CHAPTER 4

THE PIGEON PANCREATIC LIPASE

The occurrence of lipase in the breast muscle of pigeon and certain other vertebrate skeletal muscles has already been discussed in an earlier chapter. In the subsequent chapter it was shown that the vertebrate heart muscle also contains a lipase which appears to be similar to that of the pigeon breast muscle. The presence of this enzyme in these tissues was also demonstrated histochemically using "Tween 80" as substrate. The properties of the enzyme from these two different sources and from the pigeon pancreas were then studied under identical conditions to find out how far they are similar or otherwise. The properties and chemical nature of the pancreatic lipase of pigeon revealed by my study are so different from accounts on pancreatic lipase by previous workers that they merit special consideration.

Material and Methods

The material used for the enzyme was an ether defatted dry powder of the pancreas of the pigeon (Columba livia). It was observed that the lipolytic activity of the acetone powder of the pigeon breast muscle was nil and that when sections of the muscle and pancreas were treated with acetone, the lipase activity was completely destroyed. Therefore a dry powder of the material was made without treatment with

acetone. Fiore and Nord (1950) used an ether defatted dry powder of Fusarium lini Bolly as the enzyme material for the study of this mould lipase. This method of preparing a powder was adopted and found highly satisfactory. The pancreas of decapitated pigeons were removed, cut into small pieces and dried at room temperature in vacuo in a desiccator over calcium chloride. Dehydration was quick and was complete within 12 - 24 hrs. The dry tissue was then crushed into a fine powder in a mortar and the coarse parts removed by sieving through fine silk. The powder was then treated with a large quantity of cold ether for 1 hr. At the end of this period during which much of the fat was extracted, the ether was filtered off, the residue washed with more ether, spread on a filter paper and dried at room temperature, till all the ether was completely removed. An aqueous extract of this powder was used as the enzyme solution in all the experiments. The powder thus prepared keeps well in the refrigerator for months without much loss of the enzyme activity.

10 mg. of this powder was extracted in 5 ml. of water in cold for 1 hr. It was then centrifuged for 5 min. at about 2500 r.p.m. and the supernatant used as the enzyme solution. The protein content in 1 ml. of this solution was 0.6 -0.75 mg. Protein was estimated according to the micro-Kjeldahl method (Hawk et al 1954). The nitroprusside and the lead blackening test for sulphur gave negative results with this enzyme solution. A blue green precipitate

was obtained when the enzyme solution was treated with excess BAL (British-Anti-Lewisite, 2:3: dimercaptopropanol). The precipitate formed with BAL, according to the descriptions of Webb and van Heyningen (1947) and Barron et al (1947) is a BAL-iron compound. It was ashed and the ash taken up in 3 N HCl was found to contain large quantities of iron.

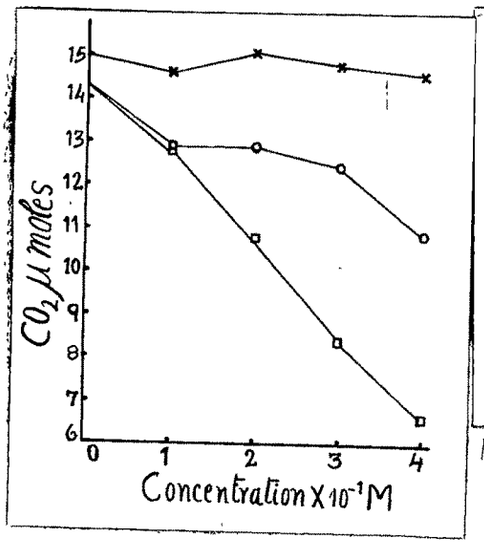
Lipolytic activity of the extract was determined manometrically in a bicarbonate-CO_2 buffer system of pH 7.4 at 37°C (Martin and Peers, 1953). An emulsion of tributyrin (4% v/v) in 0.0148 M $NaHCO_3$ prepared by shaking in a conical flask with 1 drop of "Tween 80" was used as the substrate. Each reaction flask contained 1.5 ml. 0.025 M $NaHCO_3$, 0.5 ml. of the substance under test (the various chemicals used), in concentrations to give the final concentrations as noted below, and 0.5 ml. enzyme in the main chamber and 0.5 ml. substrate in the side arm in a total volume of 3 ml. This gives a final concentration of 0.0148 M $NaHCO_3$ with a pH of 7.4 (Umbreit et al, 1951). The test solutions were introduced into the reaction flask before the addition of the enzyme, except in cases where it is otherwise stated. The flasks and manometers were gassed for 3 min with a mixture of 95% N_2 and 5% CO_2. After equilibration for 10 min in the constant temperature water bath, the substrate was tipped in and again allowed to equilibrate for another 3 min. This period is sufficient to ensure complete mixing of the contents of the flask. The readings were taken at regular intervals for 1 hr..

For each experiment a control was run in which 0.5 ml. distilled water was added in place of the solutions under test. Autohydrolysis was found to be nil. The readings given are after correction for the thermobarometer. The controls produced on the average 13 - 14 μ moles of CO_2 equivalent to the amount of butyric acid liberated.

Results

Effect of Various Substances

Inorganic salts: (Figs.1 and 2) KCl, NH_4Cl, $CaCl_2$ and $MgCl_2$ were inhibitory. Activation or inhibition of the enzyme by NaCl could not be demonstrated. Greatest inhibition was by NH_4Cl.



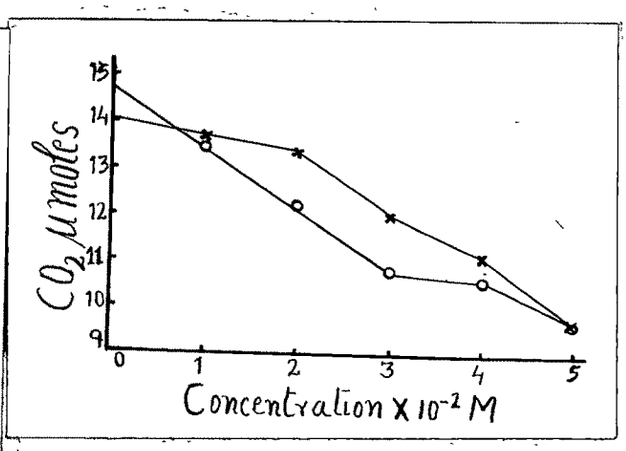


Fig.2, Effect of inorganic salts on the pigeon pancreatic lipase X, $CaCl_2$; O, $MgCl_2$

Fig.1. Effect of inorganic salts on the pigeon pancreatic lipase. x, NaCl; O, KCl; □, ■ NH_4Cl

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

was by citrate and the least by ∝ ketoglutarate.

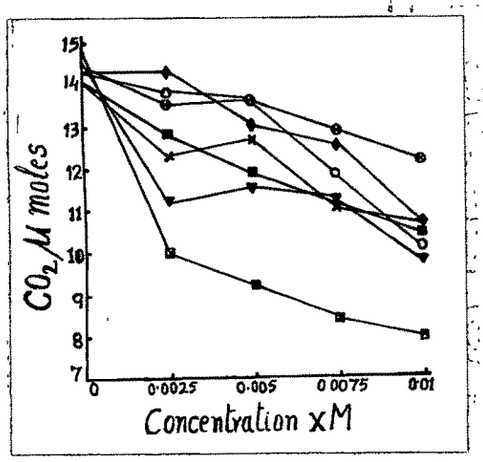


Fig.3. Effect of intermediate metabolites on the pigeon pancreatic lipase. ×, pyruvate; ○, succinate; ▼, fumarate; ■, malate; ⊗, ∝ ketoglutarate; ⊠, citrate; ♦, lactate.

Amino acids: (Fig.4) Of the two amino acids tested, histidine inhibited the enzyme whereas L-methionine activated it at low concentrations. At a concentration of 0.01 M the latter amino acid also was inhibitory.

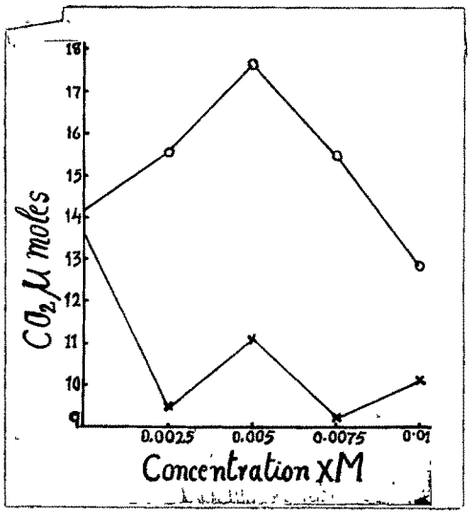


Fig.4. Effect of amino acids on the pigeon pancreatic lipase.
×, histidine; ○, L-methionine.

ATP: (Fig.5) Was found to inhibit the enzyme. Inhibition was more than 50% at a concentration of 10^{-3} M .

Heparin: (Fig.6) Showed an activation of about 7% at a concentration of 10 μg/ml. Above this concentration the activity fell sharply and the inhibition was about 30% at a concentration of 40 μg/ml.

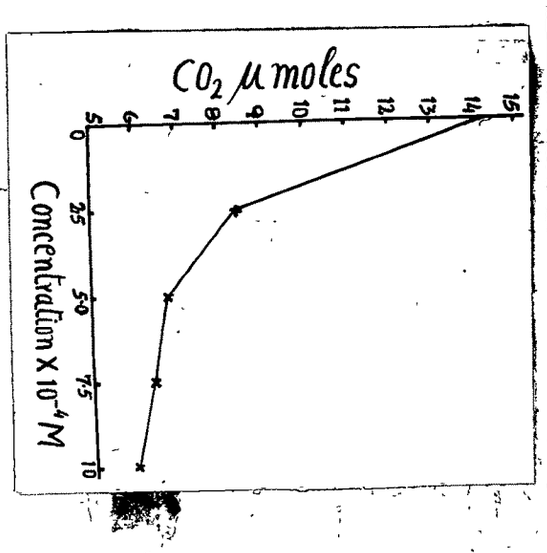


Fig.5. Effect of ATP on the pigeon pancreatic lipase.

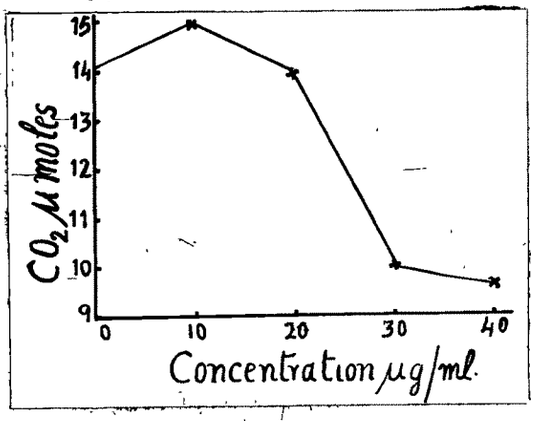


Fig.6. Effect of heparin on the pigeon pancreatic lipase.

Urethane: (Fig.7) The enzyme was slightly activated at a concentration of 0.2% . At higher concentrations the activity was considerably reduced, the inhibition being about 50% in a 1% solution of the substance.

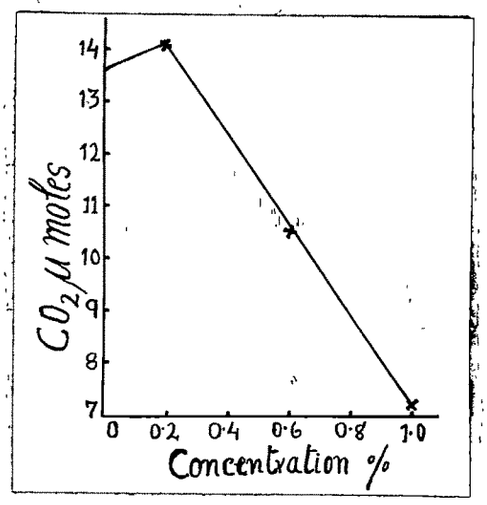


Fig.7. Effect of urethane on the pigeon pancreatic lipase.

Sodium taurocholate: (Table 1) Was found to activate the enzyme about 200% at a concentration of 0.0025 M. Further activation at higher concentrations could not be demonstrated.

Table 1

Activation of pigeon pancreatic lipase by sodium taurocholate

Addition	Activity, μmoles butyric acid produced				
	Concentration x M				
	0	0.0025	0.005	0.0075	0.01
Sodium taurocholate	13.6	39.4	38.7	35.5	37.6

Metal chelating agent: 8 - hydroxyquinoline is known to bind many 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 and incubated at 37°C for 30 min. A control was prepared by similarly incubating 1.5 ml. of the enzyme with 1.5 ml. water. 1 ml. each of these preincubated enzymes was added to each flask. The enzyme was inhibited about 84% by this chemical.

Thiol reagents: Alkylating agent - Iodoacetate was found to inhibit the enzyme about 35% at a concentration of 0.01 M. (Fig. 8)

Oxidizing agent: Potassium ferricyanide was found to be a strong inhibitor. At a concentration of 0.004 M the activity of the enzyme was decreased by about 56% (Fig.8)

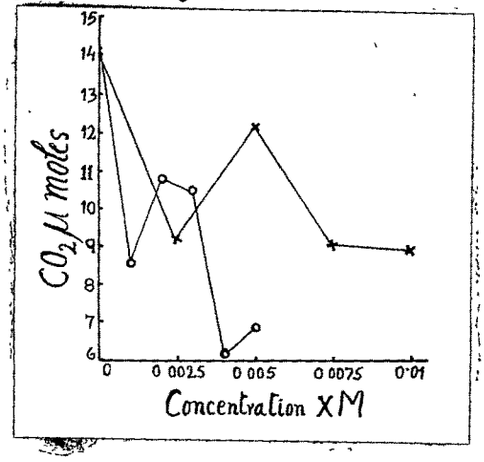


Fig.8. Effect of iodoacetate and pot.ferricyanide on the pigeon pancreatic lipase.
x , iodoacetate; 0, pot.ferricyanide

Reducing agents: Monothiols, Thioglycollate (Fig.9)

Inhibited the enzyme about 32% at a concentration of 0.1 M.

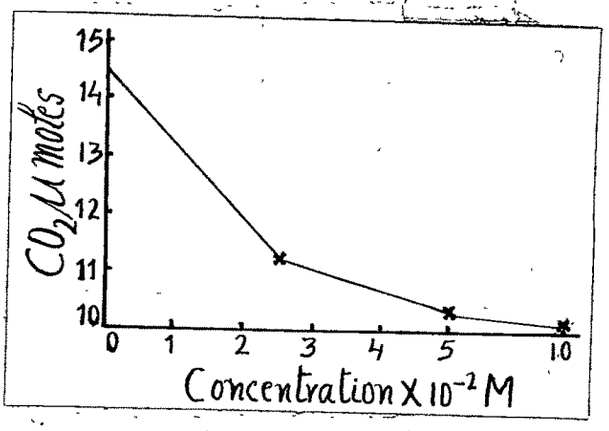


Fig.9. Effect of thioglyco - llate on the pigeon pancreatic lipase.

Glutathione and Cysteine: Both inhibited the enzyme at concentrations 0.01 to 0.05 M, the inhibition by glutathione being more pronounced (Fig.10). At lower concentrations, 0.001 to 0.003 M, however, cysteine was still inhibitory, but glutathione activated the enzyme about 32% at a concentration of 0.003 M (Fig.11).

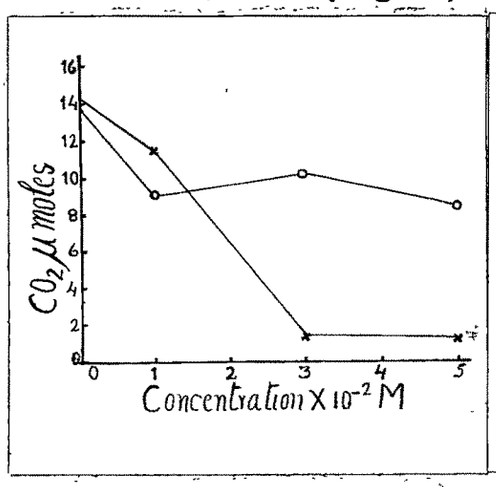


Fig. 10

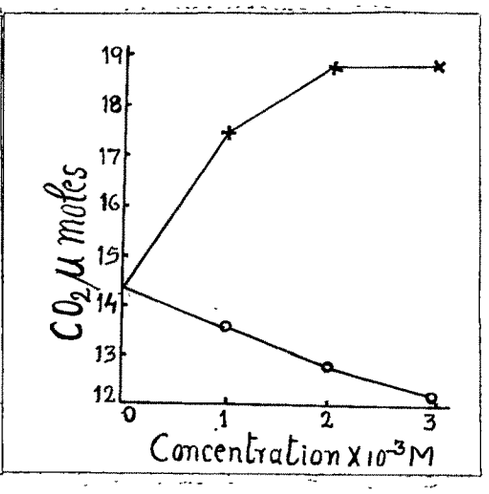


Fig. 11

Figs. 10 and 11. Effect of glutathione and cysteine on the pigeon pancreatic lipase. x, glutathione; O, cysteine.

Dithiol: BAL at concentration 0.0002 M activated the enzyme by about 24%. Higher concentrations of the reagent had a

marked inhibitory effect (Fig.12). When the enzyme was pre-incubated with excess BAL (1 drop in 3 ml.) for about 15 min at 37°C, the supernatant after centrifugation was found to be completely inactive.

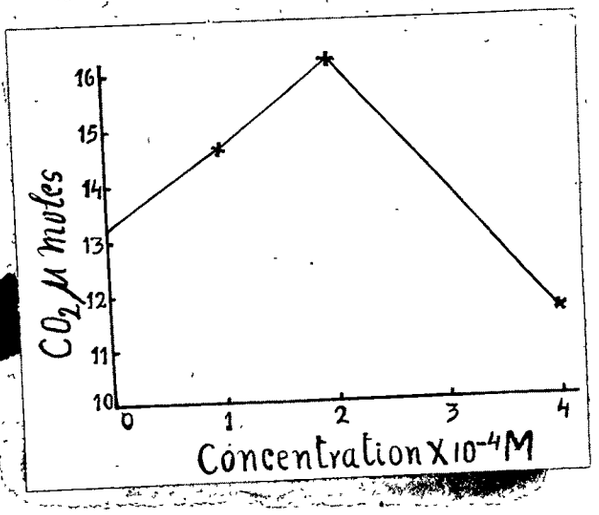


Fig. 12. Effect of BAL on the pigeon pancreatic lipase.

Mercaptide Forming Substances: PCMB (p-Chloromercuribenzoate) at all concentrations was inhibitory. Complete inhibition was obtained at a concentration of 2 x 10^{-3}M in the flask.

Mercuric chloride: Was not so effective as an inhibitor at concentrations at which PCMB inhibited the enzyme.

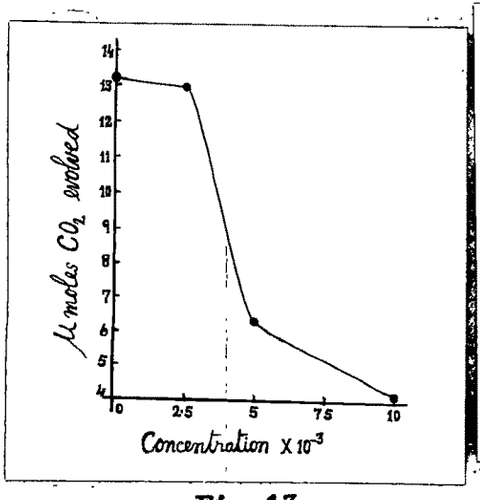


Fig.13

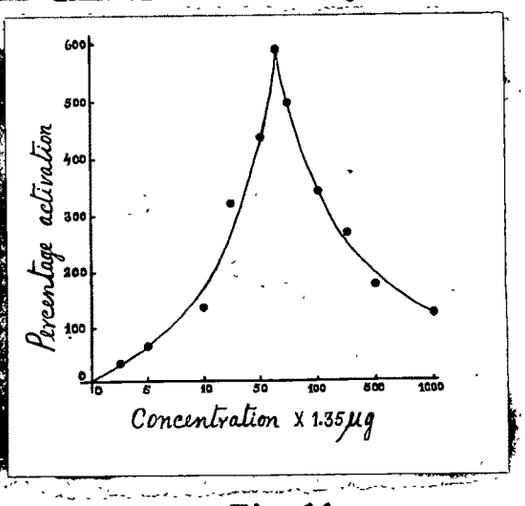


Fig.14

Figs.13 and 14. Effect of $HgCl_2$ on the pigeon pancreatic lipase.

At a concentration of 0.0025 M the inhibition by $HgCl_2$ was very slight.. Even at a concentration of 10^{-2}M the inhibition by $HgCl_2$was only about 66% (Fig.13). At a concentration of the order of micrograms of $HgCl_2$, however, the enzyme was tremendously activated. The activation was about 600% at a concentration between 75 and 100μg (Fig.14) at which concentrations also PCMB behaved as an inhibitor.

Effect of preincubating the enzyme first with $HgCl_2$ and then with BAL To 1.5 ml. of the enzyme solution was added 0.5 ml. 2.4 x 10^{-4}M $HgCl_2$ and incubated for 15 min at 37°C. A control was also similarly incubated side by side, with 0.5 ml. H_2O added to 1.5 ml. of the enzyme (tube 1). To 1 ml. of the former was added 1 ml. H_2O (tube 2) and to the remaining 1 ml. 6 x 10^{-4}M BAL. To tube 1 was added 2 ml. H_2O so as to make the concentration of the enzyme the same in all the three tubes. All the tubes were again incubated for 15 min. and 1 ml. each was added to the Warburg flasks in duplicate and the activity of the enzyme determined as usual. The results are given in table 2. 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.

Activity, μl CO_2 liberated		
Control, without addition (1)	Enzyme + $HgCl_2$, 10^{-5}M (2)	Enzyme + BAL, 10^{-4}M + $HgCl_2$, 10^{-5}M (3)
255	393	198

Table 2. Effect of preincubating pigeon pancreatic lipase with $HgCl_2$ and BAL.

$HgCl_2$ was found to activate the enzyme about 54%. The activated enzyme was inhibited about 50% by the addition of BAL.

In another set of experiments in which the concentration of $HgCl_2$ and BAL was ten times that in the previous experiments, the following results were obtained (Table 3)

Table 3
Effect of preincubating pigeon pancreatic lipase with $HgCl_2$ and BAL

Activity, μl CO_2 produced		
Control, without addition (1)	Enzyme + $HgCl_2$ 10^{-4}M (2)	Enzyme + $HgCl_2$ 10^{-4}M + BAL, 10^{-3}M (3)
294	360	37

At this concentration the lipase was still activated 22% by $HgCl_2$ and the activated enzyme was inhibited about 90% by the addition of BAL.

Effect of preincubating the enzyme first with BAL and then with $HgCl_2$. 1.5 ml. of the extract was incubated for 15 min with 0.5 ml. 6 x 10^{-3}M BAL at 37°C . A similarly incubated enzyme to which 0.5 ml. H_2O was added was used as control (tube 1). To 1 ml. of the former was added 1 ml. H_2O (tube 2) and to the remaining 0.6 ml. 2.4 x 10^{-4}M $HgCl_2$ and 0.4 ml. H_2O (tube 3). 2 ml. H_2O was added to tube 1 and the tubes incubated for a further period of 15 min. 1 ml. each of these preparations was added to the flasks and the activity noted. The final concentration of BAL and $HgCl_2$ in the flasks

was 2.5×10^{-4}M and 2.4×10^{-4}M respectively.

Table 4
Effect of preincubating pigeon pancreatic lipase with BAL and $HgCl_2$

Activity, CO_2 produced, μl		
Control, without addition (1)	Enzyme + BAL 5×10^{-4}M (2)	Enzyme + Bal 2.5×10^{-4} + $HgCl_2$ 2.4×10^{-4}M (3)
403	350	373

The enzyme was inhibited about 12.5% by BAL. The inhibition was reversed by $HgCl_2$ to the extent of 50%.

Discussion

The extensive studies on lipases and esterases by the schools of Willstatter and Rona are well known. Ammon and Jaarma recently reviewed the literature upto 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 . This is in conformity with the observations of Martin and his colleagues (Martin and Peers, 1953). They observed that oat lipase does not hydrolyse olive oil in a manometric system and that tributyrin is readily hydrolysed. They explained the non-hydrolysis of olive oil in a manometric system as due to the predominance of water in the system. It is also realized that lipases from various sources differ from each other

with regard to their solubility, specificity and kinetic properties (Martin and Peers,1953). Each enzyme may be adapted for maximal activity in the physiological environment in which it acts. From the results presented above it is clear that sodium taurocholate highly activates the enzyme and all the Krebs cycle intermediates and lactic acid inhibit the enzyme considerably. The reverse effect is observed in the case of the muscle lipase (Chapter 5). This can be appreciated when we consider that the activity of the muscle lipase is intracellular and the metabolic pool constitutes its physiological environment while it is not so with pancreatic lipase. This enzyme acts in an environment which is rich in bile salts which are known to enhance digestion. Evidently the activation of pancreatic lipase by sodium taurocholate is not due to emulsification of the substrate by the bile salt as is generally believed. Heparin and ATP are also likewise inhibitory. But a slight activation by heparin was observed at a concentration of 10μg/ml. No cation requirement if any, could be demonstrated by adding these substances . It may be that the crude enzyme contains these substances in sufficient amounts, if required ,for maximum activity.

The behaviour of the pancreatic lipase in the presence of suphydryl reagents is highly interesting because this enzyme does not contain - SH or -S -S - groups as judged from the fact that the nitroprusside reaction for -SH groups and the lead-blackening test for sulphur are negative and

still behaves as if it is a sulphydryl enzyme, according to the literature (Barron, 1951).

Potassium ferricyanide, an oxidizing agent inhibits the enzyme . Enzyme inhibition by ferricyanide is assumed to be due to the oxidation of sulphydryl groups to the disulphide (Massart, 1950). It is evident that the inhibition of this enzyme by ferricyanide is not due to oxidation of sulphydryl group. Iodoacetate, an alkylating agent, is also an inhibitor of the enzyme. According to Dickens, iodoacetate reacts by substitution of the hydrogen of the sulphydryl by the carboxymethyl group. Michaelis and Schubert (1934) however, have noted that acid halides combine with amino groups . The inhibition of pancreatic lipase by iodoacetate may be due to the latter reaction.

Barron and Singer(Barron, 1951) have 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. I could not confirm this, for it was found that small conce - ntrations of the metal activates the enzyme to the extent of 600% and is inactivated by BAL. The activation of pancreatic lipase by mercury is difficult to explain. PCMB inhibits the enzyme at concentrations at which $HgCl_2$ activates it. The toxicity of PCMB may be due to the non-metal part of the compound. Hellerman (1943) used PCMB as an effective reagent

for the detection of -SH groups in proteins. The inhibition of this enzyme by PCMB and $HgCl_2$ at high concentrations however, is not due to reaction with -SH groups. Barron (1951) has reported that all mercaptide forming substances can also react with the NH_2 groups of protein. This might perhaps explain the inactivation of lipase by PCMB and $HgCl_2$ at higher concentrations. It should be of interest to note here that Little and Caldwell (1943) have reported that free amino groups are essential for the activity of the pancreatic lipase. It appears from this study that these substances are not specific inhibitors of sulphydryl enzymes and their usefulness in the detection of -SH groups in protein is limited.

Reducing substances such as thioglycollate, cysteine, glutathione and BAL considerably affect the activity of the enzyme. This cannot be explained as due to interference with the -SH or -S -S- groups of the enzyme protein. Other explanations have to be sought. It is well known that thiols can combine with metals forming mercaptides of varying degrees of reversibility (Barron et al, 1947 ; Webb and van Heyningen, 1947)

$$Me + 2RSH \rightleftharpoons Me(RS)_2 + 2H^+$$

The inhibitory effect of BAL on metal containing enzymes is explained as due to this reaction. The enzyme solution used in this study was found to contain large quantities of iron. It is possible that they contain other metals as well. Ions of metals can inhibit the activity of enzymes

in a variety of ways depending upon the molecular structure of the enzyme protein . The activation of the pancreatic enzyme at very low concentration of BAL (0.0001 - 0.0003 M) may be due to the binding of some inhibitory metal ions present in the extract. The fact that preincubation of the enzyme with excess BAL completely inhibits the enzyme suggests that it is an enzyme which requires metals for its activity, probably a metallo-protein. The essential metal appears to be iron because it is known that BAL forms an insoluble bluish green precipitate with iron and such a precipitate was obtained and found to contain iron. The inhibition of the $HgCl_2$ activated enzyme by BAL may be explained as due to two reasons; 1) the removal of the activating effect of mercury by combining with this metal 2) inhibition of the enzyme by forming mercaptide with the metal required for the enzymic activity. On this basis it is easy to explain the reactivation by $HgCl_2$, of the enzyme inhibited by BAL. BAL leaves the emzyme metal and combines with mercury for which it has a greater affinity, bringing about a reversal of the inhibition. That the pancreatic enzyme is a metal requiring enzyme is further supported by the fact that 8-hydroxyquinoline which is a metal chelating agent (Albert and Gledhill, 1947) inhibits its activity about 85% . The inhibition of this enzyme by monothiols like cysteine, glutathione and thioglycollate also may be due to combination with the metal part of the enzyme. The oxidation of thiols by atmospheric oxygen is brought about by the

catalytic action of metals (Warburg and Sakuma, 1923) and it is known that cysteine forms a metal complex with iron previous to the oxidation of cysteine. Similar metal complexes may be formed with all thiols prior to oxidation. However, the activation of the pancreatic enzyme by glutathione at low concentration remains unexplained. Just as in the case of BAL this might be due to binding of some inhibitory metal. It is known that the efficiency of these substances in combining with different metals vary considerably (Barron, 1947).