LXXXI. THE FORMATION OF ENTEROKINASE FROM A PRECURSOR IN THE PANCREAS.

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HEIDENHAIN [1875] first observed that fresh extracts of the pancreas gland have no measurable tryptic activity, but that on keeping "spontaneous activation" occurs and the extracts develop a definite tryptic activity. Some years later Schepowalnikow [1899] also discovered that if intestinal juice, containing enterokinase, is added to fresh pancreatic juice, the mixture has tryptic activity, although each component of the mixture is inactive. From then onwards the two processes were generally studied independently of one another. The activation of pancreas extracts by direct addition of enterokinase was investigated in detail by Bayliss and Starling [1904] and by Mellanby and Woolley [1912]. Vernon [1901] worked mainly upon the "spontaneous activation" of pancreas extracts, although later [1914] he put forward a mechanism to explain the activation of pancreas extracts which applied both to the "spontaneous activation" and to that produced by direct addition of enterokinase. Waldschmidt-Leitz re-examined the whole question. He [1924] worked out the nature and mechanism of the activation of pancreas extracts by added enterokinase. Then, with Harteneck [1925], he investigated the problem of "spontaneous activation." They separated, from an extract which had undergone "spontaneous activation," the substance responsible for the activation. It was found that the properties of this substance were similar to those of enterokinase, and so it was concluded that "spontaneous activation" is a process identical with the activation of trypsin by added enterokinase. Further, Waldschmidt-Leitz and Harteneck by carrying out alumina adsorptions upon fresh pancreas extracts, were able to separate from the trypsin a fraction which apparently contained a precursor of enterokinase, since on keeping this fraction the activator was produced apart from the presence of trypsin. From these observations Waldschmidt-Leitz suggested that the enterokinase content of the intestinal mucous membrane depends upon the entry of pancreatic secretions in which the enterokinase is initially present in the form of an inactive pre-stage. This point of view was strengthened when Waldschmidt-Leitz and Waldschmidt-Graser [1927] showed that after removal

of the pancreas no appreciable amount of enterokinase appears in the intestinal mucous membrane even after several days.

The present paper records a number of experiments which have been carried out upon the formation of enterokinase from its pre-stage.

EXPERIMENTAL.

Isolation of the precursor.

This was carried out according to the method of Waldschmidt-Leitz and Harteneck, the actual procedure being as follows.

To 15 cc. of ice-cold extract (80 % glycerol) of fresh dried pancreas powder were added 15 cc. of ice-cold acetate buffer, containing 3.9 cc. *N* acetic acid, 10.5 cc. 0.2 *N* sodium acetate and 0.6 cc. distilled water. The buffered extract was then treated with 4 cc. of a suspension of alumina *C_γ* and the mixture left for 15 mins. in the refrigerator, when the alumina was centrifuged off. The clear liquid from the centrifuging was again treated in the same way with alumina, this process being carried out four times in all. The alumina from this series of adsorptions was then eluted with 25 cc. of 0.05 *N* ammonia. The precursor, together with peptidases, was contained in the elution which, after centrifuging off the alumina, was neutralised with *N* hydrochloric acid.

For the investigation of the development of enterokinase 5 cc. of a phosphate buffer were added to 20 cc. of neutralised elution, and the mixture contained in a glass-stoppered bottle was placed in a thermostat at 30°. A drop of toluene was always added to the mixture.

p_H measurements.

A sample of the buffered elution was used for the determination of its *p_H* value. This was determined at room temperature by means of the glass electrode. The circuit used was a modification of that employed by Harrison [1930] and involves the use of a special type of triode valve in conjunction with the glass electrode. The valve, which was supplied by Phillips Lamps Ltd., requires a filament current of 0.85 amp. and a plate voltage of 4–8 volts. A grid bias of at least – 1 volt is used. The glass electrodes when not in use were kept under distilled water, and immediately before use were immersed for a few minutes in boiling distilled water. It was found that this treatment helped to ensure a low value (usually less than 10 millivolts) for the “zero reading” of a particular electrode. This is in agreement with the experience of MacInnes and Belcher [1931].

The method employed for measuring the formation of enterokinase.

The principle of the method employed was to remove a sample of the elution at various time intervals and add it to a buffered solution of trypsin at 30°. Then, after allowing a period of time for activation of the trypsin by the added sample of elution, caseinogen and buffer solutions were added to the mixture. The increase in the number of free carboxyl groups, produced by

hydrolysis of the caseinogen by the activated enzyme in a given time, was then measured. The whole procedure is carried out according to the Linderstrøm-Lang and Steenberg [1929] modification of the method evolved by Willstätter *et al.* [1926] for the determination of trypsin, and the experimental details of its application to the determination of enterokinase have previously been given by the writer [1931].

In the present case, although the principle of the method remains the same as outlined above, it has been found advisable to modify certain details of the experimental practice. These modifications have been introduced because after a preliminary series of experiments it was realised that the activation effects to be measured are of very small magnitude, as revealed by the normal method of measurement. Thus it was with the object of increasing the analytical measurements of these activation effects that the procedure was modified. At the same time it was realised that the changes should not be such as to contravene the conditions which should be observed in a method for the determination of enzyme quantity.

(1) The period of activation, 30 mins. at 30°, is kept the same as before but the total volume of the mixture during activation is increased by 1 cc., *i.e.* from 9 cc. to 10 cc.

(2) The period of hydrolysis is increased from 20 mins. to 60 mins.

(3) Titrations are carried out on 15 cc. of reaction mixture instead of on 10 cc.

The composition of the mixture used is as follows:

$$\begin{array}{l} \text{During activation period} \left\{ \begin{array}{l} 2.5 \text{ cc. enzyme solution,} \\ 3.5 \text{ cc. buffer I,} \\ 4.0 \text{ cc. elution sample;} \end{array} \right. \\ \text{Then there are added ...} \left\{ \begin{array}{l} 6.0 \text{ cc. buffer II,} \\ 15.0 \text{ cc. caseinogen solution,} \end{array} \right. \end{array}$$

thus making at the start of the hydrolysis period a total volume of 31 cc.

It is also essential in every experiment to carry out a control run in which 4.0 cc. of distilled water are used instead of the 4.0 cc. of elution, the other constituents of the mixture remaining the same. This is necessary because in the longer time now employed, 60 mins., there is measurable hydrolysis by the enzyme + buffer of itself, in all probability due for the most part to the concentration of OH⁻ ion provided by the alkaline buffer.

During the course of the work enzyme solutions prepared in various ways have been used. The actual preparation used in a particular set of experiments is always stated.

Results of typical experiments in connection with the method of determination are given in Table I. In these experiments the enzyme solution used was the neutralised mother-liquor remaining over from the alumina adsorptions. It therefore contained the two components of trypsin—proteinase and carboxypolypeptidase.

Table I.

Samples for activating taken from an elution, buffered with phosphate buffer at p_H 6.98, and maintained for 32 hours at 30°. Blank and control readings have been subtracted so that the increases in acidity, recorded in terms of 0.1 N NaOH, are a measure of the hydrolysis produced by activated enzyme.

				Increase in acidity cc. 0.1 N NaOH
(1)	30 mins. activation,	20 mins. hydrolysis		0.26
(2)	30 mins. ,,	60 mins. ,,		0.80
(3)	60 mins. ,,	60 mins. ,,		0.84

The following conclusions, concerned with the method of determination, may be drawn from these experiments.

(1) With the small concentrations of activated enzyme we are dealing with, it is seen that we are in the region where the hydrolysis is a linear function of time. Thus the longer period of hydrolysis, 60 mins., may legitimately be used. For obviously we are not contravening the reason for which the short period is normally employed—namely to avoid the influence of the products of reaction upon the rate.

(2) The enterokinase formed in the elution resembles enterokinase as usually obtained from intestinal mucous membrane, in that 30 mins. at 30° is adequate time for it to produce activation at its maximum. This, of course, is in agreement with the result obtained by Waldschmidt-Leitz.

It may also be pointed out, from results previously given by the writer [1931] of the determination of enterokinase that for increases in acidity up to 1.0 cc. 0.1 N NaOH, there is direct proportionality between such increases and the “quantity” of enterokinase responsible for them.

The rate of formation of enterokinase from the precursor.

In the first place the results of a preliminary experiment upon rate of formation are given. In this experiment the enzyme solution used to measure formation of enterokinase was a glycerol extract of dried pancreas powder. The analytical readings are set out in full, in Table II, to give some idea of their magnitude, and to illustrate a point to be made later.

It will be observed from Table II that for the zero time reading the hydrolysis produced is practically identical with that observed in the control experiment. That is, there is practically no measurable quantity of enterokinase present in the elution initially.

Table III represents the results obtained with elutions prepared separately and at different times, but in each case buffered to about the neutrality point with phosphate buffer. As will be seen later this is apparently the p_H region in which formation of activator proceeds most favourably. In Table III blank and control readings have been subtracted and the increases in acidity actually due to addition of elution are given opposite corresponding times of withdrawal. All readings have been rounded off to the nearest 0.05 cc. of 0.1 N

Table II.

Elution buffered with phosphate buffer at p_H 7.2, and maintained at 30°. Glycerol extract of dried pancreas used as enzyme solution in the method of determination.

The readings are given in cc. of 0.1 <i>N</i> NaOH.			
Times of withdrawal of samples from buffered elution	0 hours	22 hours	46 hours
Analytical readings of total hydrolysis:			
(a) Titres at the end of hydrolysis period	(a) 21.29	(a) 22.16	(a) 22.08
(b) Blank titres (start of hydrolysis period)	(b) 20.78	(b) 20.80	(b) 20.82
$y = (a - b)$ = Total hydrolysis	(y) 0.51	(y) 1.36	(y) 1.26
Analytical readings of control (hydrolysis due to enzyme + buffer alone):			
(c) Titres at the end of hydrolysis period	(c) 20.77	(c) 20.80	(c) 20.81
(d) Blank titres (start of hydrolysis period)	(d) 20.30	(d) 20.33	(d) 20.35
$z = (c - d)$ = Hydrolysis due to (enzyme + buffer)	(z) 0.47	(z) 0.47	(z) 0.46
$x = (y - z)$ = Actual hydrolysis for which addition of elution is responsible	(x) 0.04	(x) 0.89	(x) 0.80

NaOH. For these experiments the enzyme solution (employed in the method of determination) was the neutralised mother-liquor from the alumina adsorptions.

Table III.

Temperature 30°

	Time of withdrawal of elution samples (hours)	Increase in acidity due to activation produced (cc. 0.1 <i>N</i> NaOH)
Elution A (p_H 7.04)	0	0.0
	10	0.50
	25	0.70
	47	0.70
	76	0.60
Elution B (p_H 6.95)	0	0.0
	7	0.45
	12	0.65
	24	0.85
	44	0.80
Elution C (p_H 6.98)	0	0.0
	10	0.45
	19	0.70
	43	0.75
	67	0.65

From Table III it is seen that, in this p_H region, maximum formation of the enterokinase is attained in about 35 hours at 30°. After this limiting value is attained there is a slow diminution, in all probability due to thermal inactivation of the enterokinase.

Although the small magnitude of the increases recorded in Table III makes it inadvisable to attach undue importance to fitting an equation to the observed results, yet the general behaviour requires some theoretical treatment. For the purposes of illustrating this treatment, an average curve from the results of Table III is given in Fig. 1.

We have two processes going on simultaneously:

- (1) formation of enterokinase from the pre-stage;
- (2) thermal inactivation of the enterokinase.

If we call the initial concentration of pre-stage a , and represent the amount of enterokinase produced therefrom, at any instant t , by x . Then we have:

$$\text{observed rate} = \frac{dx}{dt} = k(a - x) - k'x$$

where k = velocity constant of formation of enterokinase,

k' = velocity constant of thermal inactivation of enterokinase.

$$\therefore \frac{dx}{k \cdot a - (k + k')x} = dt.$$

On integration we have:

$$t = \frac{1}{k + k'} \log_e \frac{k \cdot a}{k \cdot a - (k + k')x}.$$

Fig. 1.

It will be observed that when k' is negligible compared with k the above equation reduces to that of the unimolecular expression:

$$t = \frac{1}{k} \log_e \frac{a}{a - x}.$$

Now from the original expression for the observed rate

$$\frac{dx}{dt} = k(a - x) - k'x$$

it is seen that when $(a - x) = 0$, the rate is given by $-k'x$. So that if we assume that the diminution from the limiting value at 35 hours is due to thermal destruction of the enterokinase, we can calculate from this rate of diminution a value for k' . Taking, from the curve, values of 0.80 and 0.68 as representing the quantity of enterokinase at 35 hours and at 70 hours respectively, then with t in mins. the value of k' works out to be 7.9×10^{-5} . We may now check our assumption, since we can also calculate a value for k' from previous data of the writer [1931] upon the thermal inactivation of enterokinase. From those data we have

- (1) between p_H 6.0 and p_H 7.0 the average $k_{\text{inactivation}}$ at 50° is 4.42×10^{-3} , when t is in mins.;
- (2) the observed critical increment, E , for the inactivation reaction is about 40,000 calories.

$\therefore$ substituting in the equation

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

we have

$$\log_{10} \frac{4.42 \times 10^{-3}}{k'} = \frac{40,000 \times 20}{2.303 \times 1.99 \times 323 \times 303}$$

whence $k' = 7.3 \times 10^{-5}$.

This value shows agreement with that calculated on the basis given previously. Therefore we may now examine how a value for k' of this order affects the observed rate of formation. This may be done in the following way. We may calculate the percentage diminution occurring in 15 hours from a value for k' of this order. Thus we have, putting t in mins.,

$$7.6 \times 10^{-5} = \frac{2.303}{900} \log_{10} \frac{a}{(a-x)},$$

whence $\left(\frac{a}{a-x}\right) = 1.07$. If we put $a = 1$, then it is seen that $(a-x) = 0.94$. That is the percentage diminution in 15 hours is about 6 %. It will be seen from Fig. 1 that after 15 hours the quantity of enterokinase present is represented by 0.64 cc. An error of 6 % in 0.64 cc. means a change of 0.04 cc. Over the period from 15 hours to 30 hours the increase in quantity of enterokinase present is $(0.80 - 0.64) = 0.16$. The correction on this value is 0.01 cc. Corrections of this order are no greater than the experimental error. This means then, in effect, that we may neglect k' in comparison with k , and that the limiting quantity of enterokinase formed (AB in Fig. 1) may be taken without serious error to represent a , the initial concentration of precursor. So that if, as we assumed originally, the true rate of formation of the enterokinase is a function of "active mass" of precursor alone, then, since we may neglect k' , the observed rate should agree with that predicted by the unimolecular expression.

This comparison has been made by taking as the value for a the maximum quantity of enterokinase formed (AB in Fig. 1) and taking one value of x from the curve (distance CD). From these k is worked out and found to be, t in mins., 1.57×10^{-3} . This value of k is then used to calculate x for different time intervals, and these values are then compared with those observed. This is shown in Table IV, where calculated values for a bimolecular constant $k_{bi} = \frac{1}{t} \cdot \frac{x}{a(a-x)}$ are also given.

Table IV.

Time (hours)	Increases in acidity		
	Calculated from k_{uni}	Observed	Calculated from k_{bi}
7	0.39	0.38	0.36
10	0.49	0.50	0.44
15	0.61	0.62	0.51
20	0.68	0.72	0.56
25	0.73	0.76	0.60

From Table IV it is seen that the rate of formation is apparently best represented by the unimolecular expression.

The effect of the p_H of the elution upon the formation of enterokinase.

The effect of p_H was studied in the following way. A volume of 60 cc. of neutralised elution was prepared from 60 cc. glycerol extract in the manner already described. Then 2 cc. of various buffer solutions were measured out into

each of a number of glass bottles. To each of these a volume of 8 cc. of the neutralised elution was then added, making a total volume of 10 cc. in each bottle. In every case a sample of the mixture was withdrawn for the determination of the "zero reading," and a sample for p_H determination. The bottles were then stoppered and kept in a thermostat at 30° for 16 hours. At the end of this period the quantity of activator formed in each case was measured in the way already described. Results obtained in this way are given in Table V.

Table V.

Temperature 30°. Time 16 hours.			Increase in acidity cc. 0.1 <i>N</i> NaOH
	Buffer used	p_H	
Elution X.	Acetate	5.0	0.45
	Phosphate	5.95	0.74
	"	6.57	0.85
	"	6.95	0.89
	"	7.20	0.68
	Ammonia-ammonium chloride	7.90	0.43
Elution Y.	Phosphate	5.60	0.41
	"	6.35	0.70
	"	6.92	0.76
	"	7.20	0.70
	"	7.40	0.64
	Ammonia-ammonium chloride	8.00	0.35

Blank and control readings have been subtracted. The enzyme solution used (in the method of determination) was new neutralised mother-liquor from alumina adsorptions. With the phosphate buffers the total phosphate in the mixture in each case was 0.016 *M*.

When the results given in Table V are plotted it is found that the same shape of curve is given by both elutions. The optimum region for formation of the enterokinase appears to be about p_H 6.5–7.0. The rate drops off quite sharply on the alkaline side, less so on the acid side. It now remains to examine whether the p_H effect observed is truly an effect upon formation or whether the observed effects can be explained on the grounds of increased (thermal) inactivation away from the neutrality point. This may be done by again referring to data upon the inactivation of enterokinase. We have already shown that the inactivation effect may be neglected around neutrality, and as the following values (taken from the writer's previous data) show, there is very little increase in the rate of inactivation from neutrality to p_H 5 on the one side, and to p_H 8 on the other. It is beyond these limits that the thermal inactivation becomes very markedly increased. The values now given refer to a temperature of 55°.

p_H	$k_{\text{inactivation}} \times 10^2$
5.12	1.30
6.40	1.15
7.83	1.30

It is therefore seen, by comparison of these data with those of Table V, that over approximately the same p_H range the rate of inactivation of

enterokinase only increases by about 10 % from the optimum, while the rate of formation of enterokinase decreases by about 50 % from the optimum.

Thus from these figures it may be concluded that the p_H effect observed arises in fact from the influence of p_H upon the rate of formation of the enterokinase.

Further experimental work is in progress, partly with the object of investigating whether the peptidases, present in the elution with the precursor, are concerned in the formation of the enterokinase therefrom.

SUMMARY.

(1) The separation, from pancreas extracts, of a pre-stage of enterokinase (as recorded by Waldschmidt-Leitz and Harteneck) has been confirmed.

(2) Experimental details of a modified method for measuring the formation of enterokinase from this pre-stage are given.

(3) It is found that the enterokinase produced in this way resembles enterokinase as it is usually obtained from the intestinal mucous membrane in that 30 mins. at 30° is adequate time for it to produce maximum activation of trypsin. This is in agreement with the experience of Waldschmidt-Leitz and Harteneck.

(4) It is found that the enterokinase produced is able to activate the trypsin in both purified and unpurified extracts.

(5) The rate of formation of the enterokinase at 30° has been measured, and some theoretical considerations upon the rate of formation are given. In the course of these it is shown that the rate of formation may be expected to agree with the unimolecular expression. The observed rate is compared with that predicted (*a*) by the unimolecular expression and (*b*) by the bimolecular expression. It is found that the observed rate agrees better with (*a*) than with (*b*).

(6) The effect of p_H upon the rate of formation has been observed. It is found that the optimum p_H of formation is about p_H 6.5–7.0. It is also shown that this observed effect is not due to increased thermal inactivation at points away from this region, but represents essentially the effect of p_H upon the formation of enterokinase.

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