V. ON INACTIVE LIPASE AND THE NATURE OF ITS CO-ENZYME.

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Some years ago the observation was made in this laboratory by Rosenheim [1910] that active pancreatic lipase, as contained in glycerol extracts of pig's pancreas, can be separated into two inactive fractions by mere filtration through filter paper. The insoluble moiety on the filter paper contains the thermolabile inactive enzyme, which is restored to its original activity by the addition of the filtrate. The thermostable substance contained in the latter was designated as the co-enzyme according to the current nomenclature. Rosenheim also described a convenient method by which inactive lipase may be obtained as a dry powder free from soluble proteoclastic and amyloclastic enzymes, electrolytes, fats and lipoids.

From preliminary experiments it was concluded that the co-enzyme dialyses readily, and seems to be soluble in dilute but not in absolute alcohol, and insoluble in ether. Its activity is lost on incineration of the active dialysate. Of other substances tested bile salts and glycerophosphates were also found to possess activating powers.

These observations were fully confirmed by Pekelharing [1912], who made the additional interesting statement that the ash of the co-enzyme solution produced intense lipoclasia when added to the inactive lipase, if it had previously been dissolved in hydrochloric acid. He ascribed to calcium ions the main rôle in this reaction, which he explained by the removal of fatty acid as an insoluble calcium soap. Pekelharing failed, however, to adduce proofs that calcium salts are present in any amount in the co-enzyme solution; further, the effect due to calcium salts seems to account only for a relatively small amount of the total activating power of the co-enzyme solution.

Rona and Bien [1914], whilst partially confirming Rosenheim's results, were unable to demonstrate the presence of the co-enzyme. These observers,

however, employed a phosphate mixture as a medium, in order to regulate the hydrogen ion concentration during the reaction. As it will be shown in this communication that phosphates by themselves exert a powerful activating action on inactive lipase, it is not surprising that these authors failed to find the presence of a co-enzyme in their extracts.

The observations of J. Mellanby and Woolley [1914] refer to the lipase in pancreatic juice. It is obvious that in this case a separation of enzyme and co-enzyme by mere filtration is impossible as the enzyme is in a soluble condition as distinguished from the suspended insoluble state in which it occurs in pancreatic extracts. Mellanby and Woolley made an unsuccessful attempt to effect a separation by alcohol. The impossibility of separating the lipase of pancreatic juice into enzyme and co-enzyme by means of alcohol precipitation does not, however, necessarily imply that their separation is impossible by other means.

The present investigation was undertaken in order to study in the first instance the activating power of the inorganic constituents of the co-enzyme solution. The action of electrolytes on pancreatic lipase has already been the subject of many investigations. The results have, however, been rather contradictory. Thus, to mention only two recent investigations, Terroine [1910] working with pancreatic juice and olive oil found an accelerating influence of Na, K, Mg and Ba salts, whilst Ca salts had no action. On the other hand, Mellanby and Woolley [1914] deny the influence of electrolytes altogether. Quite apart from the influence of the varying degree of emulsification of the zymolytes employed (fats or esters) it is obvious that both pancreatic juice and glycerol extracts of pancreas contain electrolytes in varying amount. Terroine has indeed attempted to remove the electrolytes by dialysis, but found that the lipase of pancreatic juice was destroyed in this process, probably by the action of the proteoclastic enzyme. Whilst therefore pancreatic juice and extracts appear to be unsuitable for the study of the action of electrolytes on their enzymes, this objection does not hold for the inactive lipase preparations made by Rosenheim's method, which can be obtained free from electrolytes and soluble enzymes by careful washing with water.

Whilst I was able to show conclusively that the inorganic constituents, especially the phosphates, contribute largely to the activity of the co-enzyme solution, the fact was incidentally established that the pancreatic co-enzyme is of a complex nature and that organic substances also are concerned in the reaction.

EXPERIMENTAL.

Preparation of inactive lipase.

2 kilos. of fresh pig's pancreas, freed as far as possible from visible fat, were minced and mixed with 4 litres of glycerol. The mixture was frequently stirred and usually allowed to stand for 24 hours. By straining through two layers of fine muslin, a turbid extract was obtained, which possessed considerable fat-splitting activity.

Portions of one litre of this extract were diluted with ten litres of distilled water and the milky fluid allowed to settle in tall cylinders in the ice box. As a rule a perfect separation is obtained at this stage, a white precipitate settling after 24 hours' standing from which the clear supernatant fluid can be easily siphoned off. The precipitate is again suspended in 10 litres of distilled water. Probably owing to the removal of the electrolytes the lipase remains now usually in a colloidal suspension, but by means of a centrifuge a separation can be easily effected.

Pekelharing recommends the addition of acetic acid until the fluid is distinctly acid to sensitive litmus paper. A very satisfactory separation is certainly produced by this modification, but there is considerable danger that trypsin and trypsinogen also are precipitated by the addition of acetic acid. I have always found that although a better yield and a better separation are obtained with the help of acetic acid, the resulting preparation is always less active lipoclastically and also possesses a marked proteoclastic activity when tested with congo-fibrin. The use of acetic acid is therefore not to be recommended when a highly active preparation is desired.

Three washings are usually sufficient to free the preparation from the co-enzyme. The flocculent precipitate is next suspended in an equal volume of alcohol and filtered through a Pukall's [1893] porous clay filter. The precipitate is washed on the filter with acetone and finally with ether. After this treatment it easily peels off in flakes from the filter and after drying in vacuo is obtained as a fine greyish-white powder.

Besides my own preparations I have used an inactive lipase which had been prepared over two years ago by the late Mr Myers-Ward and which was kindly put at my disposal by Dr Rosenheim. This preparation had not been so fully freed from co-enzyme as my own, but its activity after the addition of co-enzyme was somewhat higher than that of mine. It is interesting to note that this powder retained its potential energy although kept for two years under the ordinary conditions of the laboratory.

Preparation of co-enzyme solutions.

The fluid decanted from the precipitate obtained on diluting pancreatic glycerol extract with water, when boiled, filtered and concentrated contains the co-enzyme of lipase free from proteoclastic and other enzymes. The large percentage of glycerol, however, was thought to be in the way for the purpose of my experiments and I therefore used watery extracts of pancreas prepared according to Rosenheim's directions. 500 g. of minced pig's pancreas were boiled up with 500 cc. of distilled water, the mixture kept hot for 30 minutes and filtered after cooling. 620 cc. of extract were obtained which were preserved under toluene. On keeping, a sediment gradually formed mainly consisting of tyrosine; as this sediment was found to be devoid of any activating power, the clear supernatant fluid was used for the experiments.

The acidity of 1 cc. of the different preparations of co-enzyme solution varied from 1.4–2.5 cc. N/10 KOH. This amount, which remained constant after incubation with olive oil emulsion, was always allowed for.

Estimation of lipoclastic activity.

An emulsion of olive oil was used as the zymolyte and the degree of lipoclasia was determined by incubating the mixture of the enzyme and zymolyte for a given time (usually 17–20 hours) and titrating the fatty acids liberated with N/10 KOH. Alcohol was added in sufficient amount to keep the percentage of the titrated solution at least at 50 % alcohol and phenolphthalein was used as an indicator.

In order to obtain concordant results it is important that certain precautions are observed in carrying out these experiments. A short description of the routine followed in this laboratory may therefore not be out of place.

(a) Preparation of the oil emulsion. The quality of the olive oil employed is of considerable importance. On testing the activity of a pancreatic extract on a series of various commercial olive oils, it was found that under identical conditions some oils were easily attacked whilst others were hardly hydrolysed at all. One of these resisting oils was further investigated and was found to contain about 10 % of Arachis oil, an oil which is very resistant to the action of pancreatic lipase. The oil used in this laboratory is described as "Huile d'olive vierge" J. L. Duret & Co.

Before preparing the emulsion, the amount of N/10 KOH necessary to neutralise the free acid present is estimated by titrating 5 cc. of olive oil¹ without the addition of alcohol, until a slight permanent pink reaction to phenolphthalein is reached. Not more than 0.5 cc. N/10 KOH for 5 cc. of olive oil should be required. The calculated amount having been added to 50 cc. of

¹ For the exact measurement of small quantities of oil, emulsion or glycerol extracts, glass syringes are used made by grinding a glass rod into 5 cc. pipettes of about 25 cm. length, graduated into 1/10 cc.

oil (about 5 cc. N/10 KOH) a uniform emulsion is obtained by means of a glass syringe, the oil being alternately drawn up and discharged in a shallow glass dish. 2.5 cc. or 5.0 cc. of this emulsion, which should not be made too thick, were used for each experiment.

(b) 10 mg. of inactive lipase is sufficient to obtain a well-marked reaction. It was used in a 0.2 % or 1 % suspension in water, which is prepared by first grinding the dry inactive lipase in a small mortar with a few drops of water into a fine paste and gradually adding the necessary volume of water. The suspension was freshly prepared for each set of experiments and care must be taken not to allow it to settle before measuring off the necessary amounts.

(c) The order in which enzyme, co-enzyme and zymolyte are mixed is of importance. In my experiments the enzyme suspension was measured into a series of small flasks first, the co-enzyme (or solution to be tested) being added next. 2.5 or 5 cc. of oil emulsion were mixed with these substances by shaking each flask for exactly the same time (20 seconds), it having been found that irregular results are obtained, unless this precaution is observed. The addition of toluene during digestion is unnecessary, as the large percentage of oil prevents bacterial development.

In the following table are given the results of experiments, made at various times, with the same preparations of inactive lipase and co-enzyme. In the last column is given the activity of the mixture of inactive enzyme and co-enzyme expressed in cc. N/10 KOH, the acidity due to co-enzyme solution and that produced by the inactive lipase alone having been deducted.

	Inactive lipase (10 mg. in 1 cc. water) cc.	Co-enzyme cc.	Time of incubation, hours	N/10 KOH cc.	Activity
No. 1	1	—	17	0.5	7.1
	1	1	"	7.6	
	1	—	19	0.9	7.6
	1	1	"	8.5	
	1	—	"	0.9	7.0
	1	1	"	7.9	
No. 2	1	—	17	2.5	10.5
	1	1	"	13.0	
	1	—	16	2.5	8.9
	1	1	"	11.4	
	1	2	"	13.1	10.6
	1	—	17	2.5	9.4
	1	1	"	11.9	

In the preparation of No. 1, acetic acid had been used and the co-enzyme had been carefully removed. The preparation had a very low initial activity, but its activity after the addition of the co-enzyme solution was not as great as that of No. 2, which had been prepared by Mr Myers-Ward without the use of acetic acid. These results fully confirm Rosenheim's observations and prove that the lipase of glycerol extracts of pig's pancreas can be separated into an inactive lipase and a co-enzyme.

The action of the constituents of the ash of the co-enzyme.

After incineration of the active dialysate of a co-enzyme solution, Rosenheim found the filtered ash solution devoid of any action on the inactive lipase. This result may have been due to the alkalinity of this solution. Pekelharing observed that the ash of the co-enzyme solution (not dialysed) if neutralised with hydrochloric acid, conferred a strong lipoclastic activity on the inactive lipase. No quantitative experiments, however, are described by Pekelharing. For the purpose of my experiments I carefully incinerated portions of 5 cc. of watery pancreas extracts in a platinum dish and dissolved the ash in a small volume of water, after adding a drop of methyl red and the necessary amount (0.2–1.0 cc.) of N/10 HCl. The solution was evaporated to dryness in order to remove any excess of hydrochloric acid and the residue was dissolved again in water. The clear solution was made up to 5 cc. and its action compared with that of the original co-enzyme solution.

5 cc. of a 0.2 % suspension of inactive lipase and 5 cc. of oil emulsion were used. Time of incubation: 17 hours. The necessary corrections (in cc. N/10 KOH) were: 1 cc. co-enzyme (a) 2.0; 1 cc. ash solution (a) 0.3; 1 cc. co-enzyme (b) 1.6; 1 cc. ash solution (b) 0.4. The acidity due to inactive lipase alone was 2.5 cc. N/10 KOH. The results are given in the following table.

		Degree of lipoclasia	
1.	Co-enzyme (a) cc.	Titred value	Corrected value
		N/10 KOH cc.	N/10 KOH cc.
	1	12.2	7.7
	1	12.4	7.9
	Ash solution (a) cc.		
	1	10.3	7.5
	1	9.2	6.4
2.	Co-enzyme (b) cc.		
	1	13.3	9.2
	1	13.0	8.9
	Ash solution (b) cc.		
	1	11.2	8.3
	1	10.9	8.0

From these results it seems that the solution of the ash of the co-enzyme, when dissolved in hydrochloric acid, possesses an activating power nearly equal to that of the original co-enzyme.

Dialysis experiments.

Two different preparations of the co-enzyme solution were dialysed, one through a parchment diffusion shell (Schleicher and Schüll) and one through a sheep's appendix according to W. Wiechowski [1907]. The dialysis against distilled water was continued for four days, until a complete absence of chlorides in the dialysate was observed. The dialysate and the contents of the dialysing membranes were concentrated to the original volume. 5 cc. of each of these solutions were incinerated as above described and the activity of the ash-solutions on inactive lipase compared with that of the original solutions. 5 cc. of olive oil emulsion and 10 mg. of inactive lipase in 5 cc. of water were used in each case. Time of incubation: 18 hours. The necessary corrections were made as before.

The results are given in the following table.

		Degree of lipolysis	
		Titrated value	Corrected value
		cc. N/10 KOH	cc. N/10 KOH
1 {	Dialysate cc. 1	10.9	7.6
	2	12.3	8.4
2 {	1	10.5	7.4
	2	14.7	9.7
Ash solution of dialysate			
cc.			
1 {	1	9.8	6.8
	2	9.6	7.3
2 {	1	9.3	6.8
	2	11.8	9.0
Dialysed solution			
cc.			
1 {	1	3.0	1.0
	2	3.7	1.4
2 {	1	2.5	0.7
	2	3.5	1.5
Ash solution of dialysed solution			
cc.			
1 {	1	2.6	0.8
	2	3.2	1.3
2 {	1	2.0	0.2
	2	2.8	0.9

These results prove that nearly the whole of the activating substances passes through the dialysing membranes as stated by Rosenheim. The ash of the incinerated dialysate, if dissolved in hydrochloric acid, produces practically the same effect as the original dialysate.

From these experiments it would appear that the activity of the co-enzyme solution is not lost by incineration. This conclusion is, however, not valid in its entirety as will be seen from the following experiments. It has been found in the course of some unpublished work carried out by the late Mr Myers-Ward in this laboratory that the co-enzyme solution could be separated into two fractions by means of absolute alcohol. Both of these fractions, the alcohol-soluble as well as the alcohol-insoluble fraction, were able to activate inactive lipase. It seemed therefore of interest, to investigate the action of the ash constituents of these fractions.

Fractionation experiments with alcohol.

10 cc. of watery extract of pancreas were poured into 90 cc. of boiling absolute alcohol. A flocculent precipitate formed, from which the alcoholic solution was decanted. On the addition of more boiling alcohol the precipitate became granular and was easily filtered off, washed with alcohol and ether, and dried in vacuo.

The alcoholic solution was evaporated to dryness and the residue again

Alcohol-soluble fraction of co-enzyme cc.	Degree of lipoclasia	
	Titrated value cc. N/10 KOH	Corrected value cc. N/10 KOH
1	6.5	3.3
1	6.0	2.8
Ash solution cc.		
1	3.2	0.6
1	3.0	0.4
Alcohol-insoluble fraction of co-enzyme cc.		
1	8.5	5.0
1	8.3	4.8
Ash solution cc.		
1	6.0	3.2
1	5.8	3.0
Original co-enzyme cc.		
1	13.0	9.1
Ash solution cc.		
1	9.0	6.2

taken up with boiling alcohol. The insoluble part was added to the main insoluble precipitate.

Before use the alcohol was evaporated and the residue dissolved in 10 cc. water. The alcohol-insoluble fraction also was dissolved in 10 cc. water.

5 cc. of each of these solutions were incinerated as described above and the action of these several fractions on inactive lipase compared with that of the original co-enzyme solution.

10 mg. of inactive lipase, 5 cc. oil emulsion. Time of incubation: 19 hours. The following corrections (cc. N/10 KOH) were necessary for the various solutions: 1 cc. alcohol-soluble fraction 0.7; 1 cc. ash solution 0.1; 1 cc. alcohol-insoluble fraction 1.0; 1 cc. ash solution 0.3; 1 cc. original watery extract 1.4; 1 cc. ash solution 0.3; 10 mg. inactive lipase 2.5. The results are given in the table on p. 45.

It is evident from these results that both the alcohol-soluble and the alcohol-insoluble fraction of the co-enzyme are able to activate inactive lipase, the former, however, to a smaller degree. Moreover, the greatest part of its activating power is destroyed by incineration. It is clear, therefore, that organic substances as well as electrolytes are concerned in this reaction.

I had also at my disposal an alcoholic extract of a co-enzyme solution prepared by the late Mr Myers-Ward. This had been prepared two years ago from a large quantity of material and had been purified to such an extent that its residue was completely alcohol-soluble. The solution had a slight deposit which was filtered off. When tested on the same inactive lipase preparation as previously, it was found to have lost some of its activity. This may have been contained in the insoluble deposit which was unfortunately no longer available. For comparison the results obtained by Mr Myers-Ward are given together with my own in the following table. The necessary corrections have been made.

Alcohol-soluble fraction of co-enzyme, cc.	Activity	
	(1912)	(1914)
1	12.7	5.1
1	12.7	5.1
Ash solution of same, cc.		
1	2.2	1.1
1	2.5	1.4

As was to be expected, the percentage of ash of this alcohol-soluble fraction was very small, namely 0.09 %, and it seemed to consist to a large extent of phosphoric acid. This led me to the investigation of the action of phosphoric

acid and phosphates on inactive lipase. Except for Rosenheim's statement [1910] that glycerophosphates activate inactive lipase, no attention seems to have been given up till now to the influence of phosphates on fat splitting. In this connection it is interesting to note that according to Plimmer and Scott [1908] the pancreas alone of all the tissues examined contains phosphoproteins, and that these substances disappear from the pancreas during the secretion of pancreatic juice and are transformed to the largest extent into water-soluble phosphates which appear in the pancreatic juice [Plimmer and Kaya, 1910].

Before proceeding to carry out experiments with phosphates on inactive lipase, I estimated quantitatively the percentage of inorganic and organic phosphorus compounds in the co-enzyme solution employed.

Total P was estimated in 5 cc. of the solution by Neumann's method. For the estimation of organic P, 15 cc. of the solution were precipitated with the same volume of magnesia mixture and P estimated in the filtrate. Inorganic P was estimated by difference. The following results were obtained: Total P: 0.36 %; organic P: 0.25 %; inorganic P: 0.11 % The total ash of the co-enzyme solution was 0.59 %.

Hence the percentages of P in the ash are:

Total P	61 %.
Organic P	42 %.
Inorganic P	19 %.

It will be seen that the ash contains a very large percentage of P, 42 % of which is present in the original solution as organic phosphorus compounds (nucleic acid, organic phosphates) and 19 % as inorganic phosphates.

The action of phosphates on inactive lipase.

The action of very dilute solutions of phosphoric acid was first examined. Increasing quantities of these were added to 5 cc. of a 0.2 % suspension of inactive lipase. After bringing up to equal volume with water and adding 5 cc. of oil emulsion the mixtures were incubated for 17 hours. The acidity due to the added phosphoric acid was allowed for. The results are given in the following table.

H_3PO_4 added cc.	Molecular concentration of H_3PO_4 in mixture	Degree of lipoclasia	
		Titrated value cc. N/10 KOH	Corrected value cc. N/10 KOH
0.1 M/100	M/17000	2.6	0
0.5 "	M/1400	3.1	+0.4
1.0 "	M/700	9.3	+6.5
0.2 M/10	M/350	11.7	+8.6
0.5 "	M/140	1.6	-2.4
Inactive lipase		2.5	0
Inactive lipase + co-enzyme (1 cc.)		13.5	9.4

These experiments show conclusively that very dilute phosphoric acid solutions are able to activate inactive lipase to a marked degree, whilst an inhibition is exerted if a certain limit is overstepped. I have not investigated how far this result is connected with the optimum of hydrogen ion concentration for pancreatic lipase, but in the following experiment mixtures of primary and secondary phosphates were employed which enabled me to judge roughly the hydrogen ion concentration of the digestion mixture.

A M/15 solution of primary potassium phosphate and a M/15 solution of secondary sodium phosphate were prepared according to Sørensen [1912]. 6 cc. of definite mixtures of these were added to the usual mixture of inactive lipase and oil emulsion. The mixtures were incubated for 19 hours. In the following table are given the composition of the various phosphate mixtures, their titration values against phenolphthalein and their hydrogen ion concentration together with the effect they produced on inactive lipase.

	Phosphate mixture		Titration value of 6 cc. in cc. N/10 KOH	P_H	Degree of lipoclasia	
	Primary cc.	Secondary cc.			Titrated value cc. N/10 KOH	Corrected value cc. N/10 KOH
1.	10.0	0.0	3.6	(4.494)	6.3	1.8
2.	9.75	0.25	2.4	5.288	6.2	2.9
3.	9.0	1.0	1.7	5.906	7.1	4.5
4.	7.0	3.0	1.3	6.468	6.6	4.4
5.	5.0	5.0	0.8	6.813	8.6	9.6
6.	3.0	7.0	0.5	7.168	11.2	9.8
7.	1.0	9.0	0.2	7.731	11.3	10.2
8.	0.0	10.0	0.1 N/10 H_2SO_4	(9.182)	6.7	5.9
Inactive lipase					0.9	—
Inactive lipase + co-enzyme					8.5	7.6

It is evident that all these phosphate mixtures appear to activate lipase. The action is most marked when a mixture in the proportion of 3:7 or 1:9 of primary and secondary phosphates is used. This fact incidentally explains also the failure of Rona and Bien [1914] to detect the presence of the co-enzyme in their extracts, as its effect was hidden by that produced by the phosphate mixtures employed by them in order to regulate the hydrogen ion concentration of their mixtures.

How far the action of phosphates is due to a direct action on the enzyme itself or to the production of a favourable reaction (hydrogen ion concentration) for its action, must be left for future investigations.

The action of calcium salts on inactive lipase.

The fact that Pekelharing [1912] ascribes to calcium the greatest part of the activating effect of the ash solution of the co-enzyme, without

adducing, however, any figures to show that calcium is present in sufficient amount in this solution, induced me to estimate quantitatively the calcium contents of my extracts. Qualitative experiments showed that its quantity was very small and 200 cc. of the extract were therefore taken for the estimation, which was made by Aron's method, as modified by R. von der Heide [1914]. 200 cc. gave 0.0436 g. calcium oxalate = 0.02 % CaCl_2 .

A series of experiments was next carried out, in which increasing amounts of calcium chloride were added to 5 cc. of a 0.2 % suspension of inactive lipase. After bringing up to equal volume with water and adding 5 cc. of olive oil emulsion the mixtures were incubated for 17 hours. The action of calcium chloride was compared with that of the original co-enzyme and its ash solution. The usual controls were made.

Calcium chloride content of mixture	Degree of lipoclasia	
	Titrated value cc. N/10 KOH	Corrected value cc. N/10 KOH
M/7000	2.5	0
M/1400	2.5	0
M/700	5.0	+2.5
M/70	4.5	+2.0
M/7	3.1	+0.6
M/3.5	2.0	-0.5
Inactive lipase alone	2.5	0
Inactive lipase + co-enzyme (1 cc.)	12.4	8.3
Inactive lipase + ash solution (1 cc.)	11.0	8.1

From these results we may conclude that calcium chloride, if present in the digestion mixture to the amount of M/7000 to M/1400 has no effect on the lipoclasia. As the calcium content of 1 cc. of the ash solution of the co-enzyme corresponds to a M/4116 solution, it seems justifiable to conclude that the action of the ash solution is not due to calcium. Even if it were present in much larger proportion (M/700 to M/70) its effect would still be far below that of the ash solution itself.

I have also examined in a similar way the action of primary calcium phosphate, $\text{CaH}_4(\text{PO}_4)_2$, as this salt might possibly be present in the solution of the ash. The following results were obtained.

$\text{CaH}_4(\text{PO}_4)_2$ solution added cc.	Titration value cc. N/10 KOH	$\text{CaH}_4(\text{PO}_4)_2$ contents of the mixture	Degree of lipoclasia	
			Titrated value cc. N/10 KOH	Corrected value cc. N/10 KOH
0.1 M/100	trace	M/7000	2.8	0.6
1.0 "	0.4	M/700	4.0	1.4
2.0 "	0.8	M/350	7.6	4.6
1.0 M/20	1.3	M/140	7.1	3.6
1.5 "	1.9	M/95	5.7	1.6
2.0 "	2.5	M/70	4.9	0.2

These experiments show that primary calcium phosphate has a certain amount of activating action, which must, however, be ascribed to its phosphoric acid ions if we consider the results of the preceding experiments. But even its maximum effect (M/350) accounts for only about 50 % of that of the ash solution of the co-enzyme.

Whilst therefore admitting the possibility that calcium salts may act in the way suggested by Pekelharing¹ (*i.e.* by precipitating a small amount of fatty acid as an insoluble soap), it seems from the above experiments that its rôle as an activator of inactive lipase is in any case an insignificant one.

The action of sodium chloride on inactive lipase.

As sodium chloride forms a constituent of the co-enzyme solution, I have made a few experiments with this salt. 5 cc. of a 0.2 % suspension of inactive lipase to which increasing amounts of sodium chloride solution had been added, were brought up to equal volume with water, mixed with 5 cc. of oil emulsion and incubated for 19 hours. The results are given in the following table.

NaCl concentration of the mixture	Degree of lipoclasia	
	Titrated value cc. N/10 KOH	Corrected value cc. N/10 KOH
M/700	1.8	0.9
M/175	2.2	1.3
M/100	3.0	2.1
M/70	3.9	3.0
M/17.5	2.8	1.9
M/10	2.7	1.8
M/7	2.2	1.3
Inactive lipase alone	0.9	0
Inactive lipase + co-enzyme (1 c.)	9.5	7.0

It would seem from these experiments that the action of sodium chloride is not of any great significance. Its effect increases to a certain limit, after which it decreases again. It may be mentioned here, however, that Dr Shaw-Mackenzie and the late Mr Myers-Ward occasionally observed a considerable activating influence of sodium chloride on inactive lipase. No explanation has as yet been found for these contradictory results.

DISCUSSION.

In considering the state of our present knowledge of the conditions governing the activities of pancreatic lipase, it is essential to differentiate sharply between the lipase of glycerol extracts of pancreas and the lipase as

¹ See, however, Rona and Bien [1914], whose observations on the action of calcium on pancreatic and serum lipase do not favour this explanation.

contained in pancreatic juice. The activity of the latter is in no way impaired by ordinary filtration and it is evident that the enzyme exists in a soluble condition in pancreatic juice. In glycerol extracts of pancreas, however, the material to which its lipoclastic activity is due occurs in a colloidal suspended condition and it requires a soluble co-enzyme for its activation.

The changes by means of which the insoluble lipase of the organ is transformed into the soluble enzyme of the pancreatic juice during the process of the secretory activity of the organ, are at present still unknown. With the methods at our disposal at present, it has also been found impossible to demonstrate the presence of a co-enzyme for the soluble lipase of pancreatic juice. It is, however, to be expected that the study of substances which possess a solvent action on the inactive lipase of glycerol extracts may give us a clue as to the conditions which govern the solubility of the lipase of pancreatic juice.

Various substances are now known which are able either to activate inactive lipase or to accelerate the action of active lipase. It seems that these substances may be divided into three groups: (1) The salts of Na, K, Ca, Ba, Mg; (2) organic and inorganic phosphates; and (3) bile salts and their derivatives. Very little is known, however, with regard to their mode of action.

In the case of the inactive lipase of pancreatic extracts any explanation is complicated by the fact that we are dealing with an insoluble enzyme acting on an equally insoluble zymolyte (fat). The action of neutral salts may therefore be partly due to a physical effect either on the degree of emulsification of the fat or on the degree of colloidal suspension of the enzyme. Pekelharing has further made it probable that the relatively small effect of electrolytes may be ascribed to the removal of one of the products of reaction (fatty acid) from the system.

This explanation seems, however, hardly admissible for the substances belonging to the second group. Their action may be due to a direct action on the enzyme or perhaps in the case of phosphates to the fact that their hydrogen ion concentration corresponds to the optimum activity of the enzyme. Another explanation of the action of phosphates may be their possible rôle in linking the inactive enzyme to the fat and giving rise to the intermediate formation of glycerophosphoric acid.

In the last class of substances, the bile salts, it has been made most probable by Rosenheim's observations that their active constituent, cholalic acid, acts directly on the enzyme. A similar conclusion has also been reached by Terroine [1910].

The nature of the activating substances contained in a boiled watery extract of pancreas is not yet completely cleared up. Bile salts are absent and of the inorganic substances it seems that the main rôle is due to phosphates. The nature of the organic activator remains still to be discovered.

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CONCLUSIONS.

- (1) Phosphates represent the most active inorganic constituents of the co-enzyme of inactive lipase obtained from pancreatic extracts.
- (2) Organic substances, probably organic phosphates, are also concerned in the activation of inactive lipase.

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