CLXII. THE HEAT-INACTIVATION OF THE PROTEINASE OF THE PANCREAS.

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INTRODUCTION.

WHILE previous work of the writer, upon the heat inactivation of trypsin, was in progress, it was shown by Waldschmidt-Leitz and Purr [1929] that trypsin itself is not an individual enzyme, but that it may be resolved into two components—a proteinase and a carboxypolypeptidase.

It was found by these authors that the proteinase, although completely inactive alone, is capable, when associated with enterokinase, of hydrolysing proteins and certain of their degradation products. The carboxypolypeptidase, in the absence of enterokinase, attacks a number of polypeptides and some dipeptides, which possess a free carboxyl group in the molecule, but will not attack a protein. Enterokinase increases the activity of carboxypolypeptidase. This work of Waldschmidt-Leitz and Purr has a distinct bearing upon the interrelation of trypsin and enterokinase. It is of interest to recall, very briefly, the views which have been held about this problem.

(a) *The "classical" view.* It was considered that an inactive zymogen, trypsinogen is produced in the pancreas. The trypsinogen is enzymically transformed into trypsin by enterokinase, and the trypsin thus produced is able to hydrolyse proteins. This may be expressed thus:

(b) *The view of the Willstätter school up to 1930.* According to this view the pancreas produces an enzyme, trypsin, which, although able to hydrolyse certain of the products of protein degradation, is inactive towards proteins themselves. Trypsin forms an addition compound with enterokinase, namely, trypsin-kinase, which readily hydrolyses proteins:

(c) *The view arising from the work of Waldschmidt-Leitz and Purr.* This may be summarised thus:

Thus proteinase is analogous to the classical trypsinogen.

In the earlier work of the writer [1930] the investigation of the heat-inactivation of trypsin was carried out with solutions containing 24 % glycerol, this circumstance arising from the technique used in preparing the trypsin solutions. Further experiments were planned to study the effect of varying the glycerol concentration so that, by extrapolation, a value of the critical increment of the process in solutions containing no glycerol could be obtained, since such a value would obviously be of importance from the point of view of the behaviour of purely aqueous systems. From the work of Waldschmidt-Leitz and Purr it was apparent, however, that it would be more satisfactory to examine not "trypsin" itself but one of its components, namely proteinase, this component being chosen because it is more closely analogous to the classical trypsinogen (the carboxypolypeptidase component being more analogous to erepsin).

In the method evolved by Waldschmidt-Leitz and Purr for the isolation of the proteinase, we are left, after numerous adsorptions, with this enzyme in solution. Clearly any adjustment of the glycerol concentration would involve still further dilution, with a resulting diminution in concentration of enzyme. In view of this it was evident that as an important preliminary step an attempt should be made to carry the Waldschmidt-Leitz and Purr treatment further by adsorbing proteinase on a suitable adsorbent, say kaolin. The proteinase could then be eluted from the kaolin, always by the same volume of liquid, but the percentage of glycerol in that volume could be previously adjusted. In this way for example the enzyme could be eluted from the kaolin by an aqueous solution, containing no glycerol, and thus the critical increment of the heat inactivation process in water could be determined directly instead of by extrapolation.

EXPERIMENTAL.

(a) *The preparation of alumina C_γ.* The alumina used in the isolation of the proteinase was a sample of alumina C prepared according to the technique of Willstätter and Kraut [1923] about 12 months previously and since then kept under distilled water. It was therefore completely transformed into the C_γ modification.

(b) *Isolation of the proteinase.* This was carried out in principle according

to the method of Waldschmidt-Leitz and Purr, the actual procedure adopted being as follows. 100 cc. of ice-cold glycerol extract of dried pancreas were brought to p_H 4.0 by addition of 75 cc. of ice-cold buffer solution. (The buffer solution was made up by mixing 17.6 cc. *N* acetic acid and 2.04 cc. *N* sodium acetate and diluting to 100 cc.). 5 cc. of alumina C_γ suspension (containing 105 mg. Al_2O_3) were then added to the buffered enzyme extract. The mixture was shaken, allowed to stand in the ice-chest for about 10 minutes and then centrifuged. The clear supernatant liquid was then treated in exactly the same way with a second 5 cc. of alumina C_γ suspension. This operation, which was repeated four times in all, removes the dipeptidase and aminopolypeptidase. The clear solution from the final adsorption was then brought to p_H 7.0 by addition of *N* ammonia. To the solution, now at p_H 7.0, were added 5 cc. of alumina C_γ suspension. The mixture was shaken, allowed to stand in the ice-chest for about 10 minutes and then centrifuged. In all ten such adsorptions at p_H 7.0 were carried out upon the solution. This procedure removes the carboxypolypeptidase, and the solution from the final adsorption contained the proteinase.

This solution, which was used in the later experiments with kaolin, was kept in the ice-chest. Prior to treatment with kaolin, the proteinase content of the solution was determined by activating with enterokinase and measuring the resulting activity according to the method of Linderstrøm-Lang and Steenberg [1929]. It was found that the solution contained 0.95 enzyme unit per cc.

(c) *Adsorption of the proteinase upon kaolin.* Experiments were next carried out to determine whether the proteinase is adsorbed by kaolin, since in the experiments of Willstätter *et al.* [1926] it was found that "trypsin," under certain conditions, is adsorbed by kaolin.

To 30 cc. of the ice-cold proteinase solution at p_H 7.0 were added 5 cc. of a suspension of kaolin in distilled water, which contained 0.4 g. of B.D.H. kaolin *puriss.* The mixture was shaken and left for 10 minutes in the ice-chest, after which it was centrifuged. The clear centrifugate was treated again with 5 cc. of kaolin suspension and the above procedure repeated. This operation was carried out four times in all.

The next process was to attempt the elution of the proteinase from the kaolin and thus examine the degree of success obtained with the adsorption process. Accordingly the kaolin from the adsorption experiments was shaken up with 30 cc. of ice-cold solution E. (Solution E was made up of 57 parts by volume of 1 % diammonium phosphate solution, 3 parts by volume of *N* ammonia, and 40 parts by volume of glycerol-water solution—this solution having already been employed by Willstätter *et al.* [1926] in connection with trypsin.) The suspension of kaolin in solution E was kept in the ice-chest for 15 minutes and was then centrifuged. The clear liquid from the centrifuging was then neutralised with *N* acetic acid and kept in the ice-chest. The proteinase content of this solution was then examined, and it was found to be

0.78 enzyme unit per cc. That is, the process of adsorption on kaolin together with the subsequent elution may be considered quite satisfactory.

The heat-inactivation of the proteinase.

For the experiments in this section the starting material was the stock solution of proteinase remaining after the alumina adsorptions. Immediately before a run 30 cc. of this solution were treated with kaolin and the proteinase then eluted from the kaolin as described above. By varying the glycerol concentration in the solution employed for the elution, it was possible to examine the heat-inactivation of the proteinase in solutions differing in glycerol content, but identical in electrolyte concentration.

In the first place it was necessary to examine whether the heat-inactivation of the proteinase resembled that of "trypsin" in respect of the applicability of the unimolecular expression. The p_H measurements in this work were made by means of the glass electrode at room temperature. By way of illustration Table I gives the data obtained in aqueous solution.

Table I.

Temperature 50°. Glycerol 0 %. p_H 6.8.		
Time (mins.)	Enzyme units per cc.	$k \times 10^3$
0	0.78	—
45	0.57	7.0
90	0.42	6.9
135	0.30	7.1

It is seen that there is agreement with the unimolecular expression. With solutions containing glycerol agreement with the unimolecular expression was also found.

The critical increment of the process at different glycerol concentrations.

The critical increment of the process was determined by measuring the value of k at 50° and at 60° and then substituting into the integrated form of the equation:

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

The experiments were carried out with solutions containing 40, 20 and 0 % glycerol respectively. It was not feasible to work with a glycerol concentration greater than 40 %, because of the extreme difficulty in centrifuging the kaolin in solutions of higher glycerol content.

The results obtained are given in Table II.

Thus it is seen that the magnitude of the critical increment is sensibly independent of the glycerol concentration, lying between 35,000 and 40,000 calories per "molar unit" of enzyme.

Table II.

		Time (mins.)	Enzyme units per cc.	<i>k</i>	<i>E</i> (calories)
Glycerol 0 % <i>p_H</i> 6.9	Temp. 50°	0	0.80	7.6×10^{-3}	39,000
		70	0.47		
	Temp. 60°	0	0.80	4.7×10^{-2}	
		10	0.50		
Glycerol 20 % <i>p_H</i> 6.8	Temp. 50°	0	0.73	3.6×10^{-3}	40,000
		90	0.53		
	Temp. 60°	0	0.72	2.3×10^{-2}	
		15	0.51		
Glycerol 40 % <i>p_H</i> 6.9	Temp. 50°	0	0.76	2.1×10^{-3}	35,000
		110	0.60		
	Temp. 60°	0	0.78	1.1×10^{-2}	
		15	0.66		

DISCUSSION.

From the results given above we may make comparisons, especially in relation to the results of other work carried out in this laboratory, which may be tabulated as follows.

1. The magnitude of the critical increment for heat-inactivation of the proteinase is practically identical with that found for trypsin-kinase, enterokinase and "trypsin." Thus the separation of the carboxypolypeptidase has not influenced quantitatively the thermal stability of the proteinase.

2. The critical increment, within the limits of experimental error, is independent of the glycerol concentration of the solution, and we may conclude by inference that this holds true for "trypsin."

3. The fact that the critical increment does not vary with the glycerol concentration is in agreement with the similar result found by McGillivray [1930] for purified lipase.

4. The magnitude of the critical increment approximates to that found by McGillivray for purified lipase, namely 46,000 calories.

Probably the most striking of these facts is the resemblance between the behaviour of proteinase, trypsin-kinase, enterokinase and that of lipase. Such a resemblance between enzymes differing so markedly in specificity as do lipase and proteinase indicates that, although heat-inactivation results in destruction of their specific active groupings, the primary effect must be upon the supporting "core" of each enzyme. Further, it seems reasonable to conclude that the cores of each enzyme must be very closely similar, either in chemical structure or in the physical state of their aggregation. Their difference in specificity may be governed by the spatial distribution of active groups on the surface of the core.

In general it will be observed that the values obtained by the writer and by McGillivray for the critical increment are less than those which have been found by previous workers, who have for the most part employed cruder enzyme preparations. McGillivray showed that the purification of lipase is accompanied by a drop in the value of the critical increment of heat-inactivation from 95,000 calories to 46,000 calories. Yet it might still be considered

that the magnitude, *circa* 40,000 calories, found for the proteinase and lipase is still influenced by the presence of adventitious material associated with the true core of the enzyme. It is clear, however, by considering the case of the proteinase, that such material is so firmly attached to the enzyme that it defies separation therefrom by methods of adsorption which have been successfully applied to separate enzymes which must be regarded as very analogous in their adsorption affinities. Thus such material, if present, may very well be regarded as an integral part of the enzyme core.

SUMMARY.

1. Experiments have been carried out upon the adsorption by means of kaolin of proteinase obtained in solution by the method of Waldschmidt-Leitz and Purr [1929]. It is found that the proteinase is removed from solution by the kaolin and may be successfully eluted therefrom. This additional procedure has been incorporated with the technique of Waldschmidt-Leitz and Purr to prepare solutions of the proteinase.

2. It is found that the heat-inactivation of the proteinase proceeds in agreement with the unimolecular expression.

3. The critical increment of heat-inactivation has been determined in solutions containing 40, 20 and 0 % glycerol. It is found that the critical increment is independent of the glycerol concentration, and is of the value of 35,000–40,000 calories per molar unit of enzyme.

4. Resemblances between the heat-inactivation of proteinase, "trypsin," enterokinase and purified lipase are discussed. It is suggested that in general heat-inactivation is primarily an effect upon the enzyme core. It is pointed out in addition that it seems reasonable to conclude that different enzymes must possess cores which are closely similar, either in chemical structure or in the physical state of their aggregation.

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