I. THE EFFECT OF HEAT UPON THE ACTIVATING EFFICIENCY OF ENTEROKINASE.

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THE effect of heat upon the properties of enterokinase has not been studied in very great detail, and the work that has been done is not of a quantitative nature. Mellanby and Woolley [1913] stated that enterokinase is destroyed in 5 mins. at 65° , and is immediately inactivated in the presence of free acid. In more recent work Waldschmidt-Leitz [1924, 1925] has shown that enterokinase solutions are inactivated when heated above 50° and that they are most stable about the neutrality point. The work described in this paper deals with a quantitative investigation of the effect of heat upon enterokinase. This has been effected in principle by maintaining an enterokinase solution at a temperature of 55° , removing samples at various time intervals, and then measuring the enterokinase content of a definite volume of each sample. The procedure involves two consecutive stages.

(1) Addition of a definite volume of the solution to a constant volume of buffered trypsin solution, leaving the mixed solutions at 30° for 30 mins.¹, when formation of the compound trypsin-kinase takes place.

(2) Addition of caseinogen and buffer solution to the trypsin-kinase at the end of the above period, and then measurement of the amount of hydrolysis of the caseinogen produced in 20 mins., reckoned from the time of addition of caseinogen to the trypsin-kinase.

Before carrying out the above steps however it is necessary to define an arbitrary enterokinase unit.

The determination of enterokinase.

For the study of the effect of heat upon enterokinase it is necessary to have a method for the measurement of "quantity" of enterokinase. This measurement is arrived at by determining the activating capacity of a sample of enterokinase with respect to a known and constant quantity of trypsin. In this way both Waldschmidt-Leitz [1925] and Linderstrøm-Lang and Steenberg [1929] have defined arbitrary enterokinase units. In their determinations

¹ The diminution in the enterokinase content during this period of "activation of the trypsin" is negligible.

however the quantity of enterokinase was defined from measurements of its activating capacity with respect to dried pancreas powder or to a glycerol extract of the powder. In the present work, to avoid any possible complications from the presence of the activator in the dried gland powder, the enterokinase "quantity" has been measured by determining its activating capacity with respect to trypsin which has been purified so as to be free from enterokinase and the enterokinase pre-stage, according to the method of Waldschmidt-Leitz and Linderstrøm-Lang [1927]. Except for this modification the technique adopted for the determination of an enterokinase unit is essentially that of Linderstrøm-Lang and Steenberg [1929].

Preparations.

(a) *Enterokinase solution.* Pig's intestinal mucous membrane was treated with acetone, acetone-ether mixture and finally ether according to the procedure of Waldschmidt-Leitz [1924]. 6 g. of the dried mucosa prepared in this way were dispersed through 200 cc. of distilled water by shaking, and the dispersion was left for 24 hours in the ice chest, to allow the active material to pass into pseudo-solution. The suspension was then centrifuged and filtered. To 100 cc. of the clear filtrate were added 1.6 cc. of *N* acetic acid, and the resulting precipitate of protein was filtered off. The clear supernatant liquid containing the enterokinase was neutralised with *N* ammonia and kept in the ice chest. About 300 cc. of solution were prepared in this way and were used throughout the work described in this paper by diluting a volume of 10 cc. with 3 cc. of distilled water.

(b) *Trypsin solution.* Glycerol extract of dried pancreas powder was purified according to the method of Waldschmidt-Leitz and Linderstrøm-Lang [1927], so as to be free from enterokinase and the pre-stage of that activator. The details of this purification are given in a previous paper by the present writer [1930].

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

(d) *Buffer solutions.* Buffer (1) was prepared by mixing 300 cc. of *N* ammonium chloride and 50 cc. of *N* ammonia, giving a p_H of 8.3 at 30°.

Buffer (2) was prepared by mixing 250 cc. of *N* ammonia and 350 cc. of distilled water.

Procedure.

The determination of an enterokinase unit according to the method of Linderstrøm-Lang and Steenberg [1929] was then carried out as follows. 3.5 cc. of buffer (1) and 3 cc. of trypsin solution were measured into a 50 cc. Jena flask. There was then added a volume of distilled water such that after the addition of the required amount of enterokinase solution the total volume

was 9 cc. The flask with its contents was placed in the thermostat at 30° and the enterokinase solution was added at a time which was reckoned as the zero for the period of activation. (5 cc. of water, 65 cc. of 96 % alcohol and 1 cc. of a 1 % solution of thymolphthalein in alcohol were measured out into each of two 300 cc. flasks to be used for the titration of the mixture under investigation before and after the hydrolysis of the caseinogen.)

After the activation period of 30 mins., 6 cc. of buffer (2) (warmed to 30°) and 15 cc. of a 6 % caseinogen solution (also previously warmed to 30°) were added. The whole was shaken and, at a time reckoned as the zero for the period of hydrolysis, 10 cc. of the mixture were pipetted into one of the titration flasks containing alcohol. (The alcohol entirely stops the enzyme action without perceptibly precipitating the caseinogen, thus making it possible to keep the mixture for later titration.) At the end of the reaction period of 20 mins. the same 10 cc. pipette was rinsed with the liquid under investigation, and 10 cc. were pipetted into the second titration flask containing alcohol. Each titration flask contained one-third of the amounts used in the experiment. The contents of each flask were then titrated in the stepwise manner described by Willstätter *et al.* [1926]. The difference between the two titration values is equal to the number of carboxyl groups formed during the hydrolytic period of 20 mins. in 10 cc. of the experimental liquid, expressed as cc. of 0.2 *N* NaOH.

By using various quantities of kinase solution and making measurements in this way a relation was obtained between the quantity of enterokinase and the extent of the hydrolysis of the caseinogen. This is shown in Table I.

Table I.

Volume of enterokinase solution cc.	Increase in acidity cc. 0.2 <i>N</i> NaOH
0.05	0.53
0.10	0.80
0.20	1.04
0.30	1.12
0.50	1.19

An enterokinase unit was defined as the quantity of enterokinase which produces an increase in acidity equivalent to 1.04 cc. of 0.2 *N* NaOH under the conditions given above. By plotting the results of Table I and using this definition of an enterokinase unit the following values, set down in Table II, were obtained for fractions and multiples of the unit.

Table II.

Enterokinase units	Corresponding increase in acidity cc. 0.2 <i>N</i> NaOH
0.25	0.52
0.50	0.80
1.00	1.04
1.50	1.12
2.00	1.18
2.50	1.19

These results are plotted in Fig. 1, and the curve thus obtained is used for determining "quantity" of enterokinase.

Fig. 1.

The velocity of inactivation of enterokinase by heat.

Having now established a unit of enterokinase activity, we can proceed to measure the rate of thermal inactivation.

To 10 cc. of the enterokinase solution, prepared in the manner described above, were added *N* acetic acid or *N* ammonia to bring it to a required p_H , followed by distilled water to make the total volume 11 cc. This volume of solution was measured into a number of test-tubes already immersed in a thermostat at 55°. The test-tubes were then tightly stoppered and transferred at various time intervals from the thermostat into crushed ice. The enterokinase content of 0.2 cc.¹ of solution was then measured in the manner described above. The p_H measurements throughout the work described in this paper were made at room temperature by means of the glass electrode.

In the following tables the enterokinase content is expressed in terms of the enterokinase unit defined previously and is set down as enterokinase units per cc. of solution. The readings for each time interval were made in duplicate and the enterokinase content recorded for a particular time interval is the mean of the two readings.

The course of the reaction. There is no reference in the literature to the course of the reaction when enterokinase is inactivated by heat. It was found in this work that the effect of heat upon the activating efficiency of enterokinase could be expressed fairly well by the unimolecular expression:

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

where a is the initial concentration of enterokinase per cc., $a - x$ is the concentration of enterokinase after time t mins.

¹ This volume was chosen so as to bring the readings on the "steep" part of the curve. If too large a volume is chosen we are in the region of maximum activation and consequently there may be no apparent decrease on heating.

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The agreement with the unimolecular expression is illustrated in Table III, which gives some idea of the degree of constancy obtained in average experiments. In some of the experiments there was a tendency for the constants, worked out on the basis of the unimolecular law, to fall. It could not be decided whether this was a real effect or whether it was due solely to experimental error. It should be pointed out in this connection, however, that the experimental observations have not been confined to early stages of the reaction, but that they have covered about 75 % of its course.

Table III.

Temperature 55°.

	Time (mins.)	Enterokinase units per cc.	k^*
p_H 8.47	0	6.00	—
	20	3.56	2.61×10^{-2}
	40	2.12	2.59×10^{-2}
	60	1.37	2.46×10^{-2}
p_H 6.40	0	6.00	—
	45	3.50	1.20×10^{-2}
	95	2.00	1.16×10^{-2}
	143	1.25	1.10×10^{-2}

* In this table and throughout k represents the velocity constant of a unimolecular reaction.

The effect of p_H upon the inactivation of enterokinase by heat.

The p_H of the medium has a marked effect upon the stability of enzymes, and the following results, which show that the stability of enterokinase is also dependent upon p_H , emphasise how closely this activator resembles an enzyme in certain of its properties. In Table IV the results obtained for the inactivation of enterokinase by heat at various p_H values are summarised. The mean of the values obtained for k at a particular p_H are set down opposite that p_H value.

Table IV.

Temperature 55°.

p_H	$k \times 10^2$
4.00	3.29
4.56	2.67
5.12	1.30
6.40	1.15
7.83	1.30
8.47	2.55
9.01	6.06

The results of Table IV are presented graphically in Fig. 2, where it is seen that the optimum stability region for enterokinase is fairly broad and lies between p_H 6 and p_H 7.

The critical increment of the process of the inactivation of enterokinase by heat.

Experiments were carried out to determine the critical increment of the heat-inactivation of enterokinase by measuring k at 50° and at 60° for the

following three cases: (1) at the region of optimum stability, *i.e.* at p_H 6.5, (2) at p_H 5.12, (3) at the still more acid reaction of p_H 3.2.

Fig. 2.

Table V.

p_H	k at 50°	k at 60°	E (cals.)
6.50	4.42×10^{-3}	3.27×10^{-2}	42,600
5.12	4.95×10^{-3}	3.52×10^{-2}	41,700
3.20	1.06×10^{-2}	8.57×10^{-2}	44,000

The results are summarised in Table V 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:

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

Thus it is seen that the critical increment for the heat inactivation of enterokinase is the same over the p_H range 3–7 and is of the order of 40,000 cal. per “molar unit” of enterokinase. This value of the critical increment is substantially the same as that obtained by the writer [1930] for the heat-inactivation of trypsin itself.

It is interesting that the results obtained with enterokinase, a specific activator and not an enzyme *per se*, show such a close resemblance to those obtained with the enzyme trypsin.

SUMMARY.

Experiments have been carried out on the determination of an enterokinase unit. The method adopted was that of Linderstrøm-Lang and Steenberg, except that trypsin purified so as to be free from enterokinase and its pre-stage was used instead of a glycerol extract of the dried pancreas gland.

In this way an enterokinase unit has been defined and employed in the work described later.

The effect of heat upon enterokinase has been studied. It was found that the course of the heat-inactivation may be described fairly well by the uni-molecular expression. In this respect enterokinase resembles purified trypsin.

The effect of p_H upon the stability of enterokinase has also been investigated. It was found that the stability is influenced considerably by the p_H of the medium, but there is a fairly broad optimum region of stability lying between p_H 6 and p_H 7.

The critical increment of the process has been determined at three p_H values: 6.5, 5.12 and 3.2. It was found that the critical increment is the same at all three points and is of the order of 40,000 cal. In this respect again enterokinase exhibits a marked resemblance to purified trypsin.

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REFERENCES.

- Linderstrøm-Lang and Steenberg (1929). *Compt. Rend. Trav. Lab. Carlsberg*, **17**, No. 16.
Mellanby and Woolley (1913). *J. Physiol.* **47**, 339.
Pace (1930). *Biochem. J.* **24**, 606.
Waldschmidt-Leitz (1924). *Z. physiol. Chem.* **132**, 181.
—— (1925). *Z. physiol. Chem.* **142**, 217.
—— and Linderstrøm-Lang (1927). *Z. physiol. Chem.* **166**, 241.
Willstätter, Waldschmidt-Leitz, Dunaifurria and Kunstner (1926). *Z. physiol. Chem.* **161**, 191.