CHAPTER 6

THE PIGEON ADIPOSE TISSUE LIPASE

The occurremce of a 'true lipase' in the adipose tissue of the pigeon and certain other vertebrates has already been demonstrated employing both histochemical as well as quantitative methods. The significance of the considerable quantities of lipase present in this tissue has been discussed in the earlier chapters. The results of the investigations undertaken to elucidate the properties of the pigeon adipose tissue lipase are presented in this chapter. The properties of this adipose tissue lipase are compared with those of the same enzyme occurring in the pancreas (Scaria,1958) and the breast muscle of the pigeon, as reported by George and Scaria (1959). The experiments were conducted under identical conditions.

Material and Methods

The enzyme material used was the ether-defatted visceral adipose tissue, covering the coils of the intestine, of the pigeon (Columba livia). Acetone was found to reduce the activity of the adipose tissue lipase and according to Scaria (1958) the pigeon breast muscle lipase is totally inactivated by acetone. In the present studies wherever defatting was necessary, ether was used as the fat solvent in histochemical as well as quantitative procedures. Fiore and Nord (1950) used an ether defatted dry powder of Fusarium lini Bolly as the enzyme material for the study of the lipase of this mould. Scaria (1958) also used an ether defatted powder of the breast muscle and the pancreas of

the pigeon for a study of this enzyme. I have also found this to be highly satisfactory. The visceral adipose tissue of decapitated pigeons were removed, cut into small pieces and kept in ethyl ether at room temperature for 2 hours with two changes of the solvent. Subsequently this was dried in a vacuum desiccator at room temperature, and stored in cold (4°C). An aqueous extract of this was used as the enzyme solution in all the experiments.

40 - 50 mg of this defatted tissue was extracted in 4 ml of distilled water in cold (4°C) for 1 hour by grinding it in a mortar. It was then transferred whole to a centrifuge tube and centrifuged for 5 minutes at about 2500 r.p.m and the resulting supernatant used as the enzyme solution. The protein content in 1 ml of this enzyme solution was on the average 1.2 mg. Protein was estimated by the micro-Kjeldahl steam distillation method for total proteins (Hawk et al,1954). The nitroprusside reaction for sulphydryl groups (-SH groups) and lead-blackening test for sulphur were tried. The enzyme was also treated with excess BAL (British Anti-Lewisite, 2:3 dimercaptopropanol) to assess the presence of metals, if any. Metals like iron and copper is known to give a precipitate when treated with BAL (Barron et al,1947a; Webb and van Heyningen,1947).

Lipolytic activity of the extract was determined manometrically in a bicarbonate carbon dioxide buffer system of pH 7.4 at 37°C (Martin and Peers,1953). An emulsion of tributyrin (4% v/v) in 0.0148 M $NaHCO_3$, prepared by shaking in a small Erlenmeyer flask with a small drop of "Tween 80" was used as the substrate. Each reaction flask contained 1.5 ml of 0.025 M $NaHCO_3$, 0.5 ml of the substance under test (the various chemicals used)

in concentrations to give final concentration as noted below, and 0.5 ml substrate in the side arm in a total fluid volume of 3 ml. The test solutions were introduced into the flasks before the addition of the enzyme, unless otherwise stated. The flasks and manometers were gassed for 3 minutes with a mixture of 95% N_2 and 5% CO_2. After equilibration for 10 minutes in the constant temperature water bath (37°C), the substrate was tipped in and again allowed to equilibrate for another 3 minutes. This period was sufficient to ensure complete mixing of the contents of the flasks. The readings were taken after every 15 minutes for 1 hour. For each experiment a control was run in which 0.5 ml distilled water was added in place of the solution under test. Autohydrolysis was found to be nil. The readings given are after correction for the thermobarometer. The controls produced on the average about 218 µl CO_2, equivalent to the amount of butyric acid liberated.

Results

Effect of various substances

Inorganic salts: (Figures 1 and 2) $CaCl_2$ at all the concentrations tried, activated the enzyme. NaCl, KCl, $MgCl_2$ and NH_4Cl were inhibitory.

Intermediate metabolites: (Figure 3) All the intermediate metabolites tested had a marked inhibitory effect on this enzyme at concentrations 0.0025 M to 0.01 M.

Amino acids: Of the two amino acids tested L-methionine inhibited the enzyme at all the concentrations tried, whereas histidine activated it (Figure 4).

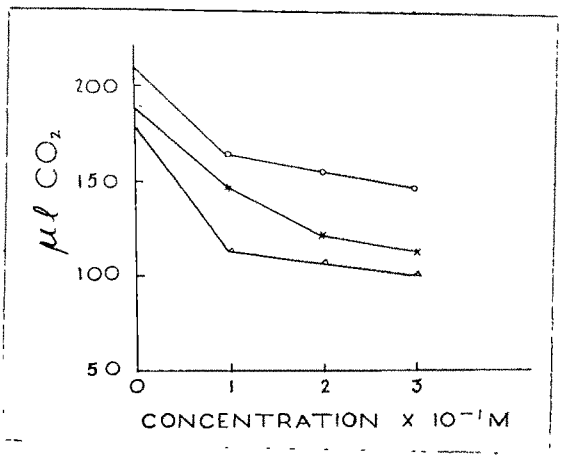

Fig. 1

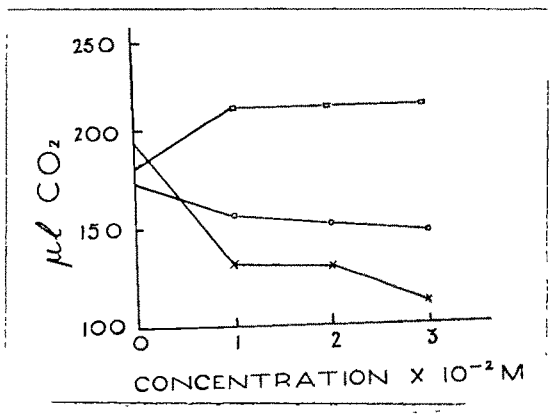

Fig. 2

Fig. 1 and 2 - Effect of inorganic salts on the pigeon adipose tissue lipase. x,NaCl; o,KCl; △,NH_4Cl; (Fig. 1). x,$MgCl_2$; □,$CaCl_2$; o,KCl (Fig. 2).

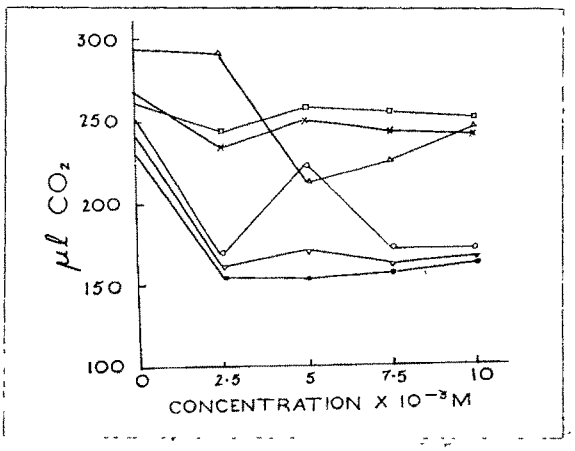

Fig. 3

Fig. 3 - Effect of intermediate metabolites on the pigeon adipose tissue lipase. x,succinate; □,pyruvate; △,malate; o,∝ketoglutarate; ▽,lactate; ●,citrate.

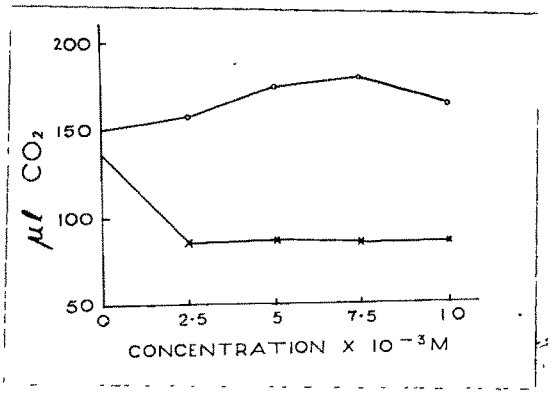

Fig. 4

Fig. 4 - Effect of amino acids on the pigeon adipose tissue lipase. x,L-methionine; o,histidine.

ATP: was found to activate the enzyme at a progressive rate from 0.00025 M to 0.001 M (Figure 5).

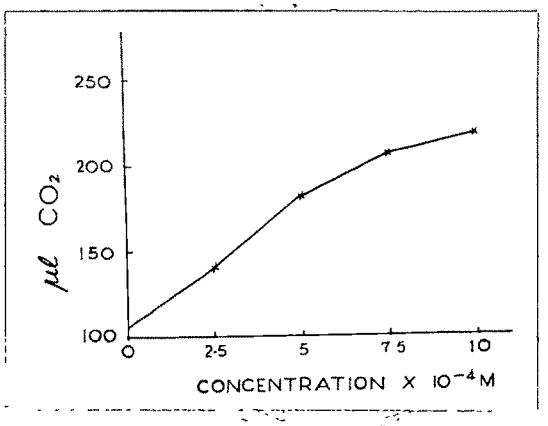

Fig. 5

Fig. 5 - Effect of ATP on the pigeon adipose tissue lipase.

Urethane: (Figure 6) At a concentration of 0.2%, the enzyme was inhibited, while at higher concentrations (0.6%, 0.8% and 1.0%) there was slight activation.

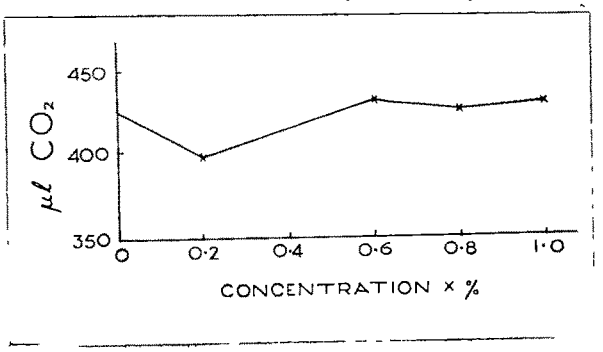

Fig. 6

Fig. 6 - Effect of urethane on the pigeon adipose tissue lipase.

Sodium taurocholate: (Figure 7) was found to inhibit the enzyme considerably, from 0.0025 M to 0.01 M, the maximum inhibition being about 80% at 0.005 M and 0.0075 M.

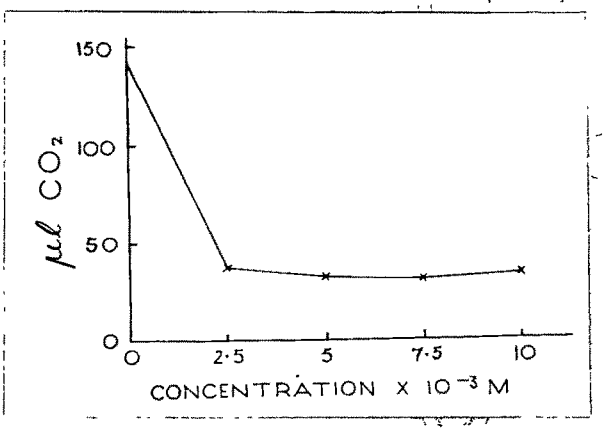

Fig. 7

Fig. 7 - Effect of sodium taurocholate on the pigeon adipose tissue lipase.

Metal chelating agent: 8-hydroxyquinoline is known to bind metals in biological materials (Albert and Gledhill,1947). The solubility of this substance in water is extremely low. A saturated aqueous solution was prepared (31°C) and 1.5 ml of this solution was added to 1.5 ml of the enzyme solution and incubated at 37°C for 30 minutes. A control was prepared by similarly incubating 1.5 ml of the enzyme with 1.5 ml of distilled water. 1 ml each of these preincubated enzymes was added to each flask. The enzyme was found to be activated about 18% by this chemical.

Thiol reagent: Alkylating agent- Iodoacetate was found to activate the enzyme at concentrations from 0.0025 M to 0.01 M. The maximum activation was about 36% at 0.0075 M (Figure 8).

Oxidizing agent: Potassium ferricyanide was found to be a potent inhibitor. At a concentration of 0.01 M the inhibition was about 39% (Figure 8).

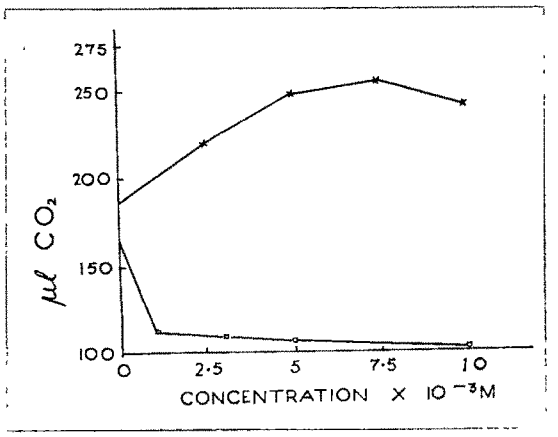

Fig. 8

Fig. 8 - Effect of iodoacetate and pot. ferricyanide on the pigeon adipose tissue lipase.
x, iodoacetate; □, pot. ferricyanide.

Reducing agents: Monothiol, Thioglycollate (Figure 9) activated the enzyme at all concentrations tested, from 0.01 M to 0.1 M.

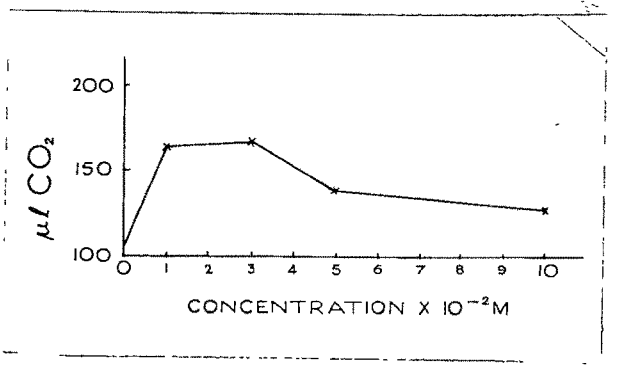

Fig. 9

Fig. 9 - Effect of thioglycollate on the pigeon adipose tissue lipase.

Glutathione and cysteine: (Figure 10) Glutathione markedly activated the enzyme from 0.0025 M to 0.01 M, while cysteine slightly inhibited it at these same concentrations. At 0.01 M, activation due to glutathione was about 195%.

Fig. 10

Fig. 10 - Effect of glutathione and cysteine on the pigeon adipose tissue lipase.
x, glutathione; △, cysteine.

Dithiol: BAL at all concentrations tested (0.0001 M to 0.001 M) was inhibitory. Maximum inhibition observed was about 63% at 0.0001 M (Figure 11).

Mercaptide forming substances: PCMB (p-chloromercuribenzoate) at all concentrations tested was highly inhibitory. Almost complete inhibition was noted at a concentration of 0.0075 M (Figure 12)

Mercuric chloride: was also an effective inhibitor (Figure 12).

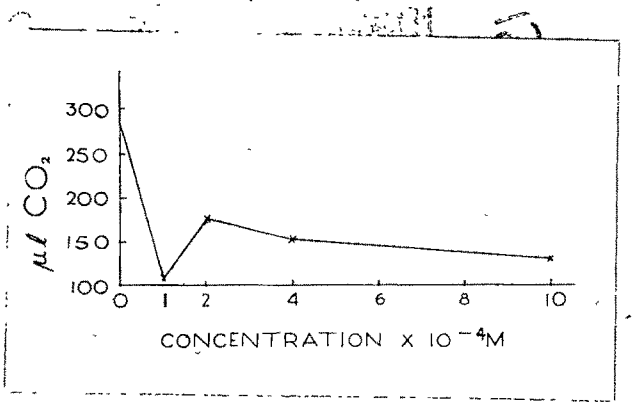

Fig. 11

Fig. 11 - Effect of BAL on the pigeon adipose tissue lipase.

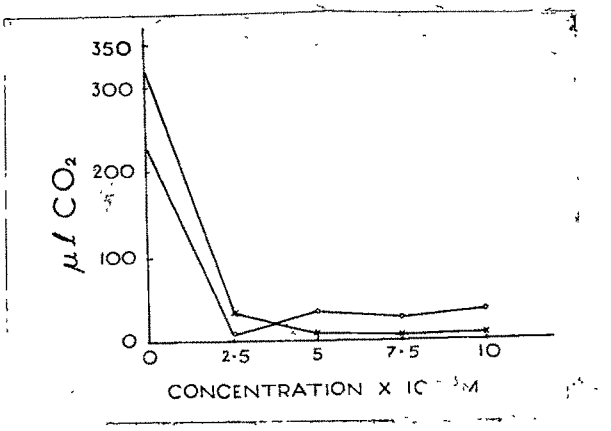

Fig. 12

Fig. 12 - Effect of PCMB and $HgCl_2$ on the pigeon adipose tissue lipase. x,PCMB; o,$HgCl_2$.

Effect of pre-incubating the enzyme first with $HgCl_2$ and then with BAL. To 1.5 ml of the enzyme solution was added 0.5 ml of 2.4×10^{-4} M $HgCl_2$ and incubated for 15 minutes at 37°C. A control was also similarly incubated side by side, with 0.5 ml distilled water added to 1.5 ml of the enzyme (tube 1). To 1 ml of the former was added 1 ml distilled water (tube 2) and to the remaining 1 ml 6×10^{-4} M BAL (tube 3). To tube 1 was added 2 ml distilled water, so as to make the concentration of the enzyme the same in all the three tubes. All the tubes were again incubated for 15 minutes and 1 ml each of these added to the Warburg flasks and the activity of the enzyme determined as usual. The results are given in table 1. Flask 2 had a final concentration of 10^{-5} M $HgCl_2$ and flask 3 contained 10^{-5} M $HgCl_2$ and 10^{-4} M BAL.

Table 1

The effect of pre-incubating pigeon adipose tissue lipase with $HgCl_2$ and BAL

Activity, µl CO_2 produced		
Control without addition (1)	Enzyme + $HgCl_2$ 10^{-5} M (2)	Enzyme + $HgCl_2$ 10^{-5} M + BAL 10^{-4} M (3)
259.9	196.4	120.9

$HgCl_2$ was found to inhibit the enzyme about 24%. A further inhibition of about 29% was brought about by the addition of BAL.

The effect of pre-incubating the enzyme first with BAL and then with $HgCl_2$. 1.5 ml of the enzyme solution was incubated for 15 minutes with 0.5 ml of 6×10^{-4} M BAL at 37°C. A similarly incubated preparation containing 1.5 ml enzyme and 0.5 ml distilled water was used as control (tube 1). To 1 ml of the former was added 1 ml distilled water (tube 2) and to the remaining 1 ml, 1 ml of 2.4×10^{-4} M $HgCl_2$ (tube 3). 2 ml of distilled water was added to tube 1 and all the tubes incubated for a further period of 15 minutes. 1 ml each of these was added to separate flasks and the activity noted. The final concentration of BAL and $HgCl_2$ was 2.5×10^{-5} M and 4×10^{-5} M respectively (Table 2)

Table 2

Effect of pre-incubating pigeon adipose tissue lipase with BAL and $HgCl_2$

Activity, µl CO_2 produced		
Control, without addition (1)	Enzyme + BAL 2.5×10^{-5} M (2)	Enzyme + BAL 2.5×10^{-5} M + $HgCl_2$ 4×10^{-5} M (3)
271.2	179.3	137.6

The enzyme was inhibited about 34% by BAL and a further inhibition of 15% was noted due to the addition of $HgCl_2$.

Effect of pre-incubating the enzyme first with PCMB and then with glutathione. 1.5 ml of the extract was incubated with 0.5 ml of 6×10^{-3} M PCMB for 15 minutes at 37°C. To another 1.5 ml portion of the extract was added 0.5 ml distilled water (tube 1) and incubated as above. This served as control. To 1 ml of the former was added 1 ml distilled water (tube 2) and to the remaining 1 ml, 1 ml 1.44×10^{-3} M glutathione (tube 3). 2 ml of distilled water was added to tube 1 and all the three tubes incubated for a further period of 15 minutes. 1 ml each of these preparations was added to separate flasks and the activity noted as usual. The final concentration of PCMB and glutathione in the flasks was, 2.5×10^{-4} M and 2.4×10^{-4} M respectively. (Table 3).

Table 3

Effect of pre-incubating pigeon adipose tissue lipase with PCMB and glutathione

Activity, µl CO_2 produced		
Control, without addition (1)	Enzyme + PCMB 2.5×10^{-4} M (2)	Enzyme + PCMB 2.5×10^{-4} M + glutathione 2.4×10^{-4} M (3)
163.9	35.3	192.2

The enzyme was inhibited about 79% by PCMB. The inhibition was completely reversed by glutathione and an activation of about 17% was also brought about.

The nitroprusside reaction for -SH groups and lead-blackening test for sulphur gave negative results. The enzyme solution when treated with excess BAL, did not yield any precipitate thereby indicating the absence at least in easily detectable quantities of metals like iron and copper.

Discussion

The extensive studies on lipases and esterases by the schools of Willstater and Rona are well known. The literature upto 1950 has been reviewed by Ammon and Jaarma (1950). It is generally agreed, that the hydrolysis of tributyrin is effected by 'true lipase' (Desnuelle,1951). In the manometric system, the pancreatic lipase hydrolyses tributyrin readily, but olive oil is not acted upon (Scaria,1958). Martin and Peers made a similar observation with regard to oat lipase. The latter authors explained the non-hydrolysis of olive oil in a manometric system as due to the predominance of water in the system. Moreover lipases from various sources differ from each other with regard to their solubility, specificity and kinetic properties (Martin and Peers, 1953). From the study of the properties of the pigeon breast muscle (George and Scaria,1959) and pancreatic lipase (Scaria, 1958), it appears that every enzyme may be adapted for maximal activity in the respective physiological environment in which it occurs. My present study also tends to support such a contention.

Sodium taurocholate considerably inhibited the enzyme. All the Krebs cycle intermediates tested and lactate, also acted as inhibitors. The pigeon pancreatic lipase has been reported to be activated by sodium taurocholate while the breast muscle

lipase of the same animal, inhibited (Scaria,1958). The Krebs cycle intermediates and lactate activated the breast muscle lipase of the pigeon while the pancreatic lipase was inhibited (Scaria,1958). The activity of the adipose tissue lipase is intracellular and its physiological environment should contain normally, only small quantities of bile salts or intermediate metabolites. Bile salts occur in appreciable quantities in the physiological environment of the pancreatic lipase while the intermediate metabolites in that of the breast muscle lipase. ATP was however found to activate the adipose tissue lipase. As far as I am aware, we have no information available on the levels of the various phosphate compounds occurring in the adipose tissue of the pigeon. But it is noteworthy that considerable quantities of ATPase was found to be present in this tissue (Chapter 2, I). No cation requirement if any, could be demonstrated by adding these substances. Added cations had only an inhibitory effect, except Ca, which showed an activation.

The negative results obtained with nitroprusside reaction for sulphydryl groups and the lead-blackening test for sulphur tend to show that the enzyme does not contain either -SH groups or disulphide (-S-S- groups) in its protein. A similar observation was made with regard to the pigeon breast muscle (George and Scaria, 1959) and the pancreatic lipase (Scaria,1958). It should however, be pointed out that there are different types of -SH groups occurring in native protein (Barron,1951). Of these, the "freely reacting -SH groups" readily react with nitroprusside and ferricyanide. The second variety, "sluggish -SH groups" react with more powerful reagents such as PCMB. In certain instances ferri-

cyanide is also known to react with the latter type. The third type, "masked -SH groups", are in general not connected with enzyme activity, since they are so protected as to require protein denaturation to become reactive. But the enzyme activity is destroyed by protein denaturation (Barron,1951). Potassium ferricyanide, an oxidizing agent, inhibited the adipose tissue lipase. Enzyme inhibition by ferricyanide is assumed to be due to the oxidation of -SH groups to the disulphide (Barron,1951). Iodoacetate, an alkylating agent, activated the enzyme. It is known that some thiol enzymes (shown to possess -SH groups necessary for enzyme activity with the aid of oxidizing and mercaptide-forming agents) are not inhibited at all by iodoacetate (Barron,1951).

Barron (1951) has classified pancreatic lipase and esterases under sulphydryl enzymes. Ions of heavy metals such as Hg are said to inactivate such enzymes by combining with reactive -SH groups, forming mercaptide compounds. Such inactivation can be reversed by the addition of BAL because of its greater affinity for the metal. It must be noted, however, that BAL because of its powerful reducing power, inhibits enzyme reactions which are independent of -SH groups in the protein (Barron,1951). Both $HgCl_2$ and BAL considerably inhibited the adipose tissue lipase. Due to the oxidation of dithiols, -SH enzymes are known to be inhibited (Barron et al,1947b). As is evident from the results (Table 1) BAL was not found to reverse the inhibition brought about by $HgCl_2$. Furthermore, due to the cumulative effect of these two substances, enhanced inhibition was the result when both were present simultaneously (Table 1 and 2). Inhibition of the adipose

tissue lipase by $HgCl_2$ and PCMB, appears to be due to their reactivity towards -SH groups. The reversal of PCMB inhibition by glutathione supports this view. It has been reported that whenever thiol groups are attacked by agents which destroy the -SH groups, glutathione will restore the -SH groups by withdrawing the heavy metal or by reducing the oxidized -SH groups (Barron, 1951). According to Scaria (1958), the inhibition of the pigeon breast muscle and pancreatic lipase is not due to reaction with -SH groups, but is due to the binding of reactive NH_2 groups of the protein. This latter possibility may be ruled out in the present instance due to the fact that PCMB inhibition is totally reversed by glutathione. PCMB is said to be the most powerful inhibiting agent for -SH groups and the most specific when accompanied by reactivation (Barron,1951). Based on Singer's observations Barron (1951) states, "whereas inhibition of D-amino acid oxidase by PCMB is dependent on the substrate used, inhibition of lipase depends on the molecular size of the substrate. The larger the substrate molecule, greater the inhibition produced by thiol reagents. He believes that in such cases as in lipase, the -SH groups do not contribute to the activity of the enzyme and that the inhibition is due to the steric interference with the approach of the larger substrate molecules to the active surface of the lipase". This explanation appears doubtful.

Of the four reducing agents tested, thioglycollate and glutathione activated the enzyme, while cysteine and BAL considerably inhibited it, especially the latter. Thiol groups of proteins which have been converted into disulphides can be easily reduced on

addition of a large excess of soluble thiol compounds (thioacids, cysteine, glutathione, BAL and other dithiols), and the effectiveness of these vary greatly (Barron,1951). The activation of the enzyme by thioglycollate and glutathione is due to this, is doubtful. Another possibility is that, activation may be due to the binding of inhibitory metal ions present in the enzyme solution. The pigeon pancreatic lipase is shown to be activated by glutathione (Scaria,1958) while cysteine activated the breast muscle lipase of the same animal (George and Scaria,1959). The ability of the above mentioned substances to combine with different metals vary considerably (Barron et al,1947b; Webb and van Heyningen, 1947). The inhibitory effect of cysteine and BAL appears to be due to their reducing property, exerting their effect on the -SH groups. The inhibition is not due to the combination with metals of the enzyme protein, forming mercaptides, is borne out by other results. First among these is the absence of any precipitate, on treatment of this enzyme with BAL. BAL in combination with a number of metals,like Fe, Pb, Bi, Cu, Co, Ni, is known to give coloured insoluble complexes (Barron et al,1947a; Webb and van Heyningen,1947). Second is that, 8-hydroxyquinoline which is a metal-chelating agent (Albert and Gledhill,1947) did not inhibit the enzyme, but curiously enough activated it. This indicates the lack of a metal in the enzyme protein. As has been mentioned earlier, activation by this chemical also may be due to the binding of some inhibitory metal ions. This however, is not conclusive.

In conclusion the following may be stated.

1. From the data presented from my experiments it is evident that aqueous extract of ether-defatted adipose tissue is active by itself and is not dependent on any added substances.
2. The adipose tissue lipase does not contain a metal in its enzyme protein and it does not require any metal ions for activity.
3. This enzyme appears to be an -SH enzyme or one requiring -SH and -S-S- for activity.

This however, is not in agreement with the conclusions reached by Scaria (1958) from his studies on the lipase of the pancreas and by George and Scaria (1959) on that of the pigeon breast muscle. According to them the pigeon pancreatic and breast muscle lipases are both metallo-proteins or enzymes requiring metal ions and reactive NH_2 groups for activity, and are devoid of reactive -SH or -S-S- groups. Nevertheless, side by side with these differences there are points of similarities between lipases obtained from different sources in the pigeon.