CXXX. FACTORS INFLUENCING THE ACTION OF PANCREATIC LIPASE.

By BENJAMIN STANLEY PLATT AND EDWARD ROMER DAWSON.

From the School of Medicine, University of Leeds.

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IN the course of a determination of the action of pancreatic lipase on certain esters, it was necessary to find a convenient method of preparing the enzyme in an active and pure condition, and to investigate the influence of certain factors with a view to obtaining the optimal conditions of action.

METHOD OF PREPARATION.

The methods which have been devised for utilising the enzyme in the organ may be divided into three main groups: (a) the whole pancreas may be used, or a simple press juice obtained from it, (b) the enzyme may be extracted from the fresh gland with suitable solvents and the extract used in the crude state or subjected to further purification, (c) the pancreas may be treated so as to obtain a solid preparation containing the lipase. The solid obtained by this last method can be worked up to yield an enzyme in a high degree of purity as in the method of Willstätter and Waldschmidt-Leitz [1923].

Most of the previous work on the action of pancreatic lipase has been carried out with an enzyme preparation containing the natural activating substances. A determination of the effect of added substances on the activity of such a preparation is obviously of little value. Accordingly, we employed a method suggested by Rosenheim [1910] and developed by Umeda [1915]. The powder obtained by this method is claimed to be free from water-soluble substances, including enzymes, and organic substances soluble in alcohol, acetone and ether. It is found that practically all the amylase originally present in the extract is removed in the preparation of the powder, which however has still considerable tryptic activity. A sample of dried pancreas powder has been prepared by the method recommended by Willstätter and Waldschmidt-Leitz [1923], and the enzyme content of the two preparations compared under identical conditions. Lipase is present to the same extent in both samples, whilst in the preparation obtained by Willstätter's method, amylase is present, and the trypsin content is greater than in the powder prepared by Umeda's method.

In agreement with Virtanen [1924], we have found that the amount of lipase extracted by glycerol varies with the time allowed for extraction.

When, however, the gland is reduced by further mincing to a fine pulp, a few hours suffice for complete extraction of the enzyme. Willstätter and Waldschmidt-Leitz [1923] object to the use of alcohol in obtaining a solid preparation of the pancreatic enzymes. They find that treatment with alcohol causes an appreciable diminution in the activity of the lipase. We find that alcohol has no influence on the lipase in the powder obtained by Umeda's method, probably because this substance has already been washed with alcohol. We would suggest that the loss in activity noticed by Willstätter is due to the removal of an activating substance soluble in alcohol. In fact, Umeda has found that the so-called "co-enzyme" of lipase is made up of two fractions, one of which is soluble in alcohol. It is noteworthy, too, that Foster and Woodrow [1924] obtain a stable mixture of pancreatic enzymes by desiccating the organ with alcohol. Also, Vollmer, Schmidt and Serebrijski [1924], from the results of experiments on the antilipolytic effect of solutions of iodine in alcohol, state that the inhibition of these solutions is not due to the alcohol.

ESTIMATION OF ACTIVITY.

The activity of the lipase obtained from the pancreas has been determined in the following experiments by estimating the amount of acid liberated from ethyl butyrate or from olive oil. When the latter substance was used as the substrate, the technique developed by Willstätter and his collaborators was adopted. No antiseptics were added to the digestion mixtures, as Umeda [1915] and Long, Hull and Atkinson [1917] found this precaution unnecessary in the case of olive oil, and we have found in our work that ethyl butyrate has a pronounced inhibitory influence on bacterial growth.

A number of workers have directed attention to the inconsistent results obtained when a series of digestion mixtures are not all shaken to the same extent [cf. Bradley, 1909; Pekelharing, 1912; Haley and Lyman, 1921; and Willstätter, Waldschmidt-Leitz and Memmen, 1923]. We have used a device similar to that described by Armstrong and Gosney [1913], viz. shaking the test mixtures while in the incubator. The shaking would appear to be essential in this case to eliminate any discrepancies which might arise due to such physical causes as diffusion. Continued shaking was found to have no deleterious effect on the action of lipase on ethyl butyrate. On the contrary, a series of mixtures shaken for different periods up to 3 hours, were (at the end of 3 hours) hydrolysed to a degree proportionate to the time of shaking.

In the experiments on the activity of lipase at different hydrogen ion concentrations, we found that the amount of action could be easily and accurately determined by titrating a sample of the digestion mixture to the initial p_H with CO_2 -free alkali, using a suitable indicator. Due regard was given to the concentration of indicator and to compensation for opalescence and colour of test solutions.

INFLUENCE OF HYDROGEN ION CONCENTRATION.

Little importance can be attached to the results of earlier work on the action of lipase owing to the failure to appreciate the necessity for regulating the reaction of the digestion mixture. Especially is this the case for the work on the influence of added substances. As Willstätter and his co-workers have shown in recent papers, a substance may have quite different effects according as the medium is acid or alkaline.

Under certain conditions, enzymes are considered to display maximal activity at a definite hydrogen ion concentration, which is termed the "optimum p_H ." This property is regarded by many as characteristic of the particular enzyme. In seeking for the conditions for maximal activity of lipase it is necessary to determine and maintain this optimum p_H . Peculiar difficulties arise in the course of lipolysis due to the liberated acid changing the p_H of the medium. After various attempts had been made to eliminate the effect of the acid formed, we found that it was not possible to keep the p_H constant; but by using buffer solutions of suitable concentrations, the amount of change could be restricted. By increasing the concentration of the buffer solution to a point consistent with the accurate determination of the end point by titration, the range of p_H over which the enzyme was allowed to work could be reduced to as little as 0.2.

There are a number of values given in the literature for the optimum p_H of pancreatic lipase. Rona [1911] determined the action of lipase in different organ extracts at p_H 7.5, in phosphate buffer mixtures. Davidsohn [1912] found a reaction optimum of p_H 8.0 and in the following year [1913] he gave the value p_H 8.5. Further work of Rona and Bien [1914] suggests that there is an optimal zone over which the lipase of a pancreatic extract is most active, namely, from p_H 8.4 to p_H 9.0. Umeda [1915] gives a value of about p_H 7.4 for the action of the lipase in his extract of pancreas on olive oil in phosphate buffer solutions. With a saline extract of pancreas or with a pancreatic fistula secretion, Rona and Pavlovic [1923] obtained for the optimum p_H a value of 6.95 to 8.0. Some of the results, when represented by curves obtained by plotting activity against increasing p_H , do not show an optimal point or even a zone of maximal activity, but simply that a limiting value is being approached. The recent values obtained by Rona and Pavlovic, however, definitely prove that at p_H 3.7 there is no lipase activity and that above p_H 8.0 there is a distinct falling off in activity. Willstätter prefers to work with a buffer solution containing ammonia and ammonium chloride at a p_H of 9.2 at 18° when using olive oil as substrate, and a similar buffer solution at p_H 8.6 when tributyrin is the substrate. Anrep, Lush and Palmer [1925] carry out their lipase estimations in a phosphate buffer solution at p_H 7.8 and allow the reaction to proceed until the p_H is not less than 7.0.

Determination of optimum p_H .

The results of typical experiments carried out to determine the optimum p_H for pancreatic lipase are given in the curves in Fig. 1. Mixtures were made up as indicated in 50 cc. flasks of Jena glass fitted with rubber stoppers. All the solutions and vessels were previously warmed up to the reaction temperature. In all cases the substrate was added last to the digestion mixture. These were shaken mechanically in an incubator kept at 37° . To stop the reaction the flasks were plunged into ice-water for 10 minutes before titration. The results given are the mean of duplicate experiments, corrected for suitable controls, and are expressed in cc. CO_2 -free alkali required to restore the initial p_H estimated colorimetrically and at room temperature. The buffer mixtures used are given by Clark [1922], Kolthoff [1925] and Palitzsch [1915].

Exp. 1. Mixture: 24.0 cc. KH_2PO_4 —NaOH buffer solution (Clark).

0.5 cc. ethyl butyrate.

1.0 cc. suspension of 5 mg. of enzyme in water.

Incubated 3 hours at 37° , then titrated with $N/20$ NaOH using either bromothymol blue, phenol red or thymol blue as indicator.

The titration values are for 10 cc. of the reaction mixture (see curve, Fig. 1).

Fig. 1. The relation between p_H and activity.

Exp. 2. Mixture: 10.0 cc. phosphate buffer solution (Clark).

0.5 cc. ethyl butyrate.

1.0 cc. suspension containing 5 mg. enzyme.

Alkali used: $N/10$ NaOH.

Other conditions as in previous experiment (see curve, Fig. 1).

Exp. 3. Conditions as in previous experiment, but 10 cc. of Kolthoff's borax-phosphate buffer used instead of the phosphate solution above (see curve, Fig. 1).

Exp. 4. Conditions as in previous experiments.

Mixture: 46 cc. borax-boric acid-NaCl buffer solution (Palitzsch).

1 cc. ethyl butyrate.

2 cc. suspension containing 10 mg. enzyme.

The results are shown in the curve, Fig. 1.

It will be seen from these results that the optimal activity of pancreatic lipase in phosphate and phosphate-borax buffer solutions with ethyl butyrate as substrate occurs at about p_H 7.0, whilst in Palitzsch's buffer mixture there is a zone of maximal activity about p_H 8.5.

The following experiments were carried out with a crude glycerol extract of pig's pancreas. 0.5 cc. of the extract, which had been strained twice through muslin, was diluted with 5 cc. of phosphate buffer solutions of various hydrogen ion concentrations, and 3 cc. of water, 0.5 cc. of ethyl butyrate and 0.5 cc. of toluene were added. After 3 hours' shaking at 37° the mixture was titrated to a pink colour with $N/10$ NaOH, using phenolphthalein as indicator.

Exp. 5.

p_H	Mean titration value (corr.) cc.
5.8	3.53
6.2	3.94
6.8	4.86
7.0	5.20
7.4	5.35
7.6	5.51
7.8	6.55

The experiment was repeated with the same quantities of extract, ester and toluene in 25 cc. of the buffer mixtures. 10 cc. portions were titrated as in the preceding experiment after 3 hours' incubation.

Exp. 6.

p_H	Mean titration value (corr.) cc.
6.2	3.4
7.0	5.6
7.4	5.6
7.6	5.75
7.8	6.1
8.0	6.25

From a consideration of the foregoing results, it is easy to understand why anomalies occur in the findings of different workers. The substances accompanying the lipase in the crude glycerol extract appear to protect the enzyme from destruction in the more alkaline media.

The effect on lipase action of a preliminary treatment with buffer solutions of different p_H .

The optimum p_H of pancreatic lipase may be considered as the outcome of two opposing tendencies. With increase in alkalinity an increase in hydrolytic activity takes place, but at the same time, the enzyme is less stable.

This idea is suggested by the work of Noyes, Suguira and Falk [1923], and is borne out by the results of the following experiments.

10 mg. of enzyme were added to 15 cc. of phosphate buffer solution (Clark) of various p_H . This suspension was shaken in the incubator for 24 hours at 37°. Each sample was then adjusted with the calculated amounts of alkali, phosphate solution or water, so that the final p_H was 7.6 and the salt concentration of the mixtures was the same in all cases. 0.5 cc. of ethyl butyrate was added and the mixture shaken again in the incubator for 24 hours, when 10 cc. aliquots were titrated with $N/10$ NaOH to p_H 7.6, using phenol red as indicator.

Exp. 7.

p_H	Mean titration value (corr.) cc.
6.6	3.35
7.0	3.25
7.2	3.29
7.4	3.33
7.6	2.88
7.8	2.35
8.0	1.90

A similar experiment was carried out using Clark's borax-boric acid-KCl buffer mixtures. All the mixtures, after 24 hours' contact with the respective solutions, were adjusted to p_H 8.6, and the activity of the enzyme estimated by its action on ethyl butyrate for 24 hours.

Exp. 8.

p_H	Mean titration value (corr.) cc.
7.8	1.92
8.0	2.19
8.2	1.66
8.6	1.42
9.2	1.40

A study of the relationship between p_H and lipase activity in the various buffer solutions, and of the effect of a preliminary treatment with buffer solutions of different p_H before estimating the activity, suggests that the "buffer value" of the mixtures determine to some extent the form of the optimum curves. The position of the optimal zone when Palitzsch's solutions are used coincides with the position of the maximum buffer value. Also, when the buffer value of the phosphate solutions is increased on the alkaline side by the addition of borax, as in the Kolthoff buffer mixtures, an increased activity is observed.

THE EFFECT OF ADDED SUBSTANCES ON THE ACTION OF PANCREATIC LIPASE.

Substances added to lipase may affect its action by establishing a favourable p_H , by maintaining this p_H because of buffer value, or by some specific influence, probably chemical in nature, on the enzyme. In an experiment

in which lipase was introduced into a solution of ethyl butyrate at a p_H suitable for lipolytic action, no lipase action could be detected by comparing the rate of change of p_H of this mixture with that of a similar solution of ethyl butyrate containing no enzyme.

The enzyme for this experiment was obtained from the powder prepared by Umeda's method, and was freed as far as possible from accompanying substances by digestion in 90 % glycerol and subsequent filtration through a hardened filter paper, with suction. A clear solution was thus obtained containing a considerable amount of lipase. This was added to a solution of pure ethyl butyrate in CO_2 -free distilled water. A control experiment was set up in which the same amounts of ester solution and glycerol were present. Samples were withdrawn at frequent intervals and the p_H estimated colorimetrically. Special precautions were taken to prevent contamination from the vessels used and from CO_2 in the atmosphere. It was found that the time taken for the change in p_H from 7.8 to 7.05, using the same indicator, phenol red, was identical in both experiments. Apparently some activating substance must be added to the enzyme before it can function even at a suitable hydrogen ion concentration.

The influence of phosphates.

In determining the most suitable concentration of buffer solution required to restrict the amount of change in p_H during the course of lipolysis, it became necessary to investigate the effect of various concentrations of phosphate solutions on lipase activity at a definite initial p_H . The following are typical of the results obtained.

A series of mixtures was prepared containing 20 mg. of enzyme in 10 cc. of phosphate buffer solution and 0.1 cc. of ethyl butyrate. Various salt concentrations were used, the initial p_H being 7.6. After shaking for 1 hour in the incubator, the mixtures were titrated with $N/20$ NaOH to p_H 7.6, using phenol red as indicator.

Exp. 9.

Molar concentration of phosphate	Mean titration value (corr.) cc.
—	0.44
0.005	0.99
0.01	1.2
0.02	1.48
0.025	1.57
0.03	1.82
0.04	2.00
0.05	2.23
0.075	2.08

The range of concentration was increased in the following experiments. Buffer mixtures at p_H 7.6 and 7.2 were made up, the concentrations of phosphate being 0.2M. These solutions were diluted to give solutions of the required salt content. The mixtures contained 25 cc. phosphate solution, 0.5 cc. ethyl butyrate and 1 cc. suspensions containing 10 mg. of enzyme.

10 cc. aliquots were titrated at the end of 3 hours' incubation with $N/20$ NaOH, using phenol red as indicator for the first and bromothymol blue for the second series. 10 cc. portions were titrated again after the reaction had proceeded for a further 21 hours.

Exp. 10. The results are shown in the curves of Fig. 2.

Fig. 2. The influence of phosphate concentration (see *Exp. 10*).

The experiment was repeated using different phosphate concentrations.

Exp. 11. p_H 7.6.

Concentration of phosphate <i>M</i>	Mean titration value (corr.)	
	3 hours cc.	24 hours cc.
0.025	1.77	5.07
0.05	1.91	7.36
0.075	1.68	7.32
0.1	1.63	7.96
0.15	1.72	8.72
0.2	1.75	9.62

It will be seen from these results that Clark's phosphate buffer solutions of the usual concentration ($0.05M$ KH_2PO_4) are suitable media for carrying out lipase tests on ethyl butyrate as substrate.

A straight line relationship between the concentration of the phosphate solution and the activity of the lipase would be anticipated if only a buffer

effect were involved. The relationship is no doubt modified by a specific salt effect similar to that which is found in the case of synthetic bile salts. When the amount of lipase action is increased by carrying out the reaction at a p_H nearer the optimum value, or by lengthening the reaction period, a closer approximation to this relationship is observed.

Solutions of sodium α - and β -glycerophosphates, when compared with a solution of potassium phosphate of the same molar concentration and at the same initial p_H , were found to activate lipase to a greater extent, when tested under the same conditions. The two glycerophosphates were identical as regards their activating powers, thus suggesting that the phosphate ion is responsible for the activation. The reason for the different effects of these salts probably lies in their buffer values, which is found to be greater for the glycerophosphates than the inorganic phosphate at the p_H employed. In view of the above results, the objection raised by Noyes, Suguira and Falk [1923] to the use of buffer solutions in the estimation of lipase cannot be sustained.

The adjuvant action of phosphates has been clearly demonstrated by Umeda [1915]. Also, Long, Hull and Atkinson [1915] adduced evidence that the salts present in the pancreas are mainly phosphates. One function of these phosphates is evidently to activate the lipase of the pancreatic juice. Willstätter, Waldschmidt-Leitz and Memmen [1923], by adding calcium chloride, albumin and an ammonia-ammonium chloride buffer mixture, claim to have produced a medium in which lipase from any source is completely activated. We find that the addition of small amounts of phosphate increases the action of pancreatic lipase, even when the system of activators recommended by Willstätter is present. Thus, in one of a number of similar experiments on the effect of small amounts of phosphate solution on the action of lipase on olive oil, we find, by using Willstätter's technique, that 4.05 cc. 0.3*M* alcoholic KOH are required to neutralise the acid formed from 2.5 g. olive oil. Increased amounts of the same alkali are required, 5.8, 4.8 and 4.7 cc., when 0.25, 0.5 and 1.0 cc. respectively of the elution mixture, used in the process of the purification of lipase described by Willstätter and Waldschmidt-Leitz [1923], are also present. The above titration figures are the mean of the results of duplicate experiments, corrected for the necessary control. The enzyme powder used in this experiment was prepared by Willstätter's method and was found to have an activity of the same order as that given in this author's paper. When in the later stages of purification of lipase Willstätter tested for the amount of enzyme, the estimations were carried out in the presence of amounts of elution mixture similar to those used above. The lipase values would therefore not be comparable with the initial values obtained in mixtures not containing phosphate.

The influence of bile salts.

It is remarkable that the results of a number of workers, de Jonge-[1917], Orbeli and Tetiaeff [1916] and Terroine [1920], on the influence of various

concentrations of bile salts on the activity of pancreatic lipase show the same relationship as we have observed for the effect of increasing amounts of phosphates. Anrep, Lush and Palmer [1925] have attributed to bile salts a specific chemical influence, distinct from their surface tension properties, which they hold in common with substances such as caprylic alcohol and saponin. We would suggest that the specific chemical effect, which both phosphate and bile salts can exert, is partly responsible for the connection observed between salt concentration and enzyme activity. It is probable also that the same factor causes the shift in optimum temperature of lipase in the presence of bile salts. Terroine [1920, p. 199] gives figures which show that an elevation of the optimum temperature of lipase in pancreatic juice occurs when bile salts are present, but he does not comment on this fact. In some preliminary work on this point, we have obtained results similar to those of Terroine.

The effect of albumin and edestin.

Emphasis is laid by Falk [1921] on the protein structure of lipase. The preparations we have obtained at different times contain amounts of nitrogen ranging from 8.5–12.3 %. Moreover, the activity of a definite amount of each sample is directly proportional to the amount of nitrogen in the powder. We do not take these results as evidence of the protein nature of the enzyme, but rather that the enzyme is associated with a protein substance and has been freed from impurities to varying extents, depending upon the efficiency of the washing. The accompanying protein, however, largely determines the properties of the enzyme. Falk [1915] has stated that he has been able to separate the ester-hydrolysing enzymes of castor bean into a "lipase" and an "esterase," the former being the globulin fraction, the latter an albumin-like substance. The following experiments were performed to show that the enzyme is not necessarily a protein, but it can manifest so-called "esterase" or "lipase" properties according as the same enzyme material is accompanied by one or other of the two proteins.

A clear, filtered glycerol extract of dried pancreas was used as the source of lipase. The albumin was crystalline egg albumin (Boots), and the globulin was edestin. The mixtures prepared were:

- (1) 18 cc. distilled water.
0.5 cc. glycerol extract of enzyme in 2 cc. of water.
0.5 cc. ethyl butyrate.
- (2) 14 cc. distilled water.
4 cc. solution containing 10 mg. albumin.
0.5 cc. glycerol extract in 2 cc. water.
0.5 cc. ethyl butyrate.
- (3) As (2) but 20 mg. albumin.
- (4) As (2) but 10 mg. edestin.
- (5) As (2) but 20 mg. edestin.

(6), (7), (8), (9) and (10) are comparable with the above, except that 2.5 g. of olive oil are used as substrate instead of ethyl butyrate. The results are expressed in terms of $N/10$ NaOH required to neutralise the butyric acid liberated in the first series after 18 hours' shaking in the incubator at 37° . In the experiments with olive oil, the titration was carried out in alcohol-ether solution with $0.397N$ alcoholic KOH. Phenolphthalein was used as indicator in both cases. The values recorded are the mean of duplicate determinations corrected for the necessary control. The results of two experiments, using different glycerol extracts, are given in the following table.

Exp. 12.

Mixture. Enzyme and substrate	Substrate			
	Ethyl butyrate		Olive oil	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
	cc.		cc.	
Plus water ...	(1) 0.43	0.48	(6) 1.6	0.99
Plus 10 mg. albumin	(2) 1.9	1.2	(7) 0.45	0.57
Plus 20 mg. albumin	(3) 2.5	1.85	(8) 0.49	0.83
Plus 10 mg. edestin	(4) 1.15	0.75	(9) 1.8	1.33
Plus 20 mg. edestin	(5) 1.4	1.0	(10) 1.8	1.90

Clearly from these results, the same enzyme, associated with albumin, hydrolyses a simple ester such as ethyl butyrate more easily than a typical glyceride, olive oil, which however is decomposed to a greater extent than ethyl butyrate when the lipase is allowed to work in association with edestin. It seems that Falk's conclusions were based on the isolation of the two different proteins, on which the same enzyme was adsorbed, rather than a separation of two ester-splitting enzymes.

The action of trypsin on lipase.

Conflicting evidence is found in the literature on this subject. We find that Lewkovitsch and Macleod [1903], Mellanby and Woolley [1914], Terroine [1920] and Mellanby [1925] state that trypsin destroys lipase. On the other hand, Shaw-Mackenzie [1915] and de Souza [1916] find that lipase and trypsin may be preserved together in extracts of pancreas containing more than 30 % glycerol. The glycerol is supposed to inhibit the action of trypsin on lipase, and on dilution the protective action of the glycerol is removed. We have found, however, that in solutions containing up to 30 % glycerol it is possible for trypsin to decompose caseinogen. Further, if trypsin is allowed to act in solutions containing active lipase, the loss of lipase is not so marked if due regard is given to the destruction of protein activating substances of the lipase. Willstätter and Memmen [1924] have remarked on the instability of lipase in aqueous extracts of pancreas. They find that the lipase is destroyed even when all the trypsin is removed from solution.

The following experiments were conducted to determine the nature and extent of the supposed destructive action of trypsin on lipase. Solutions of

lipase were subjected to the action of trypsin. The amount of lipase in these mixtures was estimated after intervals by the action on olive oil, in the presence of protein activators, or on ethyl butyrate.

Exp. 13. A mixture (A) was prepared containing:

- 5 cc. phosphate buffer solution at p_H 7.8.
- 200 mg. pancreas powder (Willstätter).
- 20 cc. neutral "liquor pancreaticus" (Benger).
- 25 cc. water.

A second mixture (B) was prepared in which the "liquor pancreaticus" had been boiled before addition.

These mixtures were maintained at a temperature of 37° . All the solutions and vessels used were previously warmed up to this temperature. To estimate the amount of lipase, 4 cc. of each mixture were withdrawn and added to 10 cc. of phosphate buffer solution at p_H 7.8, 0.5 cc. of ethyl butyrate then being added. The estimations were carried out in duplicate with the necessary controls. After incubating for 1 hour, the amount of $N/10$ NaOH required for neutralisation of the acid formed was determined in the usual manner.

Time of incubation before testing	Mean titration value (corr.)	
	Mixture A	Mixture B
	cc.	cc.
0	6.65	5.85
1	4.25	5.6
3	1.55	2.75

Exp. 14. The following results were obtained from a similar experiment, using the mixture (A):

- 100 cc. phosphate buffer at p_H 8.2.
- 200 mg. pancreas powder.
- 30 cc. neutral "liquor pancreaticus."

As before, mixture (B) contained boiled "liquor pancreaticus." For the lipase test, 10 cc. of the mixture were withdrawn, 0.5 cc. of ethyl butyrate added and the estimation carried out as above.

Time of incubation before testing	Mean titration value (corr.)	
	Mixture A	Mixture B
	cc.	cc.
0	5.8	5.95
1	2.2	4.45
2.5	0.8	2.5
4.5	0.5	1.18

Willstätter's technique for the estimation of the amount of lipase was used in Exps. 15 and 16. For each test, 2 cc. of mixture were withdrawn. 0.398 N alcoholic KOH was used for titration of the acid liberated from the olive oil.

Exp. 15. Mixture (A) contained:

- 1.25 cc. phosphate buffer solution at p_H 7.8.
- 50 mg. pancreas powder.
- 5 cc. neutral "liquor pancreaticus."
- 6.25 cc. water.

In the case of mixture (B), the "liquor pancreaticus" had been boiled before addition. The following results were obtained:

Time of incubation before testing	Mean titration value (corr.)	
	Mixture A	Mixture B
hours	cc.	cc.
0	2.6	3.15
3	1.1	1.65
24	0.22	0.23

Exp. 16. In this experiment, mixture (A) contained:

2.5 cc. phosphate buffer solution at p_H 7.8.

100 mg. pancreas powder.

20 cc. neutral "liquor pancreaticus."

2.5 cc. water.

Time of incubation before testing	Mean titration value (corr.)	
	Mixture A	Mixture B
hours	cc.	cc.
0	4.94	5.16
2	3.04	3.34
4	2.0	2.78

The amount of trypsin in the mixtures containing unboiled "liquor pancreaticus" was determined and was found to be 4-6 times as great as in the mixtures which contained boiled "liquor pancreaticus."

Experiment showed that there is no measurable tryptic decomposition of the small amounts of albumin present when Willstätter's technique is used.

From the results of the preceding experiments it is seen that there is an appreciable difference in the lipase contents of the various mixtures if no allowance is made in the estimations for the loss by tryptic action of protein activating substances of the lipase. When large amounts of trypsin are present and the lipase estimations are carried out by the ethyl butyrate method, the diminution in lipase content is very pronounced (Exps. 13 and 14). If, however, albumin is added to compensate for protein losses by tryptic action, as in the tests by Willstätter's method, the rate of loss of lipase is not very different from that of the solutions to which no further amounts of trypsin had been added (Exps. 15 and 16).

The effect of formaldehyde.

De Souza [1916] has found that formaldehyde in concentrations of 1 : 1000-1 : 500 does not destroy lipase. Palmer [1922] has shown that solutions containing 0.1-0.05 % of formaldehyde have no effect on lipase action. These results are important in view of the theory of the protein nature of lipase. We have obtained the following results which are in agreement with the foregoing.

Mixtures were made up containing:

20 cc. phosphate buffer solution at p_H 7.2.

5 cc. formaldehyde solution.

5 mg. enzyme powder in 1 cc. buffer solution.

0.5 cc. ethyl butyrate.

Test mixtures, in duplicate, with necessary controls, were shaken in the incubator for 3 and 24 hours at 37°. The results are expressed in cc. of $N/10$ NaOH required to restore the p_H of 10 cc. to the initial value, using bromothymol blue as indicator.

Exp. 17.

Concentration of formaldehyde	Mean titration value (corr.)	
	3 hours cc.	24 hours cc.
0	7.25	10.75
1 : 2500	6.16	10.4
1 : 2000	6.06	10.4
1 : 1500	5.73	9.9
1 : 1000	5.82	9.8
1 : 500	5.32	8.4
1 : 100	2.64	6.3

An experiment carried out in the same manner gave the following results:

Exp. 18.

Concentration of formaldehyde	Mean titration value (corr.)	
	3 hours cc.	24 hours cc.
0	3.7	8.8
1 : 2000	3.02	7.1
1 : 1000	3.05	7.7
1 : 500	3.29	7.6
1 : 100	1.35	5.2
1 : 50	0.90	2.9

For concentrations of formaldehyde less than 0.2 %, especially for the longer reaction periods, the formaldehyde appears to have but little inhibitory influence. If the enzyme had the structure of a typical protein the effect of the formaldehyde would no doubt be much more pronounced than the results suggest.

SUMMARY AND CONCLUSIONS.

Lipase can be conveniently estimated by titration of the acid formed from ethyl butyrate, when the reaction is carried out in a phosphate buffer medium.

The optimum p_H for the action of pancreatic lipase is determined by the substances present in the reacting mixture. With a purified pancreas powder containing lipase, the optimum p_H in phosphate and in phosphate-borax buffer solutions was at p_H 7.0, and in borax-boric acid buffer solutions there was an optimal zone about p_H 8.4. The lipase of a glycerol extract of pig's pancreas in phosphate buffer mixtures, acting on ethyl butyrate, showed maximum activity at p_H 7.8, but gave no definite p_H optimum.

The enzyme does not promote the hydrolysis of ethyl butyrate in the complete absence of activators, even if the medium has favourable hydrogen ion concentration.

Increasing concentrations of phosphates and bile salts have a similar effect on the activity of lipase. In both cases, when the values of the activities are plotted against increasing salt concentration, a curve is obtained showing

first, a rapid rise, then a plateau, followed by another rise. The form of the curve appears to be determined by both the buffer value of the solutions and a specific influence of a chemical nature on the enzyme.

Phosphates should be present in any system of activators intended for complete activation of pancreatic lipase.

Our results suggest that the protein structure which has been ascribed to lipase is characteristic of the substances associated with the enzyme rather than of the enzyme itself. The ester-hydrolysing properties of the enzyme have been definitely altered by changing the accompanying protein. The apparent destruction of lipase by trypsin is due, in part, to the removal by hydrolysis of proteins which activate the lipase.

Formaldehyde has no appreciable effect on pancreatic lipase action in concentrations less than 0.2 %.

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