CXVIII. THE KINETICS OF PROTEIN DENATURATION.

PART I. THE EFFECT OF VARIATION IN THE HY- DROGEN ION CONCENTRATION ON THE VELOCITY OF THE HEAT DENATURATION OF OXYHAEMOGLOBIN.

BY PHILIP STACEY LEWIS.

*From the Muspratt Laboratory of Physical and Electro-Chemistry,
The University, Liverpool.*

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THE generally accepted view of the heat coagulation of proteins is that of Hardy [1899], who concludes that the process takes place in two stages, the first of which is a preliminary change in the chemical structure of the protein. This stage is termed "denaturation" and is followed by a purely physical process—"flocculation." While Lepeschkin [1922] is of the opinion that flocculation is not entirely physical in nature but to some extent chemical, it is generally agreed that in the presence of electrolytes the second stage takes place at a much more rapid rate than the preliminary stage of denaturation and hence by working in solutions always containing a little electrolyte the kinetics of the heat denaturation of proteins may be studied. That the presence of electrolyte is necessary for the final flocculation is shown by the observation of Aronstein [1874] and Heinsius [1874] that in the absence of electrolytes a protein solution may be heated without flocculation, but that if salt be added after cooling flocculation takes place.

Opinion differs as to the nature of the fundamental chemical process involved in denaturation. Two opposing views are held. The one is that the reaction concerned is a chemical condensation between free amino and carboxyl groups of adjacent molecules. The other view is that the process is a mild hydrolysis. It is obvious, having regard to the relative magnitude of the protein and water molecules, that chemical analysis cannot hope to reveal the slight change in composition occasioned by either of these processes. In fact Hirsch-Pozany [1922] has attempted to differentiate between the two possibilities but without success. It was thought, however, that a study of the kinetics of the reaction and of the effect of various factors on its progress might provide some insight into the nature of the process.

The variation of the velocity of heat denaturation with the hydrogen or hydroxyl ion content of the solution (or more briefly with the p_H of the

solution) is the effect which first suggests itself for study and it is with this, in relation to the heat denaturation of oxyhaemoglobin, that the present paper is concerned.

Chick and Martin [1910] found that while the heat denaturation of oxyhaemoglobin followed the unimolecular law, that of egg-albumin did not, the latter reaction slowing off more rapidly than could be accounted for on such a basis. In later papers [1912] these authors show that when precautions are taken to keep the p_H of the solution constant the velocity of the heat denaturation of egg-albumin conforms to the unimolecular law. They attribute the slowing off in the velocity of heat denaturation to the fact that during heat denaturation of this protein a decrease of hydrogen ion concentration occurs in acid solution and of hydroxyl ion concentration in alkaline solution.

As Chick and Martin found it possible to obtain a steady unimolecular velocity constant for the heat denaturation of oxyhaemoglobin it has been implicitly assumed that no change of p_H takes place during the process. This has been verified during the course of the present work.

This, however, does not preclude variation in the rate of denaturation with p_H . Accordingly, experiments were conducted in which the velocity of the heat denaturation of oxyhaemoglobin was determined in solutions of various hydrogen ion concentrations. The results so obtained are recorded in the following section.

EXPERIMENTAL.

Materials used. The haemoglobin was prepared from fresh defibrinated ox-blood by a modification of the method of Schulz [1898]. The corpuscles were first centrifuged off and washed four times with isotonic salt solution, following which they were laked with distilled water and the haemoglobin obtained by fractional crystallisation by means of ammonium sulphate. The crystallisation was carried out five times and the resulting haemoglobin dissolved and dialysed thoroughly, the solution being constantly brought to the isoelectric point of the protein. In this way, a stock solution very rich in haemoglobin and containing very little salt was obtained. The actual composition of each stock solution was determined, the protein being estimated by the method of Devoto as modified by Hopkins [1900]. This consists in the gravimetric estimation of the coagulum formed on heating a known volume of the solution for two hours in the presence of saturated ammonium sulphate. After coagulation of the protein at the isoelectric point followed by washing and boiling of the coagulum with distilled water, the salt solution so obtained was boiled down to a very small bulk and the salt estimated gravimetrically as sulphate. As no other preservative was admissible a small, known amount of pure ammonium sulphate was added to the stock solution which was kept in the ice-chest.

The ammonium sulphate used was Merck's "puriss." thrice crystallised. The water used was well-boiled CO_2 -free water which was tested and found to be free from inorganic and organic impurities and to be of p_H 5.8 at 25°.

The solutions for use were made up by diluting the stock solution of isoelectric protein appropriately with water containing a little NH_4OH or H_2SO_4 as desired. The p_{H} of the solution was then determined by means of the quinhydrone electrode at 37° ¹ following the technique of Corran and Lewis [1924].

After each experiment the p_{H} was again determined and, as recorded above, was found to be sensibly unaltered.

Fig. 1. Apparatus used for the heat coagulation of proteins.

The apparatus employed (Fig. 1) was a modification of that used by Chick and Martin [1910]. The present apparatus is designed to prevent concentration of the solution during the reaction. The reaction vessel, *A*, is a silica beaker of capacity 250 cc., closed by a rubber bung, *B*, bearing a hollow stirrer, *C*, through which samples are withdrawn from time to time by way of a side arm, *D*. The bearing of the stirrer is mercury-sealed, *E*, thus preventing evaporation at this point. Air enters the vessel by a side arm, *F*, which leads from a flask, *G*, containing a solution of the same salt concentration and p_{H} as that under investigation. Air entering this flask does so by way of a vertical tube, *H*, which dips under the surface of the liquid. As an additional safeguard the screw clip, *K*, is kept closed save at the time of taking a sample. Both beaker and flask are kept well immersed in the water of the thermostat which is regulated to $\pm 0.02^\circ$. This apparatus is found to prevent any serious change of concentration during an experiment. The speed of the stirrer varied from 60 to 70 R.P.M.

As the samples of solution were withdrawn from the reaction vessel they were rapidly cooled in ice and it was estimated that the whole operation from the stopping of the stirrer motor to the reduction of the solution to ice cold

¹ The temperature 37° was selected as it was desired to work as near to the temperature of the experiments as was feasible. Although the p_{H} of the solution at the higher temperature would certainly be different from what it was at 37° yet working always with reference to one particular temperature the solutions used at various temperatures were more comparable.

occupied 35 seconds. The stirrer motor was off for a period of 4 to 5 seconds each time a reading was taken. In order to render the time readings more accurate—an important factor in rapid coagulation—each operation was carefully timed to the nearest second.

The samples so obtained were then filtered free from coagulum and the residual haemoglobin estimated by means of a Bausch and Lomb biological colorimeter.

RESULTS.

SECTION 1. *Heat denaturation of 1 % HbO₂ containing 0.5 % ammonium sulphate at various p_H values at 60.5°.*

Table I.

	p _H	Time in minutes	Residual HbO ₂ %	k _{uni} × 10 ⁴	
(i)	5.69	0	100	—	Mean k _{uni} 4.4 × 10 ⁻⁴ .
		5	87.7	4.4	Mean k _{uni} of duplicate experiment
		10	77.8	4.5	4.4 × 10 ⁻⁴ .
		20	60.5	4.4	Average k _{uni} 4.4 × 10 ⁻⁴ .
		30	47.8	4.3	
		50	29.5	4.1	
(ii)	5.81	0	100	—	Mean k _{uni} 3.32 × 10 ⁻⁴ .
		10	81.5	3.32	Mean k _{uni} of duplicate experiment
		20	67.1	3.31	3.32 × 10 ⁻⁴ .
		30	54.4	3.35	Average k _{uni} 3.32 × 10 ⁻⁴ .
		50	36.7	3.30	
		80	20.4	3.32	
(iii)	6.05	0	100	—	Mean k _{uni} 2.04 × 10 ⁻⁴ .
		10	88.0	2.12	Mean k _{uni} of duplicate experiment
		20	78.4	2.02	2.05 × 10 ⁻⁴ .
		30	69.0	2.06	Average k _{uni} 2.04 × 10 ⁻⁴ .
		60	48.0	2.01	
		100	29.7	2.02	
(iv)	6.43	0	100	—	Mean k _{uni} 1.25 × 10 ⁻⁴ .
		20	86.2	1.24	Mean k _{uni} of duplicate experiment
		50	68.5	1.28	1.24 × 10 ⁻⁴ .
		100	47.1	1.25	Average k _{uni} 1.25 × 10 ⁻⁴ .
		150	32.7	1.24	
		200	22.4	1.24	
(v)	6.83	0	100	—	Mean k _{uni} 1.05 × 10 ⁻⁴ .
		20	88.1	1.06	Mean k _{uni} of duplicate experiment
		40	78.1	1.04	1.06 × 10 ⁻⁴ .
		100	53.7	1.04	Average k _{uni} 1.05 × 10 ⁻⁴ .
		160	37.2	1.06	
		220	25.8	1.07	
(vi)	7.02	0	100	—	Mean k _{uni} 1.24 × 10 ⁻⁴ .
		20	86.2	1.24	Mean k _{uni} of duplicate experiment
		50	68.6	1.25	1.25 × 10 ⁻⁴ .
		100	47.2	1.25	Average k _{uni} 1.24 × 10 ⁻⁴ .
		150	32.6	1.24	
		200	22.4	1.24	
(vii)	7.21	0	100	—	Mean k _{uni} 1.67 × 10 ⁻⁴ .
		20	81.7	1.69	Mean k _{uni} of duplicate experiment
		40	67.5	1.70	1.67 × 10 ⁻⁴ .
		80	45.4	1.66	Average k _{uni} 1.67 × 10 ⁻⁴ .
		120	30.9	1.64	
		180	17.3	1.65	

Table I (*continued*).

	p_H	Time in minutes	Residual HbO ₂ %	$k_{uni} \times 10^4$	
(viii)	7.40	0	100	—	Mean k_{uni} 2.20×10^{-4} .
		10	87.4	2.24	Mean k_{uni} of duplicate experiment 2.20×10^{-4} .
		20	76.9	2.19	
		30	67.1	2.20	Average k_{uni} 2.20×10^{-4} .
		50	51.3	2.18	
		80	34.4	2.19	
(ix)	7.60	0	100	—	Mean k_{uni} 2.67×10^{-4} .
		10	85.1	2.68	Mean k_{uni} of duplicate experiment 2.68×10^{-4} .
		20	72.5	2.69	
		40	52.4	2.67	Average k_{uni} 2.67×10^{-4} .
		60	38.2	2.67	
		100	20.2	2.66	
(x)	7.96	0	100	—	Mean k_{uni} 3.33×10^{-4} .
		10	81.7	3.31	Mean k_{uni} of duplicate experiment 3.33×10^{-4} .
		20	66.6	3.33	
		40	44.8	3.31	Average k_{uni} 3.33×10^{-4} .
		60	30.5	3.35	
		80	20.6	3.33	
(xi)	8.32	0	100	—	Mean k_{uni} 3.7×10^{-4} .
		10	79.4	3.7	Mean k_{uni} of duplicate experiment 3.7×10^{-4} .
		20	60.6	3.7	
		40	40.8	3.8	Average k_{uni} 3.7×10^{-4} .
		60	26.4	3.7	
		80	17.0	3.7	

Table II. *Summarising table. Heat denaturation of 1 % HbO₂ containing 0.5 % ammonium sulphate.*Variation of k_{uni} with p_H at 60.5°.

p_H	$k_{uni} \times 10^4$
5.69	4.40
5.81	3.32
6.05	2.04
6.43	1.25
6.83	1.05
7.02	1.24
7.21	1.67
7.40	2.20
7.60	2.68
7.96	3.33
8.32	3.70

The data of Table II are plotted in Fig. 2. These results bring out an interesting behaviour on the part of the protein. They show that the velocity of denaturation passes through a minimum as p_H changes and it is not, as might have been expected, protein which was originally isoelectric which shows this minimum velocity but that which at 37° has a p_H of 6.76, the p_H of isoelectric oxyhaemoglobin at this temperature being 6.5 [Warburg, 1922; Stadie and K. A. Martin, 1924]¹.

¹ Although the p_H of the solution as measured by the quinhydrone electrode is strictly a measure of the activity of hydrogen ion, it may be taken that in the solutions employed here activity of hydrogen ion is sensibly equal to concentration of hydrogen ion owing to the solutions being dilute with respect to hydrogen ion.

Fig. 2. Heat denaturation of oxyhaemoglobin. Variation with p_H of k_{uni} at 60.5° .

SECTION 2. *Heat denaturation of 1 % HbO_2 containing 0.5 % ammonium sulphate at various p_H values at 68° .*

The results shown below are, in each case, the mean values of at least two experiments. The order of accuracy is similar to that of the previous section. Fuller details are omitted for the sake of brevity.

Table III. *Summarising table.*

Variation of k_{uni} with p_H at 68° .

p_H	$k_{uni} \times 10^3$
5.74	5.1
5.99	2.96
6.32	1.83
6.55	1.43
6.75	1.32
6.95	1.49
7.02	1.59
7.16	2.04
7.46	3.04
8.05	4.45

The data of Table III if plotted yield a graph similar to Fig. 2. Here again the velocity of denaturation passes through a minimum at p_H 6.76 and rises more rapidly on the acid than on the alkaline side. As the p_H was determined at 37° in each case this means that the same solution which denatures with the minimum velocity at 60.5° will also do so at 68° .

*The effect of temperature on the rate of the heat denaturation
of oxyhaemoglobin.*

The first workers in this field were Chick and Martin [1910] who found for this reaction a critical increment¹ of 60,000 calories. Later, Hartridge [1912] found values of 74,000 calories and 86,000 calories which confirms the order of the result of Chick and Martin.

The most noteworthy point about this critical increment is its high numerical value, from which it appears to be a composite term, the sum of several smaller critical increments, for a true critical increment of this magnitude would only be expected to accompany a process which would be immeasurably slow. This conclusion is based on general experience of the magnitude of critical increments characteristic of processes which occur with measurable velocities.

Chick and Martin say that the haemoglobin used by them was "used as prepared and contained ammonium sulphate." Further, they do not specify at what p_H they worked. The same applies to the results of Hartridge. The writer has found that both of these variables have a great effect on the velocity of denaturation. It is essential therefore, to make the comparison at the same p_H .

The critical increment has been calculated in the present instance from the minimum velocities on the p_H/k_{un1} curves. The value so obtained is 77,500 calories. It will be shown in Part III that this value is in good agreement with those obtained by two other methods.

DISCUSSION OF RESULTS.

The term denaturation is used in the literature to describe a large variety of processes whereby a protein may be converted from a soluble to an insoluble form. Such processes include the action of alkalis, acids, heavy metal salts, alkaloidal reagents, heat, light, pressure, friction, etc. There does not seem much ground for supposing that all these various processes are fundamentally the same.

Chick and Martin [1910] used the term to designate the chemical reaction which precedes flocculation on heating a solution of heat-coagulable protein. They consider this chemical reaction as taking place between the protein and hot water, and are of the opinion that it takes the form of a slight hydrolysis. This same view is taken by other authors, as Lepeschkin [1918], Wu and Wu [1925], etc.

¹ This is calculated from the integrated form of the Marcellin-Rice equation:

$$\log_e k_1 - \log_e k_2 = \frac{E}{R} \cdot \frac{T_1 - T_2}{T_1 \cdot T_2}$$

where k_1 and k_2 are the velocity constants of the reaction at the absolute temperatures T_1 and T_2 , E is the critical increment or energy of activation of the process and R is the gas constant. Actually the above authors determined the μ term of Arrhenius. The expression used for the calculation of μ is $\log_e \frac{k_1}{k_2} = \frac{\mu}{2} \left(\frac{T_1 - T_2}{T_1 \cdot T_2} \right)$ which is identical in form with that used for the calculation of E ; μ taking the place of E and the empirical figure 2 that of the gas constant R . Thus the μ value of a process will be about 1 % higher than the corresponding critical increment.

Robertson [1918], on the other hand, considers the reaction to be one of dehydration, a view originally put forward by Hofmeister [1888].

There exists also the possibility of intramolecular rearrangement. Excluding this, for which there is no experimental evidence, we are left to decide between hydrolysis (or possibly hydration) and dehydration (chemical or physical) as the underlying chemical reaction in heat denaturation.

Robertson [1918] argues from the following evidence. He himself found [1911] that on the addition of a fairly concentrated solution of cupric chloride to a solution of caseinogen in anhydrous formic acid the mixture is at first green, indicating the presence of lower hydrates of cupric chloride, but on adding more of the solution it becomes blue and simultaneously with the appearance of a pure blue colour, but not before, precipitation of cupric caseinogenate occurs. Further, if to the green solution water be added until the pure blue colour of fully hydrated cupric chloride is shown, the precipitate again forms. If formic acid be added to the mixture the precipitate dissolves as soon as the mixture becomes green. Further, the precipitate dissolves on warming, the mixture becoming green; on cooling the blue colour returns and with it the precipitate.

If instead of adding water to the green solution alcohol be added, no precipitation occurs until the colour of the mixture changes to the brown of the anhydrous salt.

Since water and alcohol are respectively hydrating and dehydrating agents, he concludes that proteins may be thrown out of solution in two grades of hydration, the one of high and the other of very low hydration. The former process he terms precipitation and the latter coagulation.

He then says that "dehydration partial or complete, leading to resolution or coagulation, may be induced by heat, by non-electrolytes possessing an affinity for water or by electrolytes."

It is doubtful, however, whether the evidence just cited can be justly applied to the phenomenon of heat denaturation. In Robertson's experiments the precipitate was one of cupric caseinogenate and the precipitation was reversible, whereas in heat coagulation the protein is precipitated as such and the process is, so far as one can determine, irreversible.

Robertson [1918], however, argues that the irreversibility of heat coagulation is only apparent and cites the observation of Corin and Ansiaux [1891] that if a solution of protein be cooled and vigorously shaken, just as the first traces of heat coagulation appear, the incipient coagula will again pass into solution. The writer has repeated this experiment with both egg-albumin and haemoglobin without success. A similar failure is recorded by Wu and Wu [1925] who, in addition, heated a solution containing the minimal quantity of acid or alkali required to prevent coagulation and cooled and kept the solution for two months in an ice-box under sterile conditions. The solution was then neutralised and the protein precipitated. It appears, therefore, that the protein cannot revert to its original condition and the process is accordingly irreversible.

Robertson considers that there exists between the protein and water an equilibrium of the type

and that the effect of increasing the temperature of the system is to displace the equilibrium from left to right. This he concludes from the fact that, at temperatures higher than 40°, there is an increase in the solubility in alkaline solutions [Robertson, 1908] and in the alkalinity and electrical conductivity [Osborne, 1901] of caseinogen.

Robertson's explanation of these facts is quite feasible and may be correct yet it seems to the writer that this increased solubility is fictitious, and in fact breakdown of the protein rather than polymerisation suggests itself. More particularly so as Robertson did not observe any tendency to reprecipitation on cooling on the part of the excess caseinogen dissolved at the higher temperature. Granting, however, the correctness of Robertson's interpretation, the transfer of these conclusions to the process of heat denaturation does not seem justifiable. In fact caseinogen, which, save in alkaline solution, is relatively insoluble [Osborne, 1901] and the solubility of which increases on heating, hardly seems a good subject for the investigation of the phenomenon of heat denaturation. It must be emphasised that, when dealing with a molecule so complex as that of the protein, changes may be observed on heating the aqueous solution other than that particular change which we term denaturation. Thus Sørensen [1925] shows that on heating a solution of egg-albumin, hydrolytic scissions take place which, as they do not proceed hand in hand with coagulation cannot be the fundamentally important processes in denaturation. It therefore seems necessary to restrict observations to the actual process of heat denaturation.

That water is necessary for the heat denaturation of proteins might be inferred from the observation of Wichmann [1899] that dry albumin may be heated to 150° for some time without change. This is confirmed by Chick and Martin [1912] who heated dry egg-albumin to 120° for five hours without change although in the presence of steam it was denatured. Robertson argues, however, that this is because in the absence of water the terminal NH_2 and $COOH$ groups of the protein are not ionised and hence no action can take place.

The noteworthy point about the results recorded in the present paper is that they show that protein which, at 37°, has a p_H of 6.76 is denatured with the minimum velocity. Now 6.76 is the p_H of "neutral" water at 37°, that is, in this solution the concentrations of hydrogen and hydroxyl ions are equal. On heating the solution to the temperature of the experiment the p_H will of course change, but it is thought that this equality of ionic concentrations will still hold. That the relative concentrations of these two catalysing ions does not change appreciably at the higher temperature is shown by the fact that the same solution has the minimum velocity of denaturation at both 60° and 68°. It is not feasible to determine the p_H of the solution at the high temperature, but in order to make more certain of this point the p_H of a haemo-

globin solution, which was of p_H 6.76 at 37°, was determined at 25°, 18° and 0° with the following results.

Table IV. *Variation with temperature of the p_H of a solution containing 1 g. of haemoglobin and 0.5 g. $(NH_4)_2SO_4$ per 100 cc.*

Temp. in degrees C.	p_H of solution	p_H at which $H^+ = OH^-$	Authority
37	6.76	6.76	Lorenz and Bohi [1909]
25	6.99	6.99	Lewis, Brighton and Sebastian [1917]
18	7.09	7.08	Kohlrausch and Heydweiler [1894]
0	7.51	7.45	

From these results it appears that in the solution containing protein and ammonium sulphate, the concentration of hydrogen ion is sensibly the same as that of hydroxyl ion over a range of 37 degrees and it is thought that it is quite likely to be the case up to 60° or 70°.

The reaction postulated by Robertson as the basis of denaturation, *i.e.* that of the chemical condensation of free amino and carboxyl groups, would have a maximum probability at the isoelectric point of the protein, as here the two groups he considers are dissociated to an equal extent. As the solution departs more and more from the isoelectric condition the dissociation of one or other of these groups becomes very rapidly depressed and on Robertson's view the reaction should be extremely slow in strongly acid or alkaline solution. This is entirely different from the behaviour observed.

That the variation of the velocity of denaturation with p_H is not connected with the ionisation of the protein *per se* is shown by the fact that the minimum velocity does not occur at the isoelectric point of the protein but at a p_H at which the concentrations of hydrogen and hydroxyl ions are equal. It might be suggested, in view of the slight difference between these two p_H values in the case of haemoglobin, that actually the solution which at 37° was of p_H 6.76 might be isoelectric at higher temperatures. This consideration is ruled out by the fact that the velocity of denaturation of egg-albumin passes through a minimum at the same p_H (*viz.* 6.76) (see Part II), the isoelectric point of egg-albumin being p_H 4.84 [Sørensen, 1917, 2].

In order to explain the rapid rise of velocity of denaturation with increasing hydrogen and hydroxyl ion concentration on either side of the minimum it is necessary to assume that the reaction is catalysed by these ions. This suggests that the process under consideration is one of hydrolysis, hydrogen and hydroxyl ion catalysis being very typical of this type of reaction.

If these were the sole influences operating, the p_H/k_{uni} curve should be symmetrical about the minimum point¹ which is contrary to experimental observation.

¹ It would follow in this case, since the initial concentration of protein is kept constant, that we could express the velocity thus:

$$\text{velocity} = k_1 C_{H^+} + k_2 C_{OH^-} \quad \dots\dots(1),$$

and hence

$$\frac{d \text{ velocity}}{d C_{H^+}} = k_1 + k_2 \frac{d C_{OH^-}}{d C_{H^+}};$$

In general shape the curve bears a strong resemblance to those obtained by Loeb [1922] for the variation with p_H of viscosity, swelling, and osmotic pressure of proteins in solution, save that the minima in the latter cases occur at the isoelectric point of the protein, which, however, is not far removed from the minimum in the *present* case. It will be seen (Part II) that, in the case of the heat denaturation of egg-albumin, where the isoelectric point of the protein is far removed from the minimum point, the asymmetry is not so pronounced.

To account for the asymmetrical nature of the curve it is suggested that the reaction is in fact a hydrolysis catalysed by hydrogen and hydroxyl ions, water being likewise involved, *the water which is effective being the water of hydration of the protein micelle*¹. This will vary with p_H in the same manner

at the minimum point this differential is zero, i.e.

$$k_1 = -k_2 \frac{d C_{OH^-}}{d C_{H^+}};$$

but since $C_{H^+} \times C_{OH^-}$ is a constant,

$$\frac{d C_{OH^-}}{d C_{H^+}} = -\frac{C_{OH^-}}{C_{H^+}}.$$

Hence at the minimum

$$\frac{C_{OH^-}}{C_{H^+}} = \frac{k_1}{k_2}.$$

But the minimum occurs at a point where $C_{H^+} = C_{OH^-}$.

Therefore

$$k_1 = k_2 = k.$$

Equation (1) may now be written

$$\text{velocity} = k \left(C_{H^+} + \frac{K_w}{C_{H^+}} \right) \quad \dots\dots(1 \Delta).$$

If the minimum velocity (i.e. the point at which hydrogen ion concentration equals hydroxyl ion concentration) occurs at $p_H = N$, then the ionic product of water (K_w) = 10^{-2N} . At any other p_H value, say that represented by $p_H = (N+x)$,

$$\text{velocity} = k \left[10^{-(N+x)} + \frac{10^{-2N}}{10^{-(N+x)}} \right] = k [10^{-(N+x)} + 10^{-(N-x)}],$$

and at $p_H = (N-x)$

$$\text{velocity} = k \left[10^{-(N-x)} + \frac{10^{-2N}}{10^{-(N-x)}} \right] = k [10^{-(N-x)} + 10^{-(N+x)}].$$

Thus the velocity at any two p_H values equidistant from the minimum will be equal. That is, the curve would be symmetrical about the minimum point.

¹ Precisely how the water term will enter into the analytical expression for the speed of the reaction is somewhat uncertain.

If it be taken that the concentration of the water of hydration enters the expression for the velocity of denaturation in the following manner

$$\text{velocity} = (k_1 C_{H^+} + k_2 C_{OH^-}) C_{H_2O},$$

then by treatment similar to that given above, the expression yields the relationship

$$\frac{k_1}{k_2} = \frac{\left(C_{H_2O} - C_{H^+} \frac{d C_{H_2O}}{d C_{H^+}} \right)}{\left(C_{H_2O} + C_{H^+} \frac{d C_{H_2O}}{d C_{H^+}} \right)}$$

at the minimum point of the p_H/k_{uni} curve.

As the minimum point under consideration is on the alkaline side of the isoelectric point $\frac{d C_{H_2O}}{d C_{H^+}}$ is negative. Hence k_1 is now greater than k_2 .

The inclusion of the water term thus renders the p_H/k_{uni} curve asymmetrical about the minimum point and further since $k_1 > k_2$ the curve will fall more steeply on the acid side of the minimum than it rises on the alkaline side; the shape of the hypothetical curve is therefore the same as that observed.

as the viscosity, etc., of the solution, but will be in thermodynamic equilibrium with the total external water of the solution. The suggestion that the water of hydration of the protein is involved appears more likely in view of the results of Part III which are concerned with the variation of k_{uni} with the concentration of neutral salt in the solution.

The balance of the evidence so far available would appear to be in favour of a hydrolysis as the fundamental reaction in denaturation.

On *a priori* grounds it might be thought that the polypeptide link ($-\text{CO}.\text{NH}-$) would be the important one for such a reaction. However, work by A. I. Escolme in this laboratory (as yet unpublished) shows that the $-\text{CO}.\text{NH}-$ link is extremely stable to hydrolysis and in view of the ease with which protein denaturation occurs the latter effect cannot be ascribed to the hydrolysis of this linkage. Further, mere dehydration of a molecule or micelle would be a process corresponding to a relatively low critical increment. The actual observed speed of denaturation would seem to involve a process of intermediate critical increment value.

Lepeschkin [1922] suggested that the chemical reaction involved in the denaturation of proteins by heat is of the same nature as the hydrolysis of polysaccharides into their constituent units. If this be so, it is necessary to assume that several such cleavages are occurring in the denaturation of the protein. The observed critical increment of the hydrolysis of sugars having the sucrose type of linkage is approximately 26,000 calories. So that here we may be dealing with a quantity of the order of 20,000 to 30,000 calories. If the suggestion of Lepeschkin is correct then, from the magnitude (77,500 cal.) of the critical increment observed for the heat denaturation of oxyhaemoglobin, it would be inferred that the reaction consists in the rupture of three or four linkages analogous to that involved in the hydrolysis of sucrose.

To avoid the difficulty which statistical considerations would introduce if denaturation were regarded as the result of collisions of three or four individuals it would seem necessary to assume that large, heavily hydrated units are already in existence in undenatured protein and that in the act of denaturation each unit underwent alteration at three or four places.

In this connection it is very suggestive that Adair [1925] and Svedberg and Fahraeus [1926] have shown, the one by osmotic pressure measurements and the others by means of the ultracentrifuge, that the molecular weight of haemoglobin in solution is four times the minimum weight as determined by analytical means.

SUMMARY.

The influence of variation in the hydrogen ion concentration on the velocity of the heat denaturation of oxyhaemoglobin has been studied. It is found that the velocity passes through a minimum, namely at that point at which the hydrogen ion and the hydroxyl ion concentrations of the solution are equal.

The critical increment of the process is found to be 77,500 calories. It is

pointed out that this value is in all probability a composite term, the sum of several smaller critical increments.

The possible mechanism of the process is considered in the light of current theories. It is suggested that the mechanism consists of a localised hydrolysis of relatively labile links (nature unspecified) situated at various points on a large, heavily hydrated molecular unit. It is considered that the cause of the variation of k_{uni} with p_{H} is the catalytic influence of hydrogen and hydroxyl ions.

In conclusion the writer desires to express his thanks to the Department of Scientific and Industrial Research for a maintenance grant which enabled this investigation to be carried out.

REFERENCES.

The references will be found at the end of Part III, p. 992.