LXVIII. THE INACTIVATION OF TRYPSIN BY HEAT.¹

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(Received April 11th, 1930.)

THE study of the heat-inactivation of trypsin contained in a crude extract of dried pancreas gland powder is complicated by the "spontaneous activation" which occurs in such extracts. Waldschmidt-Leitz and Harteneck [1925] have recently interpreted this phenomenon as due to the production of entero-kinase from its pre-stage, which occurs together with the trypsin in the pancreas. This transformation takes place in aqueous extracts of dried pancreas powder, or in the dried powder itself on prolonged storage. As a consequence, in an aqueous extract of dried pancreas powder maintained at constant temperature two processes occur, heat-inactivation of the trypsin and simultaneously "spontaneous activation" of the enzyme. Thus, if the trypsin content of the extract be measured from time to time by hydrolysis of a suitable protein, there may be, instead of the decrease to be expected from heat-inactivation of the enzyme, an apparent increase in trypsin content, if it so happens that the "spontaneous activation" effect preponderates over the heat inactivation. Furthermore, Waldschmidt-Leitz has indicated that trypsin associated with its activator is less stable than the enzyme alone. In view of this the main experiments described in this paper have been carried out with a purified extract of the dried pancreas powder, in which the trypsin is free from enterokinase and its pre-stage.

EXPERIMENTAL PROCEDURE.

To study the inactivation of trypsin at different p_H values the following procedure was adopted. To 10 cc. of the neutral solution prepared in the manner described below were added normal acetic acid or normal ammonia to bring it to a required p_H and then distilled water was added to make the total volume 11 cc. This volume of solution was divided out into a number of test-tubes already immersed in the thermostat, regulated to $\pm 0.05^\circ$, at 50° . The test-tubes were then tightly stoppered and removed at various time intervals from the thermostat into crushed ice. The trypsin content of 1 cc. of solution was then measured. For the major portion of the work the trypsin content was measured by the method of Willstätter *et al.* [1926] (activation

with enterokinase and subsequent interaction with caseinogen at p_H 8.7 at 30° for 2 minutes). While the work was in progress a modification of this method was published by Linderstrøm-Lang and Steenberg [1929]. (This modification provides for a more definite control of the activation process with enterokinase in the analytical procedure. A buffer is used and the activation is always carried out with an arbitrarily chosen "enterokinase unit." Further, the hydrolysis is made to take place at p_H 9.1.) Accordingly the measurements, already made by the writer for the determination of the critical increment of the inactivation process, were repeated using this revised technique and substantially the same results were obtained.

Preparations.

(a) *Preparation of trypsin free from enterokinase and the pre-stage of the activator.* In the earlier work of the Willstätter school a separation of trypsin from the activator and its pre-stage was accomplished by a series of selective adsorptions on alumina C_7 . Later a revised method and technique was published by Waldschmidt-Leitz and Linderstrøm-Lang [1927]. The principle of the latter method is to adsorb trypsin-kinase and the pre-stage of the activator on caseinogen, and then, by precipitating the protein, to leave the trypsin behind in the mother liquor. This process of purification, due to Waldschmidt-Leitz and Linderstrøm-Lang was carried out as follows.

Pig's pancreas, free from fat, was prepared by drying with acetone and ether according to the procedure of Willstätter and Waldschmidt-Leitz [1923]. 6 g. of the dried gland powder were added to 100 cc. of glycerol-water mixture (containing 80 % glycerol). The powder was dispersed through the liquid by shaking and the dispersion kept for 5 hours at 30°. The finely divided suspension was centrifuged, and the supernatant liquid finally filtered. To 25 cc. of the clear ice-cold glycerol extract prepared in this manner were added 15 cc. of an ice-cold 6 % caseinogen solution. The mixture was left for 5 minutes in the ice-chest and was then treated with 2 cc. of *N* acetic acid. The resulting precipitate of protein was filtered off and to 20 cc. of the filtrate was added a further 15 cc. of ice-cold 6 % caseinogen solution followed by 1 cc. of *N* acetic acid. The precipitate was again filtered off. The filtrate, now clear and colourless, was brought to neutrality by addition of *N* ammonia. This neutral solution, which contains the trypsin completely free¹ from enterokinase and its pre-stage, was kept in the ice-chest.

(b) *Enterokinase solution.* Pig's intestinal mucosa was treated with acetone, acetone-ether mixture, and finally ether according to the procedure of Waldschmidt-Leitz [1924]. An aqueous extract was prepared by suspending 6 g. of the dried mucosa in 200 cc. distilled water for 5 hours at 30°. The suspension was centrifuged and filtered. To 100 cc. of the clear filtrate were

¹ This statement is borne out by the fact that there was no hydrolysis of caseinogen in 2 hours at 30°.

added 1.6 cc. *N* acetic acid and the resulting precipitate of protein matter was centrifuged off. The clear supernatant liquid was neutralised with *N* ammonia and kept in the ice-chest.

(c) *Caseinogen solution.* The caseinogen solution was prepared by addition of 100 cc. 0.05 *N* ammonia to 6 g. caseinogen (Kahlbaum-Hammarsten), stirring and keeping for 1 hour at 30°. This solution is referred to as a 6 % caseinogen solution.

(d) *Buffer solutions.* For the method of determination according to Willstätter *et al.* [1926] the buffer solution was made up by mixing equal volumes of *N* ammonia and *N* ammonium chloride. The p_H of this buffer was measured at 20° with the glass electrode and found to be 8.6.

For the Linderström-Lang and Steenberg modification of the method two buffer solutions were made up as follows:

(i) by mixing 300 cc. *N* ammonium chloride and 50 cc. *N* ammonia;

(ii) by mixing 250 cc. *N* ammonia and 350 cc. water.

The p_H measurements of the trypsin solution were made by means of the glass electrode at room temperature.

The course of the reaction.

The heat-inactivation of a number of enzymes has been studied by various investigators and in general it has been found that the course of the reaction is "unimolecular." Thus Tammann [1895] found that the heat-inactivation of emulsin conforms to the unimolecular expression, and Madsen and Walbum [1907] obtained the same result for the proteolytic enzymes pepsin and trypsin.

It should be emphasised that all previous work on the heat-inactivation of trypsin has been carried out with material in which the enzyme has been associated with the activator, enterokinase. Since trypsin associated with the activator, *i.e.* trypsin-kinase, and non-activated trypsin are quite specific in their function as catalysts, it cannot reasonably be assumed that the loss of their catalytic efficiency on heating will occur at the same rate. Consequently previous work on the enzyme is not strictly comparable with the results described in this paper obtained with purified trypsin solution. Furthermore, most of the previous work has been carried out with unpurified material containing a considerable amount of protein matter. In such enzyme material, which contains the trypsin associated with enterokinase and therefore endowed with the capacity to hydrolyse native proteins, it is to be expected that hydrolysis of the protein matter contained in the enzyme preparation will proceed during the course of the inactivation. Thus at least two factors are introduced which may influence the temperature sensitivity of the enzyme, namely:

(1) a change in p_H , due to the formation of free carboxyl and amino-groups;

(2) the formation of reaction products which have a stabilising influence upon the enzyme, as the work of Schmidt [1911], Vernon [1904] and Northrop [1922] has shown.

Although the results obtained by Tammann and by Madsen and Walbum were in agreement with the unimolecular equation this is not the experience of other workers. Thus Northrop [1922], working with "Fairchild's trypsin," found that the course of the heat-inactivation did not conform to the unimolecular expression. He suggested that the observed decrease in the unimolecular constants with time is due to the enzyme acting upon the protein matter in the preparation used and thus forming a compound with the products of reaction which is more stable to heat than the "free" enzyme. On purifying the enzyme solution by dialysis he obtained, in one experiment, a satisfactory unimolecular constant. Vernon [1904], working with a pancreas extract containing activator, had also previously found that the constants worked out on the unimolecular basis decreased with time. He ascribed this falling off as due to the presence of several enzymes of different degrees of stability. In the light of the more recent work it is difficult to accept this explanation, for Vernon measured the quantity of enzyme by the hydrolysis of fibrin, and therefore was actually measuring the quantity of one enzyme only, namely activated trypsin, since non-activated trypsin and erepsin do not possess the property of hydrolysing native proteins.

In the experiments described in this paper, with trypsin freed from enterokinase and its pre-stage, it is found that the course of the heat-inactivation in fact follows the unimolecular expression. It would appear, as Northrop suggested, that the decrease in the unimolecular constants observed with unpurified trypsin solutions is due to the protective influence of the substances formed by the action of trypsin-kinase upon the protein in the solution.

The applicability of the unimolecular expression for the heat-inactivation of the trypsin solution employed by the writer is illustrated in Table I, for solutions at p_H values in the alkaline, acid and neutrality regions respectively.

The measurements at a particular time interval were done in duplicate and in all the following tables the magnitude of the "trypsin unit per cc." set down opposite a particular time is the mean of such duplicate experiments.

The values of k_{uni} were obtained from the equation:

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

where

a = trypsin units per cc. at zero time,

$a - x$ = trypsin units per cc. after time t mins.

The trypsin unit employed is that defined by Willstätter *et al.* [1926], namely the quantity of trypsin which produces in a 6 % caseinogen solution under the standard conditions of measurement an increase in acidity equivalent to 1.05 cc. 0.2 N NaOH.

Table I.

Temp. 50°. Glycerol content of solution 24 %.		
Time <i>t</i> mins.	Trypsin units per cc.	k_{uni}
p_{H} 8.40.		
0	1.60	—
60	1.25	3.97×10^{-3}
129	1.05	3.27×10^{-3}
161	0.905	3.54×10^{-3}
211	0.78	3.40×10^{-3}
p_{H} 5.86.		
0	1.52	—
60	1.28	2.86×10^{-3}
124	1.07	2.82×10^{-3}
159	0.94	3.02×10^{-3}
p_{H} 7.12.		
0	1.60	—
120	1.22	2.26×10^{-3}
240	0.93	2.24×10^{-3}
360	0.77	2.04×10^{-3}

THE EFFECT OF p_{H} UPON THE HEAT INACTIVATION.

The p_{H} of the medium has a very marked effect upon the stability of all enzymes. In general it is found that each enzyme has a fairly broad optimum region of stability and that outside this region the rate of destruction increases rapidly. In Table II the results obtained for the heat-inactivation of trypsin at various p_{H} values are summarised. The mean of the values for k_{uni} at a particular p_{H} are set down opposite that p_{H} value.

These results are plotted in Fig. 1, where it is seen that the enzyme is most stable at p_{H} 6.5.

Fig. 1.

Table II.

Temp. 50°. Glycerol content of solution 24 %.			
p_{H}	$k_{\text{uni}} \times 10^3$	p_{H}	$k_{\text{uni}} \times 10^3$
9.95	9.23	6.64	2.00
8.82	4.12	6.51	1.70
8.40	3.59	5.86	2.90
7.96	2.98	5.52	2.64
7.12	2.18	4.85	3.14

It is not possible to compare with certainty these results obtained for the effect of p_H with other work in which a different enzyme material has been employed. This point has been emphasised by Willstätter, Haurowitz and Memmen [1924] for the dependence of enzyme activity upon p_H , where they show that substances of various kinds may modify the influence of p_H . At the same time it is noteworthy that for many enzymes the region of optimum stability appears to lie about p_H 6. Thus Morgulis and Beber [1928] found that the optimum stability for catalase lies between p_H 6 and 6.5, Michaelis and Rothstein [1922] found the optimum stability for pepsin at p_H 6, whilst Ernstrom [1922] found an optimum stability at p_H 6.1 for salivary amylase, and Willstätter, Graser and Kuhn [1922] found the optimum stability of saccharase at p_H 5.8. Northrop [1922] found the optimum stability for the trypsin he used to be at p_H 5.0.

The shape of the p_H -stability curve is somewhat similar to that obtained by Lewis [1926] for the effect of p_H upon the rate of heat-denaturation of proteins.

THE CRITICAL INCREMENT OF THE PROCESS.

The critical increment for the process of heat-inactivation of enzymes is found to be very large and comparable with that found for the heat-denaturation of proteins by Chick and Martin [1910] and Lewis [1926].

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

$$\frac{d \ln k_{uni}}{dT} = \frac{E}{RT^2}.$$

Measurements were made in the three cases:

- (1) at the region of optimum stability, p_H 6.51;
- (2) at an alkaline p_H , 8.82;
- (3) at an acid p_H , 5.02.

The results are set out in the following tables, where the method employed for the determination of the quantity of enzyme is also stated.

Table III. *The critical increment at p_H 6.51.*

Method of determination that of Willstätter *et al.* [1926]. Glycerol content of solution 24 %.

Time <i>t</i> mins.	Trypsin units per cc.	k_{uni}
Temp. 50°.		
0	1.53	—
120	1.23	1.82×10^{-3}
295	0.95	1.60×10^{-3}
385	0.80	1.68×10^{-3}
	Mean	1.70×10^{-3}
Temp. 60°.		
0	1.51	—
18	1.23	1.14×10^{-2}
36	1.03	1.06×10^{-2}
54	0.805	1.16×10^{-2}
72	0.62	1.24×10^{-2}
	Mean	1.15×10^{-2}

Substituting the mean values of k_{uni} at 50° and 60° into the equation cited above we arrive at a value of $E=40,800$ calories for the heat-inactivation of trypsin at p_H 6.51.

Table IV. *The critical increment at p_H 6.50.*

Method of determination that of Linderström-Lang and Steenberg [1929]. Glycerol content of solution 24 %.

Time t mins.	Trypsin units per cc.	k_{uni}
Temp. 50°.		
0	0.72	—
90	0.60	2.01×10^{-3}
160	0.55	1.68×10^{-3}
200	0.49	1.92×10^{-3}
	Mean	1.87×10^{-3}
Temp. 60°.		
0	0.72	—
20	0.56	1.25×10^{-2}
40	0.45	1.17×10^{-2}
60	0.35	1.20×10^{-2}
	Mean	1.21×10^{-2}

From these values of k_{uni} at 50° and 60° we arrive at a value of $E=39,700$ calories for p_H 6.50.

Table V. *The critical increment at p_H 8.2.*

Method of determination of enzyme quantity that of Willstätter *et al.* [1926]. Glycerol content of solution 24 %.

Temp. 50°. Mean value of $k_{uni} = 4.35 \times 10^{-3}$
 Temp. 60°. Mean value of $k_{uni} = 3.47 \times 10^{-2}$

From these values of k_{uni} at 50° and 60° we arrive at a value of 44,300 calories for the critical increment at p_H 8.82.

Table VI. *The critical increment at p_H 5.02.*

Method of determination of enzyme quantity that of Linderström-Lang and Steenberg [1929].

Temp. 50°. Mean value of $k_{uni} = 2.90 \times 10^{-3}$
 Temp. 60°. Mean value of $k_{uni} = 1.75 \times 10^{-2}$

From these values of k_{uni} at 50° and 60° we arrive at a value of 38,300 calories for the critical increment at p_H 5.02.

From these results it is seen that the critical increment of the heat-inactivation of trypsin is sensibly the same over the p_H range 5 to 9.

DISCUSSION.

It may be pointed out that the critical increment of heat-inactivation of trypsin is about one-half of the critical increment of heat-denaturation of haemoglobin and about one-third of the critical increment of heat-denaturation of egg-albumin in the region of neutrality, *i.e.* the region in which the rate of denaturation is least. If heat-inactivation be connected with denaturation of the supporting or "core" protein associated with the enzyme it must follow that the protein involved is more easily denatured than is the free protein.

Again, comparing $E = 40,000$ calories for heat-inactivation with $E = 14,000$ calories for the tryptic hydrolysis of caseinogen [Moelwyn-Hughes, Pace and Lewis, 1930] and with $E = 22,000$ calories for the acid hydrolysis of acetyl-glycine [Escolme and Lewis, 1927] it is seen that inactivation is almost certainly not hydrolysis of the protein "core" of the enzyme.

It is difficult to draw any conclusions from the fact that the stability optimum of trypsin lies between p_H 6 and 7, because of the diverse results cited in the literature about the electro-chemical nature of trypsin. Thus Michaelis and Davidsohn [1911] stated that trypsin was an amphoteric body with an isoelectric point between p_H 4 and 5, while Northrop [1924] agreed that trypsin was amphoteric, but found the isoelectric point to be at p_H 10.2. Later work by Willstätter has indicated however that it is only in crude preparations that trypsin has an amphoteric character. After purification trypsin is not adsorbed by alumina suspensions having a negative charge—but is adsorbed quantitatively by kaolin suspensions which have a positive charge. From this it appears that trypsin is definitely basic in character.

It is seen that the region of optimum stability for trypsin, p_H 6–7, does not coincide with the region of optimum activity, which is p_H 7.8–8.7. In this connection, however, it should be pointed out that the rate of destruction of trypsin in a digestion mixture maintained about p_H 8.7 will not be so great as the rate of destruction found for solutions containing enzyme alone at that p_H , because in the former case the products formed during the tryptic digestion exert a considerable stabilising influence upon the enzyme.

SUMMARY.

1. Experiments have been carried out on the heat-inactivation of trypsin which has been purified so as to be free from enterokinase and its pre-stage. The importance of this purification of an extract of pancreas is emphasised on account of the complicating effect of the “spontaneous activation” of the enzyme which otherwise occurs. It is found that the course of the heat-inactivation of trypsin purified in this way follows the unimolecular equation.

2. The effect of p_H upon the heat-inactivation of trypsin prepared in the above manner has been studied. It has been found that trypsin has an optimum stability about p_H 6.5, and that the rate of destruction increases markedly on either side of this point. The p_H -stability curve is similar in shape to that obtained for the effect of p_H upon the rate of heat-denaturation of proteins.

3. The critical increment for the heat-inactivation process has been determined (a) in the alkaline region, (b) in the acid region, (c) at the region of optimum stability. It is found that the critical increment is sensibly the same at all three points and is of the order of 40,000 calories per molar unit of enzyme.

The above work was carried out under the direction of Professor W. C. M. Lewis, to whom the writer is indebted for help and advice.

The writer desires to express his thanks to the Department of Scientific and Industrial Research for a maintenance grant, and also to Imperial Chemical Industries, Ltd. for a grant to the Department of Physical Chemistry of the University of Liverpool.

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