XCIX. THE INACTIVATION OF PANCREATIC LIPASE BY HEAT.

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Very little is known about the heat-inactivation of pancreatic lipase, and the work that has been done is rather qualitative than quantitative. Terroine [1910] observed that pancreatic secretion was very sensitive to heat and rapidly lost its lipolytic activity at 45°. At 65° the lipolytic activity was completely lost in 10 minutes. According to the same author bile salts accelerated this process. Terroine also investigated the effect of activating the trypsin in the secretion with enterokinase, and observed that the lipase was then more rapidly destroyed. The addition of coagulated egg-albumin decreased the action of the trypsin-kinase on the lipase. Mellanby and Woolley [1914] have observed that pancreatic secretion, of alkalinity 0·12 *N* sodium carbonate, lost its lipolytic activity at the rate of 10 % per hour at 40°. At 50° the rate was 50 % per hour and at 60° the whole of the lipolytic activity was lost within 5 minutes. The material was found to lose its activity at a slightly slower rate when the solution was neutral. When the solution was made 0·02 *N* with respect to HCl it was found to be destroyed very rapidly. These authors also observed that the rate of inactivation was increased when enterokinase was added, and that serum-albumin and egg-albumin protected the lipase from destruction. Lombroso [1914] observed that in the presence of such substances as glycerol, glucose, sucrose and maltose (compounds which are heavily hydrated in solution) pancreatic lipase was much more stable to heat. Willstätter and Waldschmidt-Leitz [1923] employed glycerol as the most efficient stabiliser of pancreatic lipase in their experiments on the purification of this enzyme. The work described in this paper deals with a quantitative investigation of the heat-inactivation of pancreatic lipase.

The estimation of pancreatic lipase.

It is necessary in the first place to have some reliable method for the estimation of the lipase. The materials employed in the estimation were prepared as follows.

(*a*) *Glycerol extract of lipase.* Dried gland powder was prepared by drying pig's pancreas with acetone and ether according to the procedure of Willstätter

and Waldschmidt-Leitz [1923]. 6 g. of this dried gland powder were added to 100 cc. glycerol-water mixture (containing 80 % glycerol), the mixture then being kept with occasional shaking at 30° for 4 hours. After centrifuging, the supernatant liquid was clarified by filtration through two layers of filter paper. The clear liquid thus obtained is referred to as a "glycerol extract of lipase."

(*b*) *Neutral olive oil.* The method employed was a modification of that due to Corran and Lewis [1928]. Commercial olive oil (Boots's finest Lucca olive oil) was shaken with an equal volume of 1 % sodium hydroxide solution in a separating funnel at about 75°. After standing, the aqueous layer was separated, and the emulsion of oil remaining washed with hot distilled water until all soap was removed. The oil was then dried by heating on a water-bath at 2 mm. pressure. The same stock commercial olive oil was employed throughout, and the saponification number 192·5 was found to be unaltered in different preparations[1].

(*c*) *Egg-albumin solution.* 2 g. of B.D.H. egg-albumin powder were dissolved in excess ammonia, the ammonia then being evaporated by impinging a stream of warm air (30°) on the surface of the liquid. The solution was left overnight in a vacuum desiccator containing sulphuric acid and then made up to 100 cc. It is referred to as 2 % albumin solution. The p_H of such a solution was found to be approximately 7·6.

(*d*) *Calcium chloride solution.* A solution of calcium chloride was made from pure fused calcium chloride free from calcium hydroxide. The solution was adjusted to contain 1 % calcium chloride by estimating the Cl ion concentration and diluting the solution accordingly.

(*e*) *Sodium chloride solution.* A 0·1 *N* solution of sodium chloride was prepared from B.D.H. sodium chloride which had been purified by precipitation from a saturated solution with HCl gas and dried at 105° for 3 hours.

(*f*) *Buffer solution.* The buffer described by Willstätter, Waldschmidt-Leitz and Memmen [1923] was made up by mixing 66 parts of *N* ammonia and 134 parts of *N* ammonium chloride. The p_H of this solution was measured with the glass electrode and found to be 8·9 at 30°.

Willstätter, Waldschmidt-Leitz and Memmen [1923] have observed that calcium chloride and egg-albumin are excellent activators of pancreatic lipase in an alkaline medium and employ these substances in their method of estimation.

The writer has verified the excellent activations of pancreatic lipase obtained with calcium chloride and egg-albumin, and numerous experiments on the combined action of the two activators have been carried out. The combined effect of the two was found to be slightly greater than the sum of the two separate effects, again confirming the results of the above authors. The experiments were carried out as described by these investigators. The enzyme

[1] The writer is indebted to Mr Priestman, of the Department of Industrial Chemistry, the University of Liverpool, for these determinations.

material was diluted with sufficient water to keep the total volume of the water solution 13 cc. 2·5 g. olive oil, 2 cc. buffer solution and the calcium chloride solution were added and the mixture was gently shaken. Finally, the solution of egg-albumin was added and the mixture shaken vigorously by hand for 3 minutes and then placed in a thermostat at 30° for 57 minutes. The contents of the reaction vessel were washed into an Erlenmeyer flask with 96 % alcohol so that the volume of the alcoholic liquid was 125 cc. 20 cc. ether were added and the acid titrated with 0·1 *N* alcoholic NaOH in the presence of thymolphthalein. Control experiments in which the buffer and protein of the enzyme material were titrated were also carried out to determine the degree of hydrolysis of the olive oil. A few of the results are given in the table below.

Table I. *Temp. 30°. Enzyme extract 0·4 cc. Total volume of water solution 13 cc.*

mg. $CaCl_2$	mg. albumin	Hydrolysis in cc. 0·1 *N* NaOH	mg. $CaCl_2$	mg. albumin	Hydrolysis in cc. 0·1 *N* NaOH
0	0	6·5	0	10	8·2
10	0	10·95	15	10	14·4
10	10	12·8	20	10	16·4
10	15	13·6	0	20	9·5
10	20	14·6	20	20	17·1
10	40	15·4			

In the presence of a high concentration of calcium chloride (greater than 15 mg.) a thick creamy mass, which contained all the oil, separated out when the shaking was stopped, and the reproducibility of the experiments was not so good as that obtained with smaller concentrations of calcium chloride, in which case good emulsification of the oil resulted. In general, the writer considered 10 mg. calcium chloride and 20 mg. egg-albumin in a total volume of 13 cc. water solution to be a good combination of activators to employ to swamp the effect of the substances accompanying the enzyme.

An investigation of the heat-inactivation of pancreatic lipase would necessarily involve the use of glycerol extracts. This might mean a considerable variation in the concentration of the glycerol in the determination mixture during such an investigation (*e.g.* only 0·2–0·4 cc. of a fresh glycerol (80 % glycerol) extract are sufficient to give a suitable hydrolysis, whereas after heating at 55° for 30 minutes 1·0–1·5 cc. are required to give the same hydrolysis). Willstätter, Waldschmidt-Leitz and Memmen [1923] observed that glycerol accelerated the action of pancreatic lipase under the conditions of the experiments, *i.e.* an initial p_H of 8·9. They, however, only worked with 0·2–0·4 cc. of an 87 % glycerol extract, and the control of the glycerol concentration was unnecessary. The writer has investigated the effect of glycerol on the action of pancreatic lipase in the absence of other activators and also in the presence of 10 mg. calcium chloride and 20 mg. egg-albumin. The glycerol employed was B.D.H. (A.R.) glycerol. The results are given in Tables II and III.

Table II. *The effect of glycerol in the absence of other activators. Temp. 30°. Enzyme extract 0·4 cc. Initial* $p_H = 8·9$. *Total volume water solution = 13 cc.*

cc. glycerol	Hydrolysis in cc. 0·1 *N* NaOH	cc. glycerol	Hydrolysis in cc. 0·1 *N* NaOH
0	6·3	3·0	15·5
1·0	9·4	4·0	17·8
2·0	12·1	5·0	19·2

Table III. *The effect of glycerol in the presence of 10 mg. calcium chloride and 20 mg. egg-albumin. Temp. 30°. Enzyme extract 0·2 cc. Initial* $p_H = 8·9$. *Total volume of water solution = 13 cc.*

cc. glycerol	Hydrolysis in cc. 0·1 *N* NaOH	cc. glycerol	Hydrolysis in cc. 0·1 *N* NaOH
0	15·3	3·0	16·5
1·0	16·0	4·0	16·6
2·0	16·3		

(The extracts employed in Tables II and III are similar but not identical.) It will be seen that the action of glycerol is not so marked in the presence of calcium chloride and albumin as when these activators are absent. Similar results were obtained when different enzyme concentrations were employed. Although the effect in the presence of calcium chloride and albumin is small, it was considered necessary to keep the concentration of the glycerol constant in a mixture for the determination of lipase. The quantity chosen was 2 cc. of pure glycerol. This quantity allows for the use of 4 cc. of a 50 % glycerol extract without bringing in any error due to the acceleration produced by uncompensated glycerol concentration.

Another factor which must be considered is the method of regulating the p_H when carrying out the heat-inactivation of lipase at different p_H values. By employing NaOH and HCl a wide range of p_H values may be obtained. The p_H in the method of estimation of the lipase proposed to be employed is essentially a changing one, the 2 cc. of buffer employed to give an initial p_H of 8·9 being insufficient to keep the p_H constant. It is therefore to be expected that the estimation will not give true values for the amount of lipase if the enzyme material contains large varying amounts of NaOH or HCl. (The protein in the extracts, especially in the water extract, makes it necessary to employ a considerable quantity of acid or alkali to alter the p_H appreciably, *e.g.* 1 cc. water extract of p_H 5·93 requires 2·1 cc. 0·1 *N* NaOH to bring the p_H to 9·5.) If the NaOH or HCl were neutralised prior to the actual process of estimation a certain quantity of NaCl would be present. It is therefore necessary to carry out experiments to see if NaCl has any effect on the action of lipase upon olive oil in the presence of 10 mg. calcium chloride and 20 mg. egg-albumin. The results are given in Table IV.

The effect is sufficient to necessitate the use of a constant concentration of NaCl in the estimation mixture. The quantity chosen was 1 cc. of 0·1 *N* sodium chloride in a total volume of 13 cc. water solution.

Table IV. *Initial* $p_H = 8{\cdot}9$. *Enzyme extract 0·3 cc. Temp.* = 30°. *Total volume water solution = 13 cc.*

cc. 0·1 *N* NaCl	Hydrolysis in cc. 0·1 *N* NaOH	cc. 0·1 *N* NaCl	Hydrolysis in cc. 0·1 *N* NaOH
0	17·7	0·75	18·3
0·25	17·85	1·0	18·4
0·5	18·15		

The conditions for the method of determination now being fixed it only remains to find a relation between the enzyme quantity and the degree of saponification.

The reactions were carried out in Erlenmeyer flasks of 50 cc. capacity fitted with tight-fitting rubber stoppers. Sufficient water and pure glycerol were added to the enzyme material to make the volume of the water 6 cc., and that of the glycerol 2 cc. 1 cc. 0·1 *N* NaCl, 2·5 g. olive oil, 2 cc. buffer and 1 cc. 1 % (10 mg.) calcium chloride were added and the mixture was shaken for a suitably short time (5 secs.). 1 cc. of 2 % (20 mg.) egg-albumin solution was then run in, and the mixture vigorously shaken by hand for 3 minutes. The reaction flasks were then placed in a thermostat at 30° for 57 minutes and tested. The contents were washed into Erlenmeyer flasks of 300 cc. capacity with 96 % alcohol so that the volume of the alcoholic liquid was 125 cc. 20 cc. ether were added and the acid was titrated with 0·1 *N* alcoholic NaOH in the presence of thymolphthalein as indicator. The degree of hydrolysis obtained with different quantities of enzyme material, under the above conditions, was investigated and a graph plotted showing the relation between the degree of saponification and the quantity of enzyme employed.

The results, given in Table V, are means of several readings.

Table V.

Enzyme expressed in cc. glycerol extract	Hydrolysis in cc. 0·1 *N* NaOH	Enzyme expressed in cc. glycerol extract	Hydrolysis in cc. 0·1 *N* NaOH
0·05	9·70	0·25	18·25
0·10	13·80	0·30	19·1
0·15	15·25	0·40	20·75
0·20	17·1	0·50	21·60

All enzyme extracts are not of the same activity, hence one does not obtain the same hydrolysis by using the same quantity of different extracts. For purposes of standardisation Willstätter and Kuhn [1922] have suggested an arbitrarily chosen measure which is termed an enzyme unit. The writer in adopting this method defines a lipase unit as the quantity of enzyme which produces a saponification of 2·5 g. of olive oil, equivalent to 20 cc. 0·1 *N* sodium hydroxide, in 1 hour at 30° under the conditions specified above. The adoption of this unit does not mean, however, that a given enzyme extract

containing say 0·5 unit will produce just half the amount of saponification in 1 hour at 30°. The actual degree of saponification produced by 0·5 (or any other submultiple or multiple) of a unit has to be determined by experiment. This is effected in principle by starting with a given quantity of extract, and finding by trial what volume of this extract contains just one lipase unit as defined above. Aliquot portions of this amount of extract are then examined for their saponifying powers, and a graph constructed connecting number of units with cc. of alkali required (Fig. 1). Using extracts from different glands, and proceeding in the above manner, a series of identical curves was obtained, the reproducibility being satisfactory.

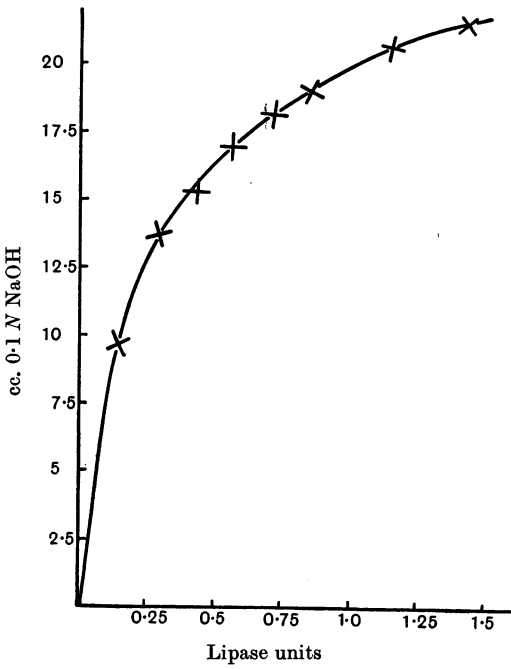

Fig. 1.

It may be mentioned that the unit as defined above is necessarily different from that of Willstätter, Waldschmidt-Leitz and Memmen, since the conditions of the experiment are different; the shape of the curve is not the same as that obtained by these authors.

The heat-inactivation of purified lipase.

(*a*) *Preparation of purified lipase.*

In order to obtain a purified lipase the writer has employed a modification of the method of Willstätter and Waldschmidt-Leitz [1923], who obtained lipase free from trypsin and amylase by carrying out two successive adsorptions on the aluminium hydroxide B of Willstätter and Kraut [1923]. They

observed that a mixture containing 57 parts by volume of 1 % diammonium phosphate, 3 parts of *N* ammonia and 40 parts of 87 % glycerol was most suitable for eluting the enzyme from the alumina-lipase adsorption complex. It was found impossible to expel the inorganic salts by dialysis against distilled water, as the lipase was completely destroyed during the process.

The method employed by the writer was as follows. Two successive adsorptions were carried out in exactly the same way as described by Willstätter and Waldschmidt-Leitz. After the second adsorption, however, the lipase was eluted from the alumina-enzyme adsorption complex with a mixture of 40 parts of glycerol, 3 of *N* ammonia and 57 of water. The ammonia was then evaporated *in vacuo* at 25°, and the solution placed in a desiccator over sulphuric acid, the p_H being thus brought to 7·0. This solution adjusted to contain 50 % glycerol was employed as a purified lipase. It was found to be free from trypsin-kinase. The yield was not as good as that obtained with the mixture containing ammonium phosphate, about 20 % of the lipase of the original glycerol extract being obtained. The absence of phosphate, however, is desirable in the present instance.

(*b*) *Apparatus employed.*

The apparatus employed is shown in Fig. 2.

The reaction vessel *A* is a silica vessel of about 70 cc. capacity, closed by a rubber bung *B*. *C* is a tube through which samples are withdrawn from time to time. Air enters the vessel *A* by means of the side arm *F*, which leads from vessel *G*, which contains the same solution as that under investigation. Air entering this vessel does so by way of a vertical tube *H* which dips under the surface of the liquid. Both vessels are kept well immersed in the water of the thermostat which is regulated to ± 0·05°. This apparatus prevents any change of concentration due to evaporation during an experiment.

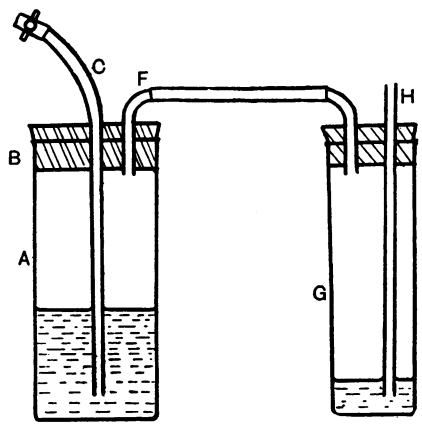

Fig. 2.

A definite quantity of lipase extract is taken and the p_H regulated by the addition of NaOH or HCl of known strength. This solution is placed in vessel *A* which is already at the temperature of the thermostat. Portions of the extract are withdrawn at various intervals of time and run into test-tubes standing in crushed ice. The first withdrawal is made 2–3 minutes after placing the extract in the thermostat. The lipase content of the various portions is estimated according to the method already described.

(c) *Course of the reaction.*

The applicability of the unimolecular expression to the heat-inactivation of this preparation of pancreatic lipase was tested by numerous experiments. The general behaviour is illustrated by Table VI.

The values of k_{uni} were calculated from the expression

$$k_{uni} = \frac{1}{t} \log_e \frac{a}{a - x},$$

where t = time in minutes, a = lipase units per cc. at zero time and $a - x$ = lipase units per cc. at time t.

Table VI.

Time in mins.	Lipase units/cc.	$k_{uni} \times 10^2$
Temp. 50°; 50 % glycerol; p_H = 6·00		
0	0·365	
5	0·250	7·57
10	0·183	6·90
15	0·130	6·90
Temp. 40°; 50 % glycerol; p_H = 8·00		
0	0·42	
30	0·29	1·23
60	0·225	0·90
90	0·15	1·14

It was observed that on the whole the constant decreased slightly in many of the experiments.

(d) *The effect of* p_H *upon the heat-inactivation.*

The effect of the p_H of the medium[1] is shown in Table VII and Fig. 3. The value for k_{uni} at a particular p_H is that obtained by taking the mean of several readings. It will be seen that the enzyme is most stable in the region of p_H 6·0.

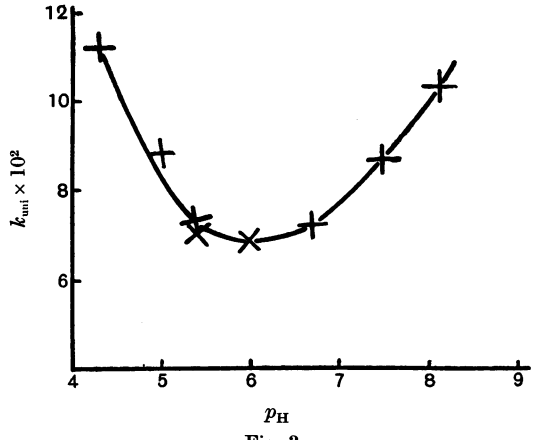

Fig. 3.

[1] The p_H measurements throughout this work were made by means of the glass electrode by the method described by Millet [1928].

Table VII.

Temp. 50°; 50 % glycerol

p_H	$k_{uni} \times 10^2$	p_H	$k_{uni} \times 10^2$
8·14	10·3	5·41	7·00
7·48	8·7	5·40	7·24
6·75	7·21	4·97	8·80
6·03	6·83	4·23	11·14

(*e*) *The critical increment of the process.*

In order to determine the critical increment for the heat-inactivation of purified pancreatic lipase, measurements were carried out at three different p_H values: (1) at the region of optimum stability, p_H 6·00; (2) at p_H 8·01; and (3) at p_H 5·00.

The results are given in Tables VIII, IX, X. The critical increment was calculated by substituting the values of k_{uni} into the integrated form of the equation

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

Table VIII.

50 % glycerol; $p_H = 6·0$

Time in mins.	Lipase units/cc.	$k_{uni} \times 10^3$
	Temp. 40°	
0	0·475	
60	0·315	6·83
120	0·21	6·80
180	0·145	6·59
		Mean = 6·74
	Duplicate exp.	Mean = 6·84
		Average = 6·80
	Temp. 50°	
0	0·365	
5	0·250	75·7
10	0·183	69·0
15	0·130	69·0
		Mean = 71·2
	Duplicate exp.	Mean = 70·7
		Average = 70·8

From these values of k_{uni} we arrive at the value of $E = 46{,}000$ calories at p_H 6·00.

Table IX.

Glycerol content of solution 50 %. $p_H = 8·01$.
Temp. 40°. Mean value of $k_{uni} = 1·14 \times 10^{-2}$.
Temp. 50°. Mean value of $k_{uni} = 10·1 \times 10^{-2}$.

From the above values of k_{uni} at 40° and 50° the value for the critical increment is found to be 44,000 calories at p_H 8·01.

Table X.

Glycerol content of solution 50 %. $p_H = 5{\cdot}1$.
Temp. 40°. Mean value of $k_{uni} = 7{\cdot}95 \times 10^{-3}$.
Temp. 50°. Mean value of $k_{uni} = 7{\cdot}80 \times 10^{-2}$.

The critical increment calculated from these values is 45,800 calories at p_H 5·1.

Table XI. *The critical increment in 25 % glycerol.*

Glycerol content of solution 25 %. $p_H = 6{\cdot}00$.
Temp. 40°. Mean value of $k_{uni} = 7{\cdot}90 \times 10^{-3}$.
Temp. 50°. Mean value of $k_{uni} = 8{\cdot}60 \times 10^{-2}$.

The critical increment calculated from these data is 47,800 calories.

The results obtained with purified lipase are summarised in Table XII.

Table XII. *Purified lipase.*

Glycerol concentration %	p_H	E in calories
50	6·00	46,000
50	8·01	44,000
50	5·1	45,800
25	6·00	47,800

Before discussing the results obtained above a very brief statement of the experiments carried out with unpurified lipase will be given.

The heat-inactivation of unpurified lipase.

Experiments were carried out with unpurified extracts of lipase in order to compare the results with those obtained with the pure enzyme.

These extracts were prepared by extracting 6 g. of the dried gland powder with 100 cc. of water or water-glycerol mixture for 2–4 hours at 30°. (Water gives a good extraction in 2 hours, while 80 % glycerol requires a longer time.) The mixture was then centrifuged and the supernatant liquid clarified by filtration.

In 80 % glycerol it was found that the course of the reaction follows the unimolecular expression quite satisfactorily except at p_H values on the acid side of the p_H of optimum stability ($p_H = 6{\cdot}0$) (Table XIII). In attaining these p_H values by the addition of HCl a precipitate was formed. (No precipitate was formed on adding NaOH.) This precipitate would carry along with it some of the lipase, and presumably this portion of the lipase is less liable to inactivation. At $p_H = 5{\cdot}0$ the velocity recorded is the initial value (since in this case the k falls with time).

Table XIII.

Temp. 50°; 80 % glycerol.

p_H	$k_{uni} \times 10^3$	p_H	$k_{uni} \times 10^3$
5·00	26·05	8·20	34·3
5·99	7·18	9·20	48·8
7·20	17·26	10·23	162·8

The results show that the enzyme is most stable in the region of p_H 6·0, as was the case with the purified enzyme in 50 % glycerol.

In the case of the water extract the unimolecular constant was found to decrease markedly with time at all p_H values. Thus at 40° and p_H 5·93 the "constant" decreased 50 % in 90 minutes. This decrease in the constant is more marked at 50°, while at 30° the decrease in 60 minutes only amounts to 15 %. The extract was most stable in the region of p_H 6·0.

The critical increment for the process was determined by measuring k_{uni} at different temperatures both in the presence and in the absence of glycerol. In the case of water extracts the initial values of k_{uni} were used to calculate the critical increment.

The results are summarised in Table XIV.

Table XIV. *Unpurified lipase.*

Glycerol conc. %	p_H	Temp. interval in ° C.	E in calories
80	5·99	45–50	101,000
80	5·99	50–55	103,000
80	9·30	45–50	92,000
50	5·98	45–50	57,500
0	5·93	30–40	35,000

Discussion of results.

In agreement with the results obtained for other enzymes the p_H of optimum stability of purified pancreatic lipase does not coincide with its "optimum p_H," *i.e.* p_H of optimum activity upon a substrate. The optimum thermal stability is in the region of p_H 6·0, whereas the "optimum p_H" is 8·0. The same behaviour is exhibited by the unpurified material.

In general it may be said that the behaviour of the purified extract is regular and reproducible. On the other hand, in the absence of glycerol the behaviour of the unpurified extract would depend upon the amount of trypsin-kinase present. The trypsin-kinase content of the unpurified material was determined according to the method of Willstätter *et al.* [1926], but without activation with enterokinase. It was found that a water extract of the "dried gland powder" contained 1·97 trypsin units per cc. and the 80 % glycerol extract 0·48 trypsin units per cc.[1]

Willstätter and Persiel [1924] have shown that glycerol inhibits the action of trypsin-kinase, hence this proteolytic enzyme would not be expected to act in 80 % glycerol. The observations of Terroine [1910] and Mellanby and Woolley [1914] indicate that trypsin activated with enterokinase (*i.e.* trypsin-kinase) destroys lipase. During the process of heat-inactivation of an impure water extract the writer has observed that a small amount of acid is formed, whereas in the 80 % glycerol extract no measurable amount results. In an 80 % glycerol extract, as would therefore be expected, the process of heat-

[1] The writer is indebted to Mr Pace, of the Department of Physical Chemistry, University of Liverpool, for these determinations.

inactivation, even of the unpurified material, is unimolecular. In a water extract, however, there are two processes going on: (1) the destruction of the lipase by heat; (2) the destruction of the lipase by trypsin-kinase. A unimolecular velocity constant for heat-inactivation is therefore not to be expected in this case.

It is also suggested that the change in the critical increment for the heat-inactivation of unpurified lipase with change in glycerol content is due to the action of trypsin-kinase, this enzyme destroying the protein carrier and thereby inactivating the active grouping of the lipase. In 80 % glycerol the trypsin-kinase effect is minimal and the observed critical increment of the heat-inactivation of unpurified lipase is very large—of the order 90,000–100,000 calories. In water the trypsin-kinase effect ought to be maximal. The critical increment of hydrolysis of caseinogen by trypsin-kinase is of the order 14,000 calories [Moelwyn-Hughes, Pace and Lewis, 1930]. This low value is probably the reason why the apparent critical increment of heat-inactivation of unpurified lipase in water is as low as 35,000 calories.

The most interesting observation is the relatively low value for the heat-inactivation of purified lipase, namely, 46,000 calories. As this value is obtained in 50 % and 25 % glycerol it seems reasonable to assume that much the same value holds in water. This value should be compared with 90,000–100,000 calories obtained for the unpurified lipase, under conditions in which the trypsin-kinase is ineffective (*i.e.* in 80 % glycerol). The effect of purification is therefore to reduce the critical increment to approximately one-half. This naturally accounts for the fact that the purified material is much less stable than the unpurified. At the same time, one is at a loss to explain precisely the manner in which this reduction in critical increment has been brought about by the act of purification. The purification consists mainly in the removal of protein, and this change in the accompanying substances in some way causes a lowering in the critical increment[1]. It is interesting to note that the critical increment of purified lipase is of the same order as that obtained by Pace [1930] for the heat-inactivation of trypsin itself, free from enterokinase and the pre-stage of this activator, the value obtained being 40,000 calories.

[1] The unpurified lipase has protective impurities adsorbed or otherwise combined with it. The extent of such adsorption will be a function of temperature, adsorption in general diminishing as temperature rises. Hence at the higher temperature the unpurified lipase is now partly purified by removal of impurity and hence is thermally inactivated at a greater rate than would have been found had the extent of adsorption remained independent of temperature. It follows that a correspondingly high value (90,000–100,000 cals.) is obtained for the apparent critical increment. If this point of view be the correct one, it may be expressed in a quantitative form. Thus if Q is the (positive) heat evolved in the adsorption of the impurities on one "molar" unit of enzyme, E_0 the true critical increment of heat-inactivation of purified enzyme and E_{obs} the observed value for the unpurified enzyme, then we would expect

$$E_{obs} = E_0 + Q.$$

Setting $E_{obs} = 95{,}000$, $E_0 = 45{,}000$ cals., it follows that $Q = 50{,}000$ cals. This is a very high value for adsorption but it has to be remembered that the "molecular unit" of an enzyme is exceedingly large and the number of molecules of adsorbed material per enzyme unit correspondingly great.

SUMMARY.

1. A modification of the method of Willstätter, Waldschmidt-Leitz and Memmen for the estimation of pancreatic lipase has been employed in investigating the heat-inactivation of this enzyme. The relation between the concentration of the enzyme and the degree of hydrolysis has been obtained under the new conditions, and a lipase unit, necessarily different from that of the above authors, has been defined and employed in the later work described.

2. A purified lipase has been prepared by two adsorptions on aluminium hydroxide. In order to obtain a preparation free from salts an elution mixture of glycerol, water and ammonia is employed in eluting the enzyme after the second adsorption.

3. Experiments have been carried out on the heat-inactivation of this purified lipase. The course of the heat-inactivation process was found to be unimolecular.

4. The effect of p_H upon the heat-inactivation of lipase prepared in the above manner has been investigated. It has been found that the optimum stability of the lipase is about p_H 6·0.

5. The critical increment for the heat-inactivation process in 50 % glycerol has been determined at three p_H values: 6·0, 8·01 and 5·0. It was found that the critical increment was sensibly the same at all three points, and was of the order of 46,000 calories.

6. The critical increment was likewise determined in 25 % glycerol and p_H 6·0 and was found to be 47,800 calories. It is concluded therefore that in the absence of glycerol the critical increment would be of this order.

7. Experiments were also carried out on the heat-inactivation of extracts of unpurified lipase. The course of the reaction in 80 % glycerol was found to be unimolecular, whereas in the water extract of the "dried gland powder" the unimolecular constant was found to decrease with time. This decrease is explained by the action of the trypsin-kinase in the water extract.

8. The effect of p_H upon an 80 % glycerol extract and water extract of unpurified lipase has been investigated. It was found that the enzyme is most stable at about p_H 6·0.

9. At p_H 6·0 and in 80 % glycerol the critical increment for the process in unpurified extracts is shown to be of the order of 100,000 calories, whereas in 50 % glycerol the value is 57,500 calories, and in water 35,000 calories. This change in the critical increment with change in the concentration of glycerol is ascribed to the action of the trypsin-kinase. The critical increment for the heat-inactivation process of an unpurified extract containing 80 % glycerol at p_H 9·3 is of the order 92,000 calories, which is sensibly the same as 100,000 calories.

10. The fall from 95,000 calories for unpurified lipase to 45,000 calories for purified lipase is in agreement with the increased instability of the purified material.

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