VI. ON THE AUXO-LIPASE OF SERUM.

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The addition of small amounts of serum to pancreatic extracts increases their fat-splitting power to a remarkable degree. This fact was established by Pottevin [1903] and independently by J. A. Shaw-Mackenzie in his joint work with O. Rosenheim on the action of lipase contained in glycerol extracts of pancreas on olive oil and ethyl butyrate [1910]. The lipase of pancreatic juice (dog) as well as that of human pancreatic juice [Shaw-Mackenzie, 1912] are similarly influenced by serum. Minami [1912], apparently without knowledge of the previous work, recorded similar observations, using monobutyryl as the zymolyte.

With regard to the chemical nature of this accelerating substance, for which I propose the name of auxo-lipase¹, Rosenheim and Shaw-Mackenzie only made some preliminary statements, according to which it seemed to withstand the temperature of boiling water and was not destroyed by putrefaction. Further, it appeared to be soluble in dilute alcohol and to dialyse through membranes².

In a recent paper J. Mellanby and Woolley [1914] made a useful suggestion with regard to the nature of this reaction, which lends itself to experimental investigation. They consider that the increase of lipoclastic power on the addition of serum to pancreatic extracts is due to the protection of the lipase from the action of trypsin by the antitrypsin contained in the added serum.

The properties of auxo-lipase, as far as they have been investigated, suggest a connection with the co-enzyme of pancreatic lipase. The purpose of this investigation was in the first instance to compare the behaviour of

¹ Similar auxo-substances in serum have already been described in literature (see M. Falk, [1914]; Guggenheimer, Minami).

² I am informed that in later experiments Rosenheim and Shaw-Mackenzie were unable to confirm this preliminary statement.

these two substances and to establish their identity or difference. Further, the relationship of auxo-lipase to the antitrypsin of serum was experimentally investigated. This relationship is of interest since it has been shown by Shaw-Mackenzie [1912, 1913] that variations in amount of the auxo-lipase of serum together with variations in amount of antitrypsin may be of diagnostic value in certain pathological conditions.

EXPERIMENTAL.

Since the action of the auxo-lipase is not specific, I have limited my work, for convenience sake, to the investigation of ox-serum. Olive oil was chosen as the zymolyte, as this fat gives much larger values than those obtained by the action of pancreatic lipase on various esters of the lower fatty acids. The method used for the estimation of the lipoclastic activity was the same as that of Rosenheim and Shaw-Mackenzie. The precautions necessary for comparable results have been fully described in the communication of Umeda [1915]. Instead of phenolphthalein, I used, however, a 2 % alcoholic solution of thymolphthalein. This indicator, introduced by Sørensen [1908], changes into a deep blue with alkali, a change which appears to afford a sharper end point in the presence of the pigments of the serum than that of phenolphthalein. All the experiments were carried out in duplicate.

The presence of auxo-lipase in serum.

In order to convince myself of the existence of auxo-lipase in serum, I carried out a large number of preliminary experiments with sera and pancreatic extracts obtained at different times. The reaction was found to be remarkably constant, and the following experiment, chosen at random from many, illustrates the result obtained.

A pancreatic extract was prepared by mixing 2.5 kg. of finely minced pig's pancreas with 5 litres of glycerol. The mixture was strained through two layers of muslin after 24 hours. Before use one part of this extract was diluted with two parts of water. 1 cc. of this diluted extract, which will be designated as "lipase" in the following, was used in each experiment, the serum being added next and the fat emulsion last. The mixture was incubated for 17–24 hours, this period being chosen for convenience sake. In the following table the effect due to auxo-lipase is shown in the last column, expressed in cc. N/10 KOH¹.

¹ The amounts of N/10 KOH required by the pancreatic extract and by the emulsion alone have in all cases been deducted from the results given in this and the following tables.

Lipase cc.	Serum cc.	Emulsion cc.	Water cc.	N/10 KOH cc.	Effect of Auxo-lipase
1	—	3	3	13.3	—
1	1	3	2	27.1	13.8
1	2	3	1	36.7	23.4
1	3	3	—	37.3	24.0

When using the same amounts of serum and pancreatic extracts of different origin and incubating for the same time practically identical figures were obtained, the variations not exceeding ± 1 cc. N/10 KOH.

In the following series of experiments a smaller amount of pancreatic extract was taken and increasing amounts of serum from 0.2 cc. to 3.0 cc. were added. The results are shown in the following table.

Lipase cc.	Serum cc.	Emulsion cc.	Water cc.	N/10 KOH cc.	Effect of Auxo-lipase
0.5	—	3	3.5	5.9	—
0.5	0.2	3	3.3	17.7	11.8
0.5	0.4	3	3.1	20.8	14.9
0.5	0.6	3	2.9	22.4	16.5
0.5	0.8	3	2.7	24.9	19.0
0.5	1.0	3	2.5	28.2	22.3
0.5	1.5	3	2.0	31.9	26.0
0.5	2.0	3	1.5	33.2	27.3
0.5	3.0	3	0.5	35.8	29.9

Fig. 1 (p. 56) gives a graphic representation of the increase of the auxo-lipoclastic effect produced on the addition of increasing amounts of serum.

The auxo-lipase of serum is not only able to accelerate the action of active lipase but also to activate inactive lipase. The few observations which I have made in confirmation of Rosenheim's [1910] statements, were carried out with a sample of inactive lipase prepared by Dr Umeda in this laboratory. 10 mg. of inactive lipase suspended in 1 cc. of water, by itself practically without any action on olive oil, produced together with 1 cc. of co-enzyme an effect nearly equal to that of 1 cc. of my own diluted pancreatic extract. The following table shows the results produced on the addition of 2 cc. of serum to 10 mg. of inactive lipase.

Inactive Lipase 10 mg. in 1 cc.	Serum I	Serum II	Serum III	Co-enzyme	Lipoclastic effect cc. N/10 KOH
1	—	—	—	—	0.3
1	—	—	—	1	10.7
1	2	—	—	—	28.1
1	—	2	—	—	27.4
1	—	—	2	—	28.7

Not only has the addition of serum activated inactive lipase, but the effect produced is even greater than that of the co-enzyme.

These experiments leave no doubt about the existence of the auxo-lipoclastic action of serum. It may be mentioned, however, that in order to observe this reaction, it is necessary that enzyme, serum, and zymolyte shall be mixed in the order given. The following experiment illustrates the effect produced by an alteration in the order of mixing. In experiment (a) lipase and serum were mixed before the addition of the fat emulsion, whilst in

Fig. 1.

experiment (b) serum was added to the mixture of lipase and fat emulsion. The mixtures were brought to an equal volume by the addition of water and incubated for 24 hours.

(a)				(b)			
Lipase cc.	Serum cc.	Emulsion cc.	Effect of Auxo-lipase	Lipase cc.	Emulsion cc.	Serum cc.	Effect of Auxo-lipase
1	1	3	14.6	1	3	1	6.7
1	2	3	26.1	1	3	2	10.2
1	3	3	27.8	1	3	3	12.7

This experiment clearly demonstrates that by altering the order of mixing, the action of the auxo-lipase is diminished by over 50 %. The significance of this fact for the explanation of the reaction will be discussed later.

The stability of auxo-lipase.

In order to test the stability of the auxo-lipoclastic substance contained in serum, its effect was estimated in fresh serum and in portions which had been kept for over a month with and without toluene. The serum kept without a preservative had become putrid.

No difference was found between the action of fresh serum and that kept under toluene. To save repetition the figures are not given. The auxo-lipase in putrefied serum, on the other hand, had not only not been destroyed, but even slightly increased. In this and the following experiments 1 cc. of lipase and 3 cc. of emulsion were used and the mixtures brought up to equal volume by the addition of water. The necessary corrections were made as before. The figures were:

Putrefied serum cc.	Effect of Auxo-lipase
1	16.0
2	23.4
3	27.2

This increase may be due to concentration of the serum owing to evaporation, as it had been kept in an open flask in order to encourage putrefaction. These experiments confirm the results previously obtained in this laboratory that serum retains its auxo-lipoclastic activity even when kept for many months.

The heat-resistance of auxo-lipase.

This was tested by keeping undiluted serum for 0.5 hour in a water-bath at a temperature of 60°, and another portion, which had been diluted with an equal volume of water, for 10 minutes in a boiling water-bath. The various sera were tested in the usual way. The results are given in the following table, showing the effect of the auxo-lipase contained in 1, 2 and 3 cc. of serum, expressed in cc. N/10 KOH.

Fresh serum	Serum kept at 60° for 0.5 hour	Diluted serum	Diluted serum kept at 100° for 10 minutes
13.8	14.2	4.6	9.1
23.4	25.9	8.3	24.8
24.0	28.0	18.4	29.5

Auxo-lipase is therefore not destroyed by heating to 60° or 100°, but its action is even increased considerably. The significance of this fact and its bearing on the relationship of antitrypsin and auxo-lipase will be discussed later.

The effect of the removal of the proteins.

It was hoped to obtain more information about the nature of the auxo-substance by the removal of the proteins, which was effected (a) by heat coagulation in the presence of acetic acid, (b) by alcohol, and (c) by means of colloidal iron. In each case, however, the active substance was either carried down together with the protein or rendered inactive, and further the protein-free filtrates exerted even an inhibitory action on pancreatic lipase.

(a) *Heat coagulation in presence of acetic acid.* It was found that the presence of acetic acid did not interfere with the reaction at 37°, but that when the serum had been heated to 100° after the addition of acetic acid, its accelerating action was converted into an inhibition of the lipase.

A few drops of glacial acetic acid were added to 25 cc. serum, until an acid reaction to litmus was distinct.

Quantities of 2 cc. and 3 cc. were measured into a series of flasks. One series was kept for 10 minutes at 100° in a water-bath. The action of heated and unheated acidified serum was compared with that of normal serum in the usual way. The figures represent the increase or decrease (expressed in cc. N/10 KOH) of the lipoclastic action of 1 cc. of lipase after the addition of 2 and 3 cc. of the sera.

Normal serum	Serum + acetic acid (unheated)	Serum + acetic acid (heated for 10 minutes at 100°)
+16.4	+17.8	-5.5
+21.1	+21.2	-6.0

It seemed to be interesting to analyse the inhibitory action of the heated acidified serum further and to investigate whether the auxo-lipase was actually destroyed or if it was possible to restore its action by subsequent neutralisation.

25 cc. of serum were diluted with an equal volume of water, acidified with a few drops of acetic acid and kept at 100° for 10 minutes. The coagulated protein was filtered, finely ground in a mortar, boiled up with distilled water and again filtered and washed. It was then suspended in 50 cc. of water and one half of it neutralised with N/10 KOH.

The combined filtrates were concentrated, made up to 25 cc. with water and one half of the solution was neutralised with N/10 KOH.

The following results were obtained when testing the action of 2 cc. and 3 cc. of these fluids on lipase as before.

Protein suspension		Filtrate	
Washed	Neutralised	Acid	Neutralised
-6.4	+ 7.0	-6.0	-1.2
-5.7	+10.8	-5.8	-0.9

It will be seen that both the coagulated protein and the filtrate acquire an inhibitory action, when serum is heated in the presence of acetic acid. It seems further that the auxo-lipase is carried down with the coagulated protein, and is set free again by careful neutralisation, whilst the neutralised filtrate still remains inhibitory.

It may be mentioned in this connection that I have found in some preliminary experiments that milk also contains a substance which accelerates lipoclastic action. In this case also it is carried down by acetic acid with the caseinogen of milk, and set free again by the addition of a small amount of alkali.

(b) *Removal of proteins by alcohol.* 40 cc. of serum were poured into 400 cc. of boiling absolute alcohol, kept on a water-bath for half an hour and filtered. The coagulated protein was freed from alcohol by drying at 40°, finely powdered and divided into four parts. One part was suspended in 20 cc. of water. Another part was suspended and boiled with 20 cc. of water and finally to another part, suspended in 20 cc. of water, a few drops of N/10 KOH were added so as to make its reaction faintly alkaline to litmus.

The alcoholic filtrate was evaporated to dryness on the water-bath and the residue taken up with 200 cc. of absolute alcohol. After filtration the alcohol was evaporated and the residue taken up with 40 cc. of water.

The action of the various fractions on lipase was tested as before, 1 cc. and 2 cc. of each fraction being used. The results are given below.

Alcohol-Coagulum			Alcohol Extract
Suspended	Boiled	Neutralised	
+1.0	+3.4	+ 7.2	-0.1
+4.7	+7.6	+20.8	+0.7

Here again we see that the auxo-lipase is carried down with the coagulated protein, but in distinction from the acetic acid coagulum it retains its activity. Further, in analogy with its behaviour in the original serum, its activity is increased by boiling and still further by the addition of a small quantity of

alkali. The protein-free alcoholic extract is practically inactive. The insolubility of auxo-lipase in absolute alcohol differentiates this substance from the co-enzyme of lipase contained in watery pancreatic extracts.

(c) *Removal of proteins by colloidal ferric hydroxide.* By the use of this method [Michaelis and Rona, 1908] every trace of protein is removed from serum in the cold, without the introduction of any foreign substance into the filtrate. To 25 cc. of serum, diluted with 250 cc. of water, 20 cc. of colloidal ferric hydroxide solution were gradually added. The precipitated colloids were filtered off and washed until the wash-water was free from chlorides. The clear filtrate and wash-water were evaporated to 25 cc. The solution, containing the electrolytes and crystalloids of serum, was found to possess an inhibitory action on lipase.

When tested in the usual way the inhibitory action (expressed in cc. N/10 KOH) was: for 1 cc., — 0.5, and for 2 cc., — 1.1.

Evidently the auxo-lipase is removed together with the proteins by colloidal iron¹, a fact which speaks in favour of the colloidal nature of this substance. In order to test this still further, the behaviour of the auxo-lipase on dialysis was investigated.

The behaviour of auxo-lipase on dialysis.

As all the experiments which I carried out in this direction gave identical results, it will be sufficient to describe one of them in full. 30 cc. of serum were dialysed for seven days in a parchment diffusion shell (Schleicher and Schüll), toluene being used as a preservative. The distilled water was changed twice daily until it was free from chlorides. The dialysate was concentrated to 30 cc.

The dialysed fluid, which measured 48 cc., was divided into two parts. One half was used as such, whilst the other half was centrifugalised in order to test the action of the euglobulin fraction separately. Further, one part of the centrifugalised fluid, containing the serum albumin and pseudoglobulin, was heated on a water-bath for 10 minutes. The various fractions were tested as before, 1 cc., 2 cc. and 3 cc. being used of each and allowance being made for the dilution of the serum during dialysis. The results are given in the following table, the increase or decrease of the lipoclastic action being expressed in cc. N/10 KOH.

¹ The colloidal precipitate was not tested, as the large amount of iron in it was expected to render the result inconclusive.

Original serum	Dialysed serum	Dialysate	Albumin + Pseudoglobulin		Euglobulin
			Unheated	Heated	
+ 13.8	+ 16.4	- 3.2	+ 18.4	+ 21.1	+ 2.0
+ 23.4	+ 21.3	- 3.8	+ 21.4	+ 28.3	+ 4.2
+ 24.0	+ 24.1	- 6.5	+ 24.4	+ 32.2	+ 4.3

The colloidal nature of the auxo-lipase is strikingly confirmed by this experiment, the action of the dialysed serum being little different from that of the original serum. Here again we meet with a characteristic difference between the behaviour of auxo-lipase and the co-enzyme of lipase, which diffuses easily [Umeda, 1915]. The experiments on fractionation of the serum proteins, as far as they go, point to an association of the auxo-lipase with the albumin and pseudoglobulin fraction. I was unable, owing to lack of time, to complete the fractionation.

The inhibitory action exerted by the dialysate suggests the question whether the inhibition is due to a substance of organic nature, or whether the electrolytes are responsible for it. As it has been shown by Umeda [1915] that the inorganic constituents of the co-enzyme of lipase are largely concerned in the activation of inactive lipase, an investigation of this question was likely to throw further light on the question of the identity of auxo-lipase and the co-enzyme.

The action of the mineral substances of serum.

The mineral substances remaining after the incineration of the whole serum as well as those of the dialysate were examined. 10 cc. of serum and 10 cc. of the dialysate (see above) were incinerated, the usual precautions being taken to avoid loss. The ash was in each case taken up with water and neutralised with N/10 HCl, using methyl-red as an indicator. The solution was evaporated to dryness, in order to drive off any excess of hydrochloric acid and the residue made up to 10 cc. with water. The following table shows the results, expressed in cc. N/10 KOH. For comparison the results obtained from the dialysate before incineration are added.

	1 cc.	2 cc.	3 cc.
Dialysate	- 3.2	- 3.8	- 6.5
Ash of dialysate	- 1.1	- 1.7	- 4.8
Ash of serum	- 2.5	- 2.9	- 4.2

From these results it would seem that a large part of the inhibitory action of the dialysate is due to its mineral constituents, but that a certain amount of it is of an organic nature and lost by combustion. In sharp distinction

from the inorganic constituents of the co-enzyme of lipase, those of the serum not only fail to produce an accelerating effect but even inhibit lipoclastic activity, thus furnishing further evidence against the identity of auxo-lipase and co-enzyme.

The effect of serum lipoids.

In view of the fact that auxo-lipase is non-dialysable the possibility of its lipid nature had to be considered. It has already been shown above, that an alcoholic extract of serum is practically inactive. I have further investigated the solubility of auxo-lipase in ether. For this purpose I made use of a serum powder obtained by drying serum in Petri dishes at 40° by means of an electric fan. I have convinced myself that a solution of this powder possesses the same effect as the original serum. 2 g. serum powder, corresponding to 20 cc. serum, were extracted in a Soxhlet apparatus with ether for four days. The residue of the ether extract was suspended in 20 cc. of water and tested in the usual way. Its effect on lipase (expressed in cc. N/10 KOH) was: 1 cc., 0; 2 cc., - 1.8; 3 cc. - 2.6.

This result together with the negative result produced by the alcohol extract shows that auxo-lipase is not of lipid nature.

The relationship of auxo-lipase to antitrypsin.

It has already been mentioned above that the well-known presence of antitrypsin in serum has been suggested as affording an explanation of the auxo-lipoclastic action of serum. According to this view antitrypsin by inhibiting the action of trypsin prevents also the supposed destructive action of this enzyme on lipase.

The above described experiments on the heat resistance of auxo-lipase (p. 57) make it impossible to accept this view. According to Cathcart [1904] antitrypsin of serum—the albumin fraction—is destroyed on boiling and “is rapidly injured by heating. A temperature of 55° for half an hour suffices both in the presence and absence of alkali to destroy to a large extent the anti-action.” Further, Hedin [1906] showed that it is readily destroyed at blood-heat by dilute acetic acid. In the above described experiments serum was kept at 60° for 0.5 hour and at 100° for 10 minutes, under which conditions antitrypsin is undoubtedly injured and destroyed. In spite of this the effect of the auxo-lipase was found in both cases considerably increased. Further, the action of auxo-lipase was not affected by dilute acetic acid at blood temperature.

Further experimental evidence against the assumed interaction of trypsin, antitrypsin and lipase is furnished by the following observation. Diluted pancreatic extract (1:3) was incubated for 24 hours, toluene having been added. The solution had acquired a powerful tryptic action which was found on comparison to be equal to that of a 1 % solution of Kahlbaum's trypsin. Under these conditions the lipase of pancreatic juice is entirely destroyed. It was therefore to be expected, if the lipase of pancreatic extract behaved like that of pancreatic juice, that the subsequent addition of serum to incubated pancreatic extract would fail to produce any effect, if its action were merely due to the antitrypsin contained in it. The following results were obtained on comparing the activity of freshly diluted and of incubated pancreatic extract. The effect due to auxo-lipase is expressed in cc. N/10 KOH.

Pancreatic extract		Serum	N/10 KOH cc.	Auxo-lipase
Freshly diluted	Incubated for 24 h.			
1	—	—	9.6	—
—	1	—	10.3	—
—	1	1	29.2	18.9
—	1	2	34.8	24.5

It will be seen that in distinction from the lipase of pancreatic juice, that of pancreatic extract is in no way affected by incubation for 24 hours, in spite of the development of trypsin. This protection may possibly be due to the presence of glycerol. Further, on addition of serum the action of auxo-lipase is even somewhat more strongly marked than in the case of freshly diluted extract (see p. 55).

DISCUSSION.

The experimental material now available enables us to define the general properties of auxo-lipase somewhat more closely and to compare them with those of the co-enzyme of lipase.

Auxo-lipase of serum is thermostable, non-dialysable, insoluble in absolute alcohol and ether, and destroyed by incineration. It is associated with the serum-proteins and carried down with them by alcohol, by heat coagulation or by colloidal ferric hydroxide. Of the serum proteins the albumin and pseudoglobulin fraction appear to have a greater affinity to auxo-lipase than the euglobulin fraction. It is also noteworthy that serum contains an organic anti-lipoclastic substance the action of which is, however, masked by that of the auxo-lipase.

From its inability to pass through a dialysing membrane, one may conclude that auxo-lipase is of a colloidal nature, a conclusion which is strengthened by its behaviour towards colloidal ferric hydroxide.

This property sharply differentiates it from the co-enzyme of lipase, which dialyses easily and the activity of which, moreover, is not greatly diminished by incineration. The experimental evidence seems therefore to justify the conclusion that the auxo-lipase of serum and the co-enzyme of lipase are not identical.

With regard to the relationship of auxo-lipase and antitrypsin my experiments make it unfortunately impossible to accept the very plausible explanation advanced by Mellanby and Woolley. It may further be pointed out that from the experimental work of Shaw-Mackenzie [1912, 1913] it follows clearly that the antitryptic index of serum does not run parallel to its content in auxo-lipase and that, for instance, in the same individual a high antitryptic index may be associated with a low auxo-lipase content or a high auxo-lipase content of the serum associated with a low antitryptic index.

In dealing with this complex reaction the important factor of the physical condition of enzyme and zymolyte must not be lost sight of. As both these are insoluble, the presence of a colloidal solution like serum may influence the degree of emulsification of the zymolyte (fat) or the colloidal suspension of the enzyme (lipase).

From the experiment recorded above, however, in which the importance of the order of mixing was emphasised, we may conclude that this reaction is due to a direct action of the auxo-lipase of serum on the enzyme.

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CONCLUSIONS.

1. The auxo-lipase of ox-serum is non-dialysable, thermostable and destroyed by incineration.
2. The auxo-lipase of serum and the co-enzyme of lipase are not identical.
3. No relationship could be demonstrated between the antitrypsin and the auxo-lipase of serum.

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