CCXL. THE HEAT-INACTIVATION OF CRYSTALLINE PEPSIN; THE CRITICAL INCREMENT OF THE PROCESS.

By WILFRID JAMES LOUGHLIN.

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

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In a recent series of publications Northrop [1930, 1, 2; 1931] has advanced very strong evidence that pepsin is a protein. Starting from commercial pepsin, by suitably regulating the p_H and precipitating with the aid of magnesium or ammonium sulphate a crystalline product was obtained which had the general properties of a protein. In particular, solutions of this crystalline pepsin were coagulated by heat, and Northrop states that the inactivation of the enzyme either by heat or by alkali is quantitatively proportional to the denaturation of the protein. A successful effort was made [1931] to reactivate denatured inactivated pepsin along the lines used by Anson and Mirsky [1929; 1931, 1, 2] for the reversal of the denaturation of certain proteins, and the product obtained was identical with the original freshly prepared material, both in general properties and in its specific proteolytic activity. It seems safe to conclude therefore that the crystalline pepsin is in fact a protein. More recently Northrop [1932, 2] has shown that trypsin can similarly be obtained in a crystalline form and has the properties of a protein, whilst Sumner [1926] claims that the crystalline urease prepared by him is also a protein.

Work has been proceeding in this Department for a number of years with a view to determining the mechanism of the process of the denaturation of proteins. In this connection the conclusions mentioned above are of great importance, for if the processes of inactivation and denaturation of the crystalline enzymes do go hand in hand, then an investigation of the heat-inactivation of the enzymes might yield valuable information regarding the mechanism of the process of denaturation, especially as the rate of inactivation can be determined experimentally with great accuracy by a number of different methods. Consequently an investigation of the heat-inactivation of crystalline pepsin as prepared by Northrop was undertaken, and the results obtained are given in the present communication.

EXPERIMENTAL.

The crystalline pepsin used in the present investigation was prepared from Parke Davis pepsin U.S.P. 1 : 10,000 in the manner described by Northrop [1930, 1] using magnesium sulphate. The product was then purified by several recrystallisations in the manner given by Northrop, and the material thus obtained was tested exhaustively as described below, to ensure that it was identical with that obtained by Northrop himself.

When viewed under a microscope the pepsin was found to consist of transparent, colourless dihexagonal bipyramids, the slight yellow colour observed in the first crop of crystals having gradually been removed in the recrystallisation process. After drying, the crystals dissolved with difficulty, and the solution obtained was coagulated by heat. The pepsin was precipitated on

saturating the solution with either magnesium or ammonium sulphate. Positive results were obtained for the biuret reaction and for the xanthoproteic reaction. Polarimetric measurements gave a value for $[\alpha]_D^{22}$ of -70.5° compared with Northrop's values of between -68° and -72° . The nitrogen content as obtained by several micro-Kjeldahl determinations was 15 %. Reference to Northrop's paper [1930, 1] will show that all these tests are in agreement with those obtained using his preparations. It must also be shown, however, that the writer's pepsin has the same proteolytic activity as that of Northrop's preparation.

The method recommended by Northrop for its accuracy and rapidity and selected by the author is that in which a given quantity of the enzyme is allowed to act on a caseinogen solution, and the rate of hydrolysis is followed by measuring the change produced in the viscosity of the protein solution at various time intervals.

Briefly, the method consists of adding different known weights of pepsin dissolved in a given volume of water to a known volume of a caseinogen solution and measuring the viscosities of the mixtures at definite time intervals. From the results obtained a series of viscosity-time curves are plotted and from these the gradients at zero time are measured. From the gradient of the curve it is a simple matter to obtain the percentage decrease in viscosity produced per minute, and the arbitrary unit of activity is then described as this value divided by the number of mg. of pepsin-nitrogen present in the mixture. The value of this unit should of course be the same (within the limits of experimental error) independently of the curve from which it was obtained. The mean value of the unit obtained from determinations carried out with five different concentrations of pepsin was 1350. Similar treatment when applied to four curves given by Northrop [1932, 1] gave a value of 1250. As a considerable experimental error is possible both in calculating the time interval after mixing the pepsin and protein solutions and also in the extrapolation of the viscosity curves to zero for the measurement of the gradient, this agreement may be taken as being satisfactory. Furthermore the value 1350 agrees with the various values obtained by Northrop from different preparations over a number of years. It may therefore be taken that the activity of the writer's preparation as measured by the caseinogen viscosity method is the same as that of the various Northrop preparations. A further indication that the activities are identical will be given in a later section. For the present, however, it is sufficient to say that a study of the properties of the pepsin used in the present investigation has shown them to be identical with the properties of the crystalline pepsin prepared by Northrop.

Determination of the pepsin unit by the alcohol titration method.

From what has been said above it will be seen that although the caseinogen viscosity method for measuring the activity of the pepsin is comparatively rapid, it is at the same time not particularly accurate. Furthermore the interpretation of the figures obtained is obscure as no definite chemical significance can be attached to them. It is therefore advisable to use some other method which, although perhaps not so convenient, does at least have a chemical meaning. One such method is the formaldehyde titration method. Another similar method is the alcohol titration method of Willstätter and Waldschmidt-Leitz [1931].

This method is somewhat tedious and requires great accuracy. It serves, however, to give a measure of the number of carboxyl groups liberated by the pepsin in a given time and therefore is not subject to the same criticism as the viscosity method given above. This method was examined by the writer, and the following figures which were obtained for the pepsin unit will serve as an indication of its accuracy.

The optimum p_H for the hydrolysis of caseinogen by pepsin is given by Northrop as 1.8. Consequently it is at about this p_H that the alcohol titration method will be most sensitive. For the purpose of obtaining this p_H a citrate buffer was used. The final mixture was as follows: 5.5 cc. $M/3$ sodium citrate, 34.5 cc. $M/3$ HCl and 10 cc. water. The caseinogen solution used was prepared by the addition of 100 cc. 0.05 N ammonia to 6 g. Kahlbaum-Hammarsten

caseinogen, stirring and warming to 40° until all the caseinogen dissolved. 15 cc. of this solution were run slowly into 10 cc. of the buffer with constant shaking. By this means, although the caseinogen first precipitated slightly, it then redissolved to give a clear solution with just a slight blue colour. The requisite amount of pepsin was weighed out and dissolved in 10 cc. of the buffer. The two solutions were placed in the thermostat at 35°, and when temperature equilibrium had been established 5 cc. of the pepsin solution were measured into the caseinogen solution. A 10 cc. sample of the reaction mixture was immediately withdrawn and run into a known volume of alcoholic caustic soda solution sufficient to stop the reaction by attaining p_H about 8. After exactly one hour a second 10 cc. sample was also withdrawn and treated similarly. 1 cc. of thymolphthalein solution was added to each of the samples which were then titrated with the same alcoholic caustic soda solution as was used to stop the reaction until the faint blue colour of the indicator appeared ($p_H = 9.5$). 100 cc. of boiling alcohol were then added causing the blue colour to disappear and at the same time precipitating the caseinogen in a finely divided state. The titration was continued until the blue colour reappeared, showing that the titration of the protein was complete. The increase in alkali titration due to the hydrolysis of the caseinogen by the pepsin was noted. The p_H of the reaction mixture was also noted during the course of the reaction, and was always in the neighbourhood of 1.9.

A large number of determinations was made using different concentrations of pepsin and the figures obtained are collected in Table I, and are plotted as a

Table I. *Amount of hydrolysis produced in a caseinogen solution under the conditions specified above by different concentrations of crystalline pepsin.*

p_H of reaction mixture = 1.9. Time of reaction, 1 hour at 35°.			
Weight of pepsin present in reaction mixture (mg.)	Increase in titration (cc. N/50 alc. NaOH solution)	Weight of pepsin present in reaction mixture (mg.)	Increase in titration (cc. N/50 alc. NaOH solution)
1.2	1.03	4.8	3.67
1.9	1.62	5.7	3.79
1.9	1.78	5.7	4.00
3.0	2.65	7.6	4.43
3.0	2.80	7.6	4.66
3.3	3.00	8.9	4.69
3.8	3.24	10.8	5.12
4.1	3.52	18.6	6.05
4.5	3.60		

curve in Fig. 1. It is intended to use this curve as a standard in the subsequent work on the heat-inactivation in a simple manner to be described later. It should be noted that the values given were obtained with several different preparations, yet all lie on a smooth curve.

It will be seen from the figure that the hydrolysis produced is directly proportional to the concentration of pepsin at low concentrations, but at higher concentrations the rate of hydrolysis produced is less than it would be if the linear function continued to hold.

A series of determinations was carried out on Parke Davis pepsin to obtain comparative data between the activity of the newly-prepared crystalline pepsin and that of the commercial material. This comparison is of interest since it affords confirmation of the comparability of the activity of the writer's prepara-

tion with that of Northrop, even though Northrop did not use the alcohol titration method.

From the figures obtained it is possible as will be seen below to give a pepsin unit expressed in an analogous way to those already published by Northrop.

Fig. 1. Variation of activity with concentration of crystalline pepsin.

From the gradient of the curve at zero time one finds that 0.002 g. of pepsin in the reaction mixture liberates in one hour an amount of carboxyl groups requiring 1.8 cc. *N*/50 alcoholic caustic soda for neutralisation, *i.e.* 1.8 $\times$ 1/50 milli-equivalents are liberated per hour, per 2 mg. pepsin in the reaction mixture, or per 0.3 mg. pepsin-nitrogen. Hence the activity [*PU*] per mg. pepsin-nitrogen, may be represented

$$[PU] = \frac{1.8}{50 \times 0.3} \\ = 1.20 \times 10^{-1}.$$

A rough experiment with Parke Davis pepsin gave the following figures: 0.024 g. pepsin in the reaction mixture corresponded to a titration of 3.95 cc. *N*/50 NaOH.

From which

$$[PU] = 2.2 \times 10^{-2}.$$

Hence

$$\frac{\text{Activity of own preparation}}{\text{Activity of Parke Davis}} = \frac{120}{22} \\ = 5.5.$$

Northrop, using the caseinogen-formaldehyde method for obtaining the activity of his preparations gives the values 14 units for his own preparations, and 2.5 for Parke Davis pepsin, a relative activity of 5.6. This affords further evidence that the writer's pepsin is identical with that of Northrop.

The heat-inactivation of crystalline pepsin.

Although the process of heat-inactivation of the crystalline pepsin was not examined in any detail by Northrop, he performed one or two experiments with the object of determining whether the process followed the course of a simple unimolecular reaction. The conclusion drawn from the results obtained was that the reaction did follow the theoretical unimolecular course quite closely, thus

confirming the conclusion reached earlier by Tammann [1895] and Arrhenius [1907]. This result is confirmed in the present work, and unimolecular constants have been obtained for the reaction at various p_H values and at different temperatures.

The method of following the inactivation reaction used by the writer was as follows. About 0.03 g. of pepsin was dissolved in distilled water, and the solution was made up to 30 cc. with a solution of HCl of such a concentration as to give the required p_H . The solution was placed in a thermostat at 65°, and after temperature equilibrium had been established 7 cc. were withdrawn and run into a thin-walled test-tube immersed in an ice-water mixture, the time of this action being noted. After suitable time intervals further 7 cc. samples were withdrawn and the reaction stopped in the same way. The activity of each of the samples was then obtained by the alcohol titration method as before, but certain slight alterations had to be made in order to obtain conditions which were identical with those used in obtaining the standard activity curve.

In the first place the pepsin was now dissolved in water or a very dilute HCl solution whereas before it was dissolved in the buffer solution. To obtain therefore the same reaction mixture as before the following quantities and concentrations were used. The caseinogen solution used was 6.923 %, and the buffer solution contained 5.5 cc. *M*/3 sodium citrate to 34.5 cc. *M*/3 HCl, but no water was added. 13 cc. of the caseinogen solution were slowly run into 12 cc. of the new buffer solution with stirring, and when the pepsin solution (5 cc. unbuffered) was added it will be seen that the reaction mixture was identical with that used previously. The only difference was in the small concentration of HCl added with the pepsin which had been used to adjust the p_H for the inactivation period, but this was so small compared with the acid present in the buffer that it had no appreciable effect on the p_H of the reaction mixture used for the activity determination. The remaining 2 cc. of pepsin solution were used for the p_H determination by the quinhydrone method of Corran and Lewis [1924] at 25°.

As before 10 cc. samples were withdrawn at the beginning of the reaction and after exactly one hour at 35°. The increase in titration was measured, and from the activity curve the quantity of active pepsin present in the reaction mixture which corresponded to this increase was read off. The concentration in the initial sample was taken as 100 % and the percentage inactivation at each time interval was calculated. From each of these values it was possible to obtain a value of the unimolecular reaction velocity constant from the equation

$$k_{\text{un1}} = \frac{2.303}{t} \log_{10} \frac{a}{a-x},$$

where a = concentration of active pepsin at zero time (100 %); $a-x$ = concentration of active pepsin remaining at time t (% activity at time t); t = time in secs.

The fact that concordant values for k_{un1} were obtained showed that the heat-inactivation was indeed following the unimolecular reaction rate. Values of k_{un1} were obtained at 65° for a series of different p_H values, and the figures obtained are given in Table II.

From these figures it is seen that the effect of p_H on the rate of inactivation is considerable except between p_H 3.0 and 4.5 between which values the curve shows a fairly flat minimum. On the acid side of p_H 3, and the alkaline side of p_H 4.5, however, the rate increases exceedingly rapidly and in fact at p_H 6.5 the rate is too great to be measured. The approximate value given for p_H 6.9 was obtained as follows. The reaction mixture was prepared in the usual way and

Table II. *Heat-inactivation of crystalline pepsin at 65°.*

p_H	Mean value of $k_{uni} \times 10^4$	$\log_{10} k_{uni}$
2.30	19.9	3.2989
2.56	5.2	4.7160
2.65	4.7	4.6721
2.98	2.2	4.3424
3.30	2.1	4.3222
4.04	(4.0)	(4.6021)
4.50	3.6	4.5563
4.60	3.3	4.5185
5.43	15.2	3.1818
6.90	>700.0	2.8451

It will be noted that 0.03 g. of pepsin in 30 cc. is equivalent to 5 mg. in 5 cc., i.e. the initial weight of pepsin in the reaction mixture is 5 mg. Reference to Fig. 1 shows that this is at the head of the steep straight portion of the curve so that as the pepsin becomes inactivated a rapid change in titration will be observed. In short, using this concentration causes the method to be particularly sensitive in measuring the degree of inactivation produced.

Fig. 2.

Fig. 2. Heat-inactivation of pepsin. Variation with p_H of k_{uni} at 65°.

○ Represents value obtained by Northrop.

Fig. 3.

Fig. 3. Heat-inactivation of pepsin. Variation with p_H of $\log k_{uni}$.

○—○ Variation with p_H of $\log k_{uni}$ at 65°. ×—× Variation with p_H of $\log k_{uni}$ at 55°.
— Variation with p_H of $(\log k_{uni} \text{ at } 65^\circ - \log k_{uni} \text{ at } 55^\circ)$.

placed in the thermostat at 65°. A sample was removed and cooled 3 minutes later. The activity determination gave an increase in titration of 0.05 cc. 0.073 *N* NaOH showing that the pepsin was almost completely inactivated by this short heating. Making a calculation on the assumption that maintenance for

1½ minutes at 65° has produced something over 95 % inactivation it is seen that the value of k_{uni} must be greater than 7×10^{-2} at this p_{H} .

The figures in Table II are plotted in Fig. 2 together with one value of k_{uni} obtained by Northrop, viz. 0.58 at 65° and p_{H} 3.0 (calculated with time measured in hours), i.e. 1.6×10^{-4} if t is measured in seconds. It is seen that this point lies very near the writer's curve. In Fig. 3 the curve is given in the form $p_{\text{H}} - \log k_{\text{uni}}$ to enable a wider range of p_{H} to be shown, and also for the purpose of showing how the critical increment of the process varies with p_{H} .

Effect of temperature on the heat-inactivation process.

To examine the effect of temperature, a second series of determinations of the values of k_{uni} for various p_{H} values was carried out exactly as described above but at a temperature of 55°. Since the technique is identical it is sufficient merely to give the data obtained; these are shown in Table III.

Table III. *Heat-inactivation of crystalline pepsin at 55°.*

p_{H}	$k_{\text{uni}} \times 10^5$	$\log_{10} k_{\text{uni}}$
2.30	8.2	5.9138
3.06	0.7	6.8451
4.15	0.3	6.4771
4.89	1.5	5.1761
5.42	5.4	5.7324

The behaviour with respect to alteration in p_{H} is therefore the same at 55° as it is at 65°. There is a flat minimum between p_{H} 3.5 and 4.5 and on either side of these values a sharp rise. The similarity is brought out by plotting the values in the third column ($\log_{10} k_{\text{uni}}$) against p_{H} in Fig. 3 on the same scale as the values obtained at 65°. The use of this diagram will be mentioned later.

The critical increment of the heat-inactivation process.

Consideration of the figures in Tables II and III shows that the process of heat-inactivation is one which possesses a very high temperature coefficient. This point is emphasised when the critical increment is calculated from the integrated form of the equation

$$\frac{d \log k}{dT} = \frac{E}{RT^2},$$

$$\text{viz.} \quad 2.303 (\log_{10} k_1 - \log_{10} k_2) = \frac{E}{R} \left(\frac{T_1 - T_2}{T_1 T_2} \right),$$

where k_1 and k_2 are unimolecular constants at temperatures T_1 and T_2 , E is the critical increment and R the gas constant.

The value in the bracket on the left-hand side of this equation is obtained at any given p_{H} by the difference in the ordinates of the two curves in Fig. 3. For the sake of simplicity a third curve has been drawn in Fig. 3 which represents at each p_{H} the difference between the ordinates of curves *A* and *B*. If we represent this difference by d , then the critical increment at any given p_{H} is given by

$$E \text{ (cals.)} = \frac{2 \times 2.303 \times d \times (328 \times 338)}{10},$$

i.e. $E \propto d$.

Consequently from the diagram we see that E is maximum at p_{H} 3.7 to 4.0 and falls off on both sides of this value until on the acid side of p_{H} 3 and the alkaline side of p_{H} 5.5 it attains an approximately constant value which is about the same in both cases. To bring this out more clearly several values of the critical increment have been calculated and are appended in Table IV.

Table IV.

p_H	Critical increment (55 to 65°)	p_H	Critical increment (55 to 65°)
2.3	71,000	4.5	91,000
2.7	68,000	4.9	80,000
3.0	73,000	5.43	74,000
3.5	88,000	6.0	69,000
4.15	96,000	6.5	67,000

These figures cannot be taken as absolute but they are probably correct to within ± 5000 , and the variation with p_H is probably a real one. It is quite certain, however, that the critical increment of the heat-inactivation process may be taken as being of the order 80,000 cal.

DISCUSSION OF RESULTS.

Let us consider in the first instance how the results obtained for pepsin agree with those already obtained by various workers for other enzymes. The process of the heat-inactivation of pepsin has been found to be one which follows the theoretical unimolecular course. Other enzymes for which the heat-inactivation process has been shown to be unimolecular are trypsin by Pace [1930], pancreatic lipase by McGillivray [1930], enterokinase by Pace [1931, 1], trypsin-kinase by Pace [1931, 2], pancreatic proteinase by Pace [1931, 3], and pancreatic amylase by Giri [1932].

The figures given in Tables II and III show that for pepsin the rate of heat-inactivation is minimum and almost constant over a p_H range from about 3 to 4.5. As the p_H is altered away from this range, however, the rate of inactivation increases rapidly for comparatively small alterations in p_H . Similar behaviour for the alteration in the rate of heat-inactivation with p_H is reported for yeast saccharase by Euler and Laurin [1919] the minimum rate being at p_H 4 to 5.

In the case of trypsin, a new method of preparation has been developed by Northrop [1932, 2] which gives a very pure crystalline trypsin having the properties of a protein. The rate of inactivation for the trypsin prepared in this way is minimum at p_H 1.8.

It is therefore seen that although the general behaviour of the rate of heat-inactivation with respect to change in p_H is similar in all these cases, it is not identical, the minimum rate obtained in the different cases being at different p_H values. In the case of the heat-denaturation of oxyhaemoglobin and egg-albumin (the proteins upon which the most reliable investigations have been made) it has been shown by P. S. Lewis [1926, 1, 2] that the rate of heat-denaturation is minimum at the neutrality point of water p_H 6.8.

It may be noted in passing that for neither process does the isoelectric point of the protein or the enzyme appear to have any special importance.

A striking similarity between the two processes, *viz.* protein denaturation and enzyme inactivation, is found in the magnitude of the critical increments. The value obtained for crystalline pepsin above is of the order 80,000 cal., agreeing with the value 75,000 given by Arrhenius [1907]. A collection of such data made by Haldane [1930] for other enzymes shows values ranging from 26,000 for lipase to 189,000 for peroxidase. The critical increment for the denaturation of oxyhaemoglobin was found by Lewis [1926, 1, 2] to be 77,000, and for egg-albumin 130,000 cal., so that the unusually high values are common to both processes.

A further point observed in the present investigation is that the critical increment varies with p_H , the value being maximum, as one would expect, at the range of p_H for which the rate of inactivation is minimum. In general, other investigators have not determined how the critical increments have varied with p_H , but have been content to obtain a mean value which gave them only the order of magnitude of the critical increment. For yeast saccharase, however, Euler and Laurin [1919] have shown that the critical increment is maximum at the p_H range over which the rate of inactivation is minimum and falls off rapidly as the p_H is altered away from this range. Booth [1930], working in this Laboratory, has shown, by examination of the denaturation results of Lewis [1926, 1, 2] and Cubin [1929] supplemented by further experimental determinations, that the critical increment of the heat-denaturation of oxyhaemoglobin behaves similarly with respect to change in p_H , being maximum at the p_H for which the rate of denaturation is minimum (6.8), and decreasing as the p_H is altered away from this value.

The comparisons made above therefore show that not only does pepsin behave as a protein, thus confirming the conclusion of Northrop, but that the heat-inactivation of pepsin is analogous to the heat-denaturation of egg-albumin or oxyhaemoglobin.

SUMMARY.

1. Crystalline pepsin has been prepared by the method of Northrop, and tests have been made to ensure that the product obtained was identical with that of Northrop both in general properties and in specific proteolytic activity.

2. An activity-concentration curve for this pepsin has been obtained using the alcohol-titration method of Willstätter and Waldschmidt-Leitz, and this curve has been used as a standard in the subsequent investigation of the heat-inactivation process.

3. The process of heat-inactivation is found to follow the theoretical unimolecular course.

4. At a given temperature the rate of heat-inactivation varies with p_H , being minimum at a p_H range from 3 to 4.5 and increasing rapidly when the p_H is altered from this range towards either the more acid or alkaline regions.

5. The effect of temperature on the rate of heat-inactivation has been examined and is found to be very marked. The critical increment of the process is found to be of the order 80,000 calories.

6. The critical increment is found to vary with p_H , being maximum (96,000 calories) at the p_H range for which the rate of inactivation is minimum and decreasing to a much lower value (70,000 calories) in the more acid and alkaline regions (p_H 2.3 and p_H 6.0).

7. The results obtained are compared with those obtained by other investigators for various enzymes and also with the results obtained for the heat-denaturation of proteins. It is shown that the two processes, the heat-inactivation of enzymes and the heat-denaturation of proteins, are analogous, in all but one point, *viz.* the location of the minimum speed, for which no explanation can be offered at present.

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