CLXXXVI. THE FORMATION OF ENTEROKINASE FROM A PRECURSOR IN THE PANCREAS: A SUPPLEMENTARY NOTE.

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In a recent paper [1932] the writer presented data upon the rate of formation of enterokinase from its precursor in the pancreas. It was found that the observed rate conforms to the unimolecular expression and that it is a function of the hydrogen ion concentration of the medium, exhibiting a maximum at about p_H 6.90. Certain additional experiments which have been carried out are now described.

The critical increment of the process.

It was considered that a knowledge of the critical increment (or energy of activation) might be of value in discussing the mechanism of the process. This was therefore determined by measuring the rate of formation at 20° and at 30° and then substituting the observed values into the equation for the critical increment:

$$E = \frac{2.303 RT_1 T_2}{(T_1 - T_2)} \log_{10} \frac{k_{T_1}}{k_{T_2}},$$

where k_{T_1} and k_{T_2} are the velocity constants at the temperatures T_1 and T_2 respectively.

The experimental details were as follows. The solution containing the precursor (neutralised elution from alumina adsorptions of glycerol extract of dried pancreas powder) was prepared in the way previously described by the writer. To 40 cc. of neutralised elution were added 10 cc. of phosphate buffer such that the p_H of the mixture was 6.9. Then 20 cc. of the buffered mixture were measured out into each of two glass bottles. One of these was then placed in a thermostat at 20° and the other in a thermostat at 30°, and after samples had been removed for the determination of the zero reading the bottles were stoppered and kept in their respective thermostats.

It has already been shown that the rate of formation may be described by the unimolecular expression:

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

where a is the initial concentration of precursor, and $(a - x)$ is the concentration of precursor after time t .

In the writer's previous paper it was also shown that a may be taken to be the maximum amount of enterokinase formed—i.e. the "total extent" reading, which is reached after about 35 hours at 30° . Therefore in the present experiments a was determined in this way and a value of $(a - x)$ was obtained by measuring the quantity of enterokinase formed after 18 hours and then subtracting this from the a value. This difference is a measure of the quantity of precursor remaining unchanged after 18 hours.

The quantity of enterokinase formed in each case was measured by the method described previously. Table I gives the results of two separate experiments. Blank and control readings have been subtracted and the increases in acidity recorded are therefore direct measures of the quantity of enterokinase formed.

Table I.

	Increases in acidity cc. 0.1 <i>N</i> NaOH		$\frac{a}{(a-x)}$	k (t in mins.)	E (calories)
	18 hours	Total extent			
Exp. 1			0.92		
Temp. 20°	0.54	0.92	0.38	8.2×10^{-4}	9600
Temp. 30°	0.72		0.92	1.4×10^{-3}	
			0.20		
Exp. 2			0.96		
Temp. 20°	0.55	0.96	0.41	7.9×10^{-4}	9500
Temp. 30°	0.74		0.96	1.36×10^{-3}	
			0.22		

The significance of a value for the critical increment of this order, viz. 9500 calories, is considered later.

Adsorption experiments.

The solution which has been used so far in this work for observing the formation of enterokinase from a precursor has been the neutralised elution from alumina adsorptions carried out upon a glycerol extract of fresh dried pancreas gland powder. A solution prepared in this way is known to contain the two enzymes aminopolypeptidase and dipeptidase. These may be separated by ferric hydroxide adsorptions at p_H 4 (see for example Waldschmidt-Leitz and Purrr [1929]). The dipeptidase is adsorbed by the ferric hydroxide under these conditions, while the aminopolypeptidase remains in solution. In view of this it was decided to examine whether the precursor of enterokinase also exhibited any specific adsorptive behaviour towards ferric hydroxide.

The ferric hydroxide was prepared by the method of Willstätter, Kraut and Fremery [1924] as follows. Two solutions were first made up, (a) a solution of 50 g. of ferric ammonium sulphate in 100 cc. of water, (b) 400 cc. of an aqueous solution containing 5.4 g. NH_3 and 13.75 g. of ammonium sulphate. Solution (b) was stirred vigorously with a mechanical stirrer and solution (a) then added as quickly as possible. The stirring was continued for half an hour. At the

end of this time the ferric hydroxide was in the form of a uniform reddish-brown precipitate. This precipitate was washed by diluting with distilled water to 2 litres, allowing it to settle again, and then decanting. This was carried out twelve times in all. At the second and third decantations 0.5 cc. of concentrated ammonia (sp. gr. 0.88) was added to decompose any unchanged sulphate.

The gel-like ferric hydroxide remaining after the final decantation was used for the adsorption experiments, which were carried out in the following way.

Four alumina adsorptions were first carried out upon 20 cc. of glycerol extract in the way previously described by the writer. The alumina from these adsorptions was then eluted with 20 cc. of 0.05 *N* ammonia, the alumina centrifuged off and the clear elution then neutralised with *N* HCl.

The neutralised elution was then brought to p_H 4 by addition of 5 cc. of an acetate buffer (made up by mixing 17.6 cc. *N* acetic acid and 2.4 cc. of *N* sodium acetate). The solution was then treated with 5 cc. of the ferric hydroxide suspension, the mixture left for about 15 minutes, and the ferric hydroxide then centrifuged off. The mother-liquor from this first adsorption was then treated again with 5 cc. of the ferric hydroxide suspension, and the process repeated. The final mother-liquor from the centrifuging was neutralised with *N* ammonia. The ferric hydroxide precipitate was eluted with 25 cc. of a 1 % Na_2HPO_4 solution; the ferric hydroxide then centrifuged off and the elution neutralised with *N* acetic acid. Samples of the neutralised mother-liquor and the neutralised elution were then kept at 30° and the formation of enterokinase was measured in the usual way by taking initial readings and readings after 20 hours at this temperature. Table II shows the results of a number of experiments. These are separate and individual experiments—in each case the whole adsorption procedure was carried out on different original samples of glycerol extract. Blank and control readings have been subtracted.

Table II. *Ferric hydroxide adsorption experiments. Increases in acidity due to enterokinase formed after 20 hours at 30°.*

cc. 0.1 *N* NaOH.

	Mother-liquor	Elution
<i>Exp. a</i>	0.00	0.36
<i>Exp. b</i>	0.06	0.50
<i>Exp. c</i>	0.08	0.48
<i>Exp. d</i>	0.07	0.40

The results given in Table II are of interest when it is remembered that the dipeptidase is also adsorbed by ferric hydroxide under these conditions and is therefore present together with the precursor in the elution. From the detailed scheme for the separation of the pancreatic enzymes of the tryptic and ereptic type given recently by Waldschmidt-Leitz [1931] it will be seen in fact that dipeptidase is the only known enzyme of this type which is present in the elution from the ferric hydroxide adsorptions. This raises the question of whether the transformation of precursor to enterokinase is itself dependent on

the dipeptidase. Waldschmidt-Leitz and Harteneck [1925] inferred the transformation to be an enzymic process, and Haldane and Stern's "Allgemeine Chemie der Enzyme" [1932] contains the suggestion that traces of "trypsin" may be responsible, but it is pointed out that this view is speculative. Although the balance of evidence in the present investigation appears to be more in favour of dipeptidase than of "trypsin," it is insufficient to settle the question decisively.

The effect of glycine.

Experiments were also carried out to examine the effect of added glycine upon the transformation process, since the action of dipeptidase upon glycylglycine is inhibited by this amino-acid [Euler and Josephson, 1926]. As Table III shows, glycine has apparently no measurable effect upon the formation of enterokinase.

Table III. *Increases in acidity due to formation of enterokinase after 18 hours at 30°.*

cc. 0.1 N NaOH.		
Without glycine		With glycine
<i>Exp. a</i>	0.78	0.74
<i>Exp. b</i>	0.72	0.70

The experiments were carried out with neutralised elution from the alumina, buffered with phosphate buffer to p_H 6.9. To a sample of 10 cc. of the buffered mixture was added 1 cc. of *N* glycine solution, and to a second sample of 10 cc. was added 1 cc. of distilled water. The formation of enterokinase in each of these samples after 18 hours at 30° was measured in the usual way.

If dipeptidase is assumed to be the responsible agent, the most that may be inferred from the above result is that glycine is not a product of the reaction. It is therefore of little value in helping either to confirm or disprove the suggestion.

DISCUSSION.

Discussion of the mechanism of the change precursor $\rightarrow$ enterokinase is made especially difficult because at the present time the chemical nature of both precursor and enterokinase is unknown.

We do know, however, that enterokinase is thermolabile and has a critical increment for heat-inactivation of the same order as is observed with enzymes [Pace, 1931]. From this fact it seems reasonable to conclude that enterokinase possesses some form of colloidal support or carrier, which presumably therefore is also present in the precursor. If this supposition is correct it may be presumed that the transformation process itself is essentially some change occurring in the surface configuration of the carrier, for the magnitude of the critical increment of the process, 9500 calories, indicates that no very profound disruption is taking place. If an enzyme is responsible, it thus appears that the process probably involves the opening-up of relatively simple

surface linkages. But the experimental facts are insufficient to indicate the nature of the linkages involved, or the enzyme responsible. For although it has been shown that the process occurs in a solution containing dipeptidase alone (of the known pancreatic enzymes of the tryptic and ereptic type) the evidence available does not permit us definitely to attribute the change to this enzyme. Further experiments devised to test whether dipeptidase is responsible seem, however, to be a very suitable method of approach in any further investigation of this problem.

These results have been presented at a rather inconclusive stage of the investigation because circumstances prevent the writer from continuing work in this field.

SUMMARY.

1. The critical increment of the process precursor $\rightarrow$ enterokinase is found to be 9500 cals per mole.
2. The precursor is shown to be adsorbable by ferric hydroxide, as dipeptidase is known to be. The elution following a ferric hydroxide adsorption contains therefore precursor and dipeptidase together. Enterokinase is ultimately formed in this elution.
3. The presence of glycine does not affect the rate of formation of enterokinase.
4. It is tentatively suggested that of the known pancreatic enzymes of the tryptic and ereptic type dipeptidase appears to be the one to which the formation of enterokinase might conceivably be ascribed. The low value of the critical increment suggests that the colloidal carrier is not involved, the change being restricted to certain surface groupings.

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REFERENCES.

- Euler and Josephson (1926). *Z. physiol. Chem.* **157**, 122.
Haldane and Stern (1932). *Allgemeine Chemie der Enzyme*, p. 214. (Leipzig.)
Pace (1931). *Biochem. J.* **25**, 1.
— (1932). *Biochem. J.* **26**, 240.
Waldschmidt-Leitz (1931). *Z. angew. Chem.* **44**, 573.
— and Harteneck (1925). *Z. physiol. Chem.* **149**, 221.
— and Purr (1929). *Ber. deutsch. chem. Ges.* **62**, 2217.
Willstätter, Kraut and Fremery (1924). *Ber. deutsch. chem. Ges.* **57**, 1491.