LI. THE INACTIVATION OF TRYPSIN-KINASE BY HEAT, AND THE EFFECT OF ADDED PROTEIN THEREON.

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IN previous papers the writer has described experiments on the inactivation of trypsin [1930] and of enterokinase [1931] by heat. The first section of the present paper deals with the inactivation by heat of trypsin-kinase, the complex formed by union of the enzyme trypsin with its specific activator enterokinase. The examination of the heat-inactivation of trypsin-kinase involves in principle the three following operations:

- (1) the formation of trypsin-kinase by mixing solutions of trypsin and enterokinase and leaving the mixture for 30 mins. in a thermostat at 30°;
- (2) the transference of the trypsin-kinase, resulting after the above period of activation, to a thermostat at 50°, and the removal of samples at various time intervals;
- (3) the measurement of the trypsin-kinase content of the various samples by the extent of the hydrolysis produced in a caseinogen solution under standard conditions.

The general experimental procedure involved in these operations may now be briefly described.

Experimental procedure.

For comparison with the work on the heat-inactivation of trypsin free from enterokinase, where the glycerol concentration was 24 %, it is necessary to arrange in the present investigation that the solution of trypsin-kinase undergoing inactivation should have the same glycerol concentration. This is arrived at in the following way.

5 g. of dried intestinal mucosa, prepared according to the method of Waldschmidt-Leitz [1924], were dispersed through 200 cc. of a glycerol-water mixture (containing 23.6 % of glycerol) by shaking and the dispersion was left for 24 hours in the ice-chest to allow of the activator passing into pseudo-solution. The dispersion was then centrifuged and filtered, and to 160 cc. of the filtrate were added 3 cc. of *N* acetic acid. The resulting precipitate of protein matter was centrifuged off, and the clear supernatant liquid neutralised

with N ammonia and kept in the ice-chest. This kinase solution now contained 23 % of glycerol. The experiments were then carried out as follows.

15 cc. of trypsin solution prepared as described in a previous paper of the writer [1930] (and containing 26.4 % of glycerol) were added to 10 cc. of the kinase solution described above. Formation of trypsin-kinase was allowed to occur by maintaining the mixed solutions for 30 mins. at 30°. At the end of this period 1 cc. of p_H -adjusting liquid (N acetic acid, N ammonia and distilled water) was added, thus producing in the final solution a glycerol content of 24 %. Of this final solution 3 cc. were measured out into each of a number of flasks already immersed in a thermostat at 50°. These flasks were withdrawn into crushed ice at various time intervals and the quantity of trypsin-kinase, *i.e.* the number of "units" therein, determined by the method of Linderstrøm-Lang and Steenberg [1929] at 30°, as described by the writer in a previous paper [1931].

The p_H measurements were carried out at room temperature by means of the glass electrode.

It was found that the course of the reaction, using this solution of trypsin-kinase, is in fair agreement with the unimolecular expression¹:

$$k = \frac{2.303}{t} \log_{10} \frac{a}{a-x}$$

where

a = initial content of trypsin-kinase,

$a - x$ = content of trypsin-kinase after time t mins.

Table I, which is typical of the results in general, illustrates the degree of agreement with the unimolecular expression.

Table I.

Temperature 50°. p_H 8.77. Glycerol 24 %.

Time (mins.)	Trypsin-kinase content units per cc.	$k \times 10^3$
0	1.00	—
30	0.83	6.21
60	0.67	6.67
100	0.54	6.16
220	0.25	6.30

The effect of p_H upon the heat-inactivation.

The stability of all enzymes is influenced very markedly by the p_H of the medium, and Table II shows the effect of p_H upon the heat-inactivation of trypsin-kinase. In the second column the mean of the values of k for a particular p_H are set down opposite that p_H . The third column shows "smoothed values" of k obtained from the most probable curve resulting on plotting the experimental values of k against p_H . This curve is shown in Fig. 1.

¹ Throughout this paper k represents the velocity constant of a unimolecular reaction.

Table II.

Temperature 50°. Glycerol 24 %.

p_H	$k \times 10^3$	
	Experimentally found	"Smoothed" from curve
4.10	4.9	4.9
4.20	4.7	4.7
4.97	3.3	3.4
5.09	3.3	3.2
5.90	2.1	2.3
5.98	2.4	2.2
6.50	1.9	2.0
6.57	2.2	2.05
7.88	3.4	3.5
8.15	4.6	4.3
8.67	6.6	6.3
8.77	6.3	6.9
8.92	9.6	9.6

It is seen that the optimum stability for trypsin-kinase lies about p_H 6.5.

Fig. 1.

The critical increment for the heat-inactivation of trypsin-kinase.

Experiments were carried out to determine the critical increment for the heat-inactivation of trypsin-kinase. This was determined by measuring k at 50° and 60° and then substituting the mean values in the integrated form of the equation:

$$\frac{d \log_e k}{dT} = \frac{E}{RT^2}.$$

Measurements were made:

- (1) in the region of optimum stability, p_H 6.5,
- (2) in the acid region at p_H 4.1, p_H 5.1, p_H 5.9,
- (3) in the alkaline region at p_H 8.8.

The results are summarised in Table III in which the figures in the last column represent the critical increments calculated by substituting the mean values of k at 50° and 60° into the integrated form of the equation.

Table III.

p_H	k (at 50°) $\times 10^3$	k (at 60°) $\times 10^3$	E (cals.)
8.8	6.34	4.77	43,000
6.5	1.90	1.55	44,600
5.9	2.14	1.35	39,000
5.1	3.27	2.80	45,000
4.1	4.95	2.90	38,000

The critical increment appears to vary with p_H in a fortuitous manner. It may be concluded that over the p_H range 4–8.8 it is of the order of 40,000 calories per “molar unit” of trypsin-kinase.

Comparison with the results obtained for trypsin¹ free from enterokinase.

It will be seen, on comparing these results with those obtained by the writer [1930] for the heat-inactivation of trypsin free from enterokinase, that the behaviour is similar in two important respects:

- (1) in the position of the region of optimum stability, namely about p_H 6.5;
- (2) in the order of magnitude of the critical increment, namely about 40,000 calories, being sensibly the same at the region of optimum stability and at points on the acid and alkaline sides in each case.

This similarity suggests that the active enzymic groupings may still be of the same nature in trypsin-kinase as they are in trypsin. Since however trypsin-kinase has more extensive proteolytic activity than trypsin alone it is to be inferred that the attachment of the kinase, necessarily at points other than those proteolytically active, serves to intensify the latter.

A comparison of the actual rates of heat-inactivation in both cases is of interest. This may possibly be best effected if, instead of comparing unimolecular constants, we compare the times of “half decomposition.” This time interval is obtained from the unimolecular expression by writing

$$k = \frac{2.303}{t_{\frac{1}{2}}} \log_{10} 2.$$

The comparison is made in Table IV. The values of k corresponding to the

Table IV.

Temperature 50°. Glycerol 24 %.

p_H	Time for “half decomposition” (mins.)	
	Trypsin	Trypsin-kinase
5.0	231	210
6.0	346	277
7.0	346	300
8.0	247	193
8.5	198	128
9.0	144	74

¹ The word trypsin is used here in the sense employed by the Willstätter school up to 1930, *i.e.* the enzyme inactive towards true proteins but capable of hydrolysing certain protein degradation products. It has since been shown that trypsin itself may be resolved into a proteinase and a carboxy-polypeptidase. It is the latter fraction which is responsible for the hydrolysis of protein degradation products.

p_H - k curves for trypsin and trypsin-kinase respectively are calculated and then substituted into the equation for $t_{\frac{1}{2}}$ given above.

It is seen from Table IV that the rates of heat-inactivation of trypsin and of trypsin-kinase are fairly close together. On the whole it is to be concluded that trypsin is slightly more stable when free from its activator than when associated with it. This superior stability of trypsin over trypsin-kinase is most marked in the alkaline region.

The effect of the addition of protein upon the heat-inactivation of trypsin-kinase.

Proteins, peptones and amino-acids have been found to have a stabilising influence upon a number of enzymes. Luers and Lorinser [1922] found that egg-albumin and gelatin have a stabilising influence upon malt amylase, and Willstätter, Graser and Kuhn [1922] showed that leucylglycine and yeast gum protect saccharase to some extent from heat-inactivation. When the proteins and their degradation products are the substrates and products of reaction respectively, as with the proteolytic enzymes, the stabilising effect is most pronounced, for enzymes generally are found to be protected by their specific substrates and their products of reaction. Thus Biernacki [1891] found that albumins protected trypsin from heat-inactivation, and Bayliss and Starling [1904] have observed a similar effect with caseinogen peptones and egg-albumin. Northrop [1922] has stated that the products formed by the action of trypsin on proteins have a protective action upon the enzyme, but that gelatin itself has no such stabilising action.

In the following experiments, carried out by the writer, caseinogen was added to the solution of trypsin-kinase undergoing inactivation at two different p_H values:

- (1) at p_H 8.5, *i.e.* in the region of optimum activity for the enzyme, where the products of reaction are most readily formed;
- (2) at p_H 4.7, *i.e.* well outside the optimum region of activity for the enzyme.

The experimental procedure was as follows.

At p_H 8.5. To 25 cc. of trypsin-kinase, prepared as described above, were added 2 cc. of a buffer, prepared by mixing equal volumes of N ammonia and N ammonium chloride, and 2 cc. of a 6 % caseinogen solution.

As a control the same solution was made up except that instead of 2 cc. of a 6 % caseinogen solution, 2 cc. of distilled water were used.

At p_H 4.7. The same solution and control were made up as at p_H 8.5 except that 2 cc. of a buffer prepared by mixing equal volumes of 0.066 N sodium acetate and 0.066 N acetic acid were used instead of the alkaline buffer.

The solutions containing the added protein and also the control solutions were maintained at 50° for 90 mins. and the trypsin-kinase content was measured initially and at the end of this period of time. The results are shown in Tables V and VI.

Table V.

Temperature 50°. p_H 8.5.

	Time (mins.)	Trypsin-kinase units per cc.		$k \times 10^3$
		Run A	Run B	
With added protein	0	1.06	1.00	Run A 3.3
	90	0.76	0.73	Run B 3.2
Without added protein	0	1.06	1.01	Run A 5.8
	90	0.63	0.61	Run B 5.6

From these results it follows that the mean decrease in k due to the added protein is 43 %.

Table VI.

Temperature 50°. p_H 4.7.

	Time (mins.)	Trypsin-kinase units per cc.	
		Run A	Run B
With added protein	0	1.09	1.05
	90	0.85	0.81
Without added protein	0	1.09	1.05
	90	0.83	0.80

It is seen that at this p_H of 4.7 the difference between the degree of inactivation of the solution containing added protein and that of the control is within the experimental error.

It is to be concluded from these experiments that it is the products of hydrolysis which exert a stabilising influence upon the enzyme, the protein as such having little effect.

A further experiment was carried out in which the heat-inactivation was again determined at the alkaline p_H 8.5, but at 60° instead of at 50°. Since the critical increment for the tryptic hydrolysis of caseinogen is about 14,000 calories [Moelwyn-Hughes, Pace and Lewis, 1930] while the critical increment for the heat-inactivation of the enzyme is seen to be of the order of 40,000 calories, a rise of 10° in temperature will result in a relatively smaller increase in the velocity of hydrolysis as compared with the increase in the velocity of inactivation. So that if, as we have inferred, the products of hydrolysis exert a stabilising influence upon the enzyme, this effect should be relatively less at 60° than it is at 50°. The results of the experiment are seen in Table VII.

Table VII.

Temperature 60°. p_H 8.5.

	Time (mins.)	Trypsin-kinase units per cc.	$k \times 10^2$
With added protein	0	1.02	3.6
	90	0.71	
Without added protein	0	1.02	4.0
	90	0.68	

The decrease in k owing to added protein is now 10 %, compared with the decrease of 43 % in the corresponding experiment at 50°. This result is therefore in agreement with the conclusion that it is the products of reaction rather than the protein itself which stabilise the enzyme to some extent against heat-inactivation.

In experiments carried out by the writer on the heat-inactivation of a sample of commercial "active trypsin" (*i.e.* trypsin-kinase) it was found that the constants calculated on the basis of the unimolecular expression decreased with increase in time. Northrop [1922] found the same behaviour in experiments with commercial trypsin. He put forward the explanation that the slowing up of the reaction is due to the enzyme acting upon the protein matter in the preparation used, when the resulting products of hydrolysis unite with the enzyme to form compounds which are more stable to heat than the free enzyme. This conclusion, arrived at by Northrop, is substantially confirmed by the results described in this paper.

SUMMARY.

1. A solution of trypsin-kinase has been prepared by the interaction of trypsin and enterokinase solutions for 30 mins. at 30°. The solution was used to study the heat-inactivation of trypsin-kinase. At the degree of purity employed it is found that the course of the reaction is in fair agreement with the unimolecular expression.

2. The effect of p_H upon the heat-inactivation has been investigated and it is found that the optimum stability is about p_H 6.5.

3. The critical increment of the heat-inactivation process has been determined at five p_H values, lying between p_H 4 and 9. It is found that the critical increment is of the order of 40,000 calories per "molar unit" of enzyme.

4. Experiments have been carried out upon the effect of adding caseinogen to the solution of trypsin-kinase undergoing inactivation. The caseinogen was added to solutions of the enzyme at p_H 8.5 (in the optimum region of activity for trypsin-kinase) and at p_H 4.7 (well outside the optimum region of activity) respectively. It was found that at p_H 8.5 the effect of the caseinogen, added initially, is to slow up the heat-inactivation of the enzyme, but that at p_H 4.7 there is no marked stabilisation. Thus it is concluded that it is the products of hydrolysis of the caseinogen which stabilise the enzyme. Further support for this conclusion was obtained by conducting the heat-inactivation of the enzyme, with caseinogen added initially, again at p_H 8.5 but at a temperature 10° higher. It was found that under these conditions the relative stabilising effect is reduced.

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