oxyhaemoglobin at 68°. The effect of increase in the concentration of ammonium sulphate.

It will be seen that the present results are in entire accord with this hypothesis. Furthermore, they appear to be directly antagonistic to the alternative view, namely that denaturation is a chemical condensation between the carboxyl and amino groups of adjacent molecules, for were this the case the higher the concentration of neutral salt the greater would be the velocity of denaturation, owing to dehydration and consequent "exposure" of these groups.

THE CRITICAL INCREMENT OF THE HEAT DENATURATION OF OXYHAEMOGLOBIN.

In this case the critical increment has been calculated in two ways. First, the values of the velocity constants have been obtained by extrapolation to zero salt content on the graphs which show the variation of k_{uni} with concentration of ammonium sulphate. Secondly, the values given by the maxima on the same graphs have been used. It is argued, in the latter case, that the points chosen represent states in which the two opposing effects balance each other and hence the velocities to be used for a true comparison are those which correspond to this similar condition, and not the velocities at the *same* salt concentration.

In addition we have the value of the apparent critical increment as determined in Part I by utilising the velocity of the minimum point on the curves of p_H/k_{uni} (where the amount of ammonium sulphate present was small, namely 0·5 %).

Table VI. *The observed critical increment of the heat denaturation of oxyhaemoglobin.*

Source of data	Temp. in ° C.	k_{uni}	E in calories
Minima of p_H/k_{uni} curves	68·0	$1{\cdot}32 \times 10^{-3}$	77,500
	60·5	$1{\cdot}03 \times 10^{-4}$	
Maxima of Am_2SO_4/k_{uni} curves	70·0	$5{\cdot}4 \times 10^{-3}$	80,000
	64·0	$5{\cdot}68 \times 10^{-4}$	
Extrapolation to zero salt	70·0	$2{\cdot}68 \times 10^{-3}$	79,500
	64·0	$3{\cdot}42 \times 10^{-4}$	

It will be seen that the agreement is quite good. The significance of this critical increment has been discussed in Part I.

SUMMARY.

The influence of varying concentrations of ammonium sulphate, sodium chloride and sodium sulphate on the velocity of denaturation of oxyhaemoglobin at constant hydrogen ion activity has been studied.

It is shown that the neutral salts appear to have a dual effect. The first, predominating in lower concentrations of salt, acts so as to displace the minimum of the curve of k_{uni} against p_H to one side. The second, which predominates in higher concentrations, acts so as to displace this same curve downwards.

It is shown that these results bear out an hypothesis advanced in Part I as to the mechanism of denaturation.

Values of the apparent critical increment of the heat denaturation of oxyhaemoglobin are calculated from the results obtained in presence of ammonium sulphate. These values are in good agreement with that previously found (Part I).

In conclusion the writer wishes to express his thanks to Prof. W. C. M. Lewis who suggested the subject of this investigation and whose helpful criticism has proved invaluable. In addition the author's gratitude is due to the Department of Scientific and Industrial Research for a grant which enabled this investigation to be carried out.

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