CHAPTER 9

CERTAIN BIOCHEMICAL PROPERTIES OF THE INSECT (COCKROACH) MUSCLE LIPASE

The occurrence of lipase in the flight muscles of certain insects has already been established employing both histochemical as well as quantitative methods. The significance of the considerable quantities of lipase in these muscles has been discussed in the earlier chapters. George and Scaria (1959) has studied certain biochemical properties of lipase in the pigeon breast muscle and showed that this enzyme is very much similar to that of the pancreatic lipase of the same animal in most of its properties. They were also able to show that the pigeon breast muscle lipase is a 'true lipase! Recently several investigations have been carried out on lipase from different sources. This chapter deals with a similar investigation on the cockroach muscle lipase to elucidate its properties. The properties of the insect muscle lipase are also compared with that of pigeon breast muscle lipase and of the pancreatic lipase. The experiments were carried out in identical conditions.

Materials and Methods

Ether defatted muscle powder of the cockroach thoracic muscles was used as the enzyme material. It was observed by earlier workers that acetone reduces the enzyme activity (Fiore and Nord, 1950; George and Scaria, 1959;

George and Eapen, 1960). Fiore and Nord (1950) used an ether defatted dry powder of Fusarium lini Bolly., as the enzyme material. From the observations cited in Chapter 6 it is evident that the concentration of lipase is considerably more in the male cockroaches than in the female. Hence, muscle from the male cockroaches kept under suitable conditions, were used. The muscles were removed from their thoracic attachments and spread into thin layers in petri dishes and dried in vacuo in a desiccator over calcium chloride. The dehydrated muscle was then ground into a fine powder in a mortar and sieved through fine silk to remove the chitinous particles. The powder was then defatted with cold ether for one hour during which most of the fat was removed. It was then filtered, washed with more ether and dried at room temperature till the ether was completely removed and stored at 4°C. in a refrigerator. This powder remains active for this enzyme for a number of days. Aqueous extracts of this powder was used as the enzyme material for the present investigations.

90 to 100 mg. of the dry powder was extracted in 5 ml distilled in an ice cold mortar for 15 minutes. The extract was centrifuged for 3 minutes at 2500 r.p.m. and the supernatent was used as the enzyme solution. One ml of this solution contained 12 to 14 mg. protein. Protein was estimated according to the micro-Kjeldahl steam distillation method (Hawk, et al, 1954). Lipase activity was determined

manometrically using the conventional Warburg apparatus in a bicarbonate-carbon dioxide buffer system of pH 7.4 at 37°C. The substrate used was 4% (v/v) emulsion of tributyrin in 0.0148 M sodium bicarbonate solution prepared by shaking with a drop of "Tween 80" in a small conical flask. Each reaction flask contained 1.5 ml of 0.025 M sodium bicarbonate solution, 0.5 ml of the substance under test in concentrations to give the final concentrations as indicated below and 0.5 ml of the enzyme solution in the main chamber and 0.5 ml of the substrate in the side arm, thus making a total fluid volume of 3 ml in the flask. This gives a final concentration of 0.0148 M sodium bicarbonate with pH 7.4 (Umbreit, 1957). The test solution was introduced in the reaction flask before the addition of the enzyme in all the cases except otherwise stated. The experiments were carried out under a gas phase of 95% nitrogen and 5% carbon dioxide. After the equilibration of the flasks and manometers in the constant temperature water bath (37°C.) of the Warburg apparatus for 10 minutes the substrate was tipped in and allowed 3 minutes for the proper mixing of the solutions before the initial readings were noted. Readings were taken for every 15 minutes for 1 hour. For each experiment a control also was kept in which 0.5 ml distilled water was added in place of the test solution. The readings were corrected for the thermobarometer.

Results

Effect of various substances

Inorganic salts: (Figs. 1 and 2). Calcium chloride at all concentrations below 0.005 M tried, activated the enzyme. NaCl, KCl, NH_4Cl and $MgCl_2$ were found to be inhibitory.

Intermediate metabolites: (Figs. 3 and 4) Except pyruvate and lactate all the intermediates of the tricarboxilic acid cycle were slightly inhibitory at concentrations 0.0025 to 0.01 M. Pyruvate activated the enzyme slightly at all the above concentrations.

Amino acids: (Fig. 5) Both the amino acids tested namely histidine and L-methinoine were inhibitory.

ATP: (Fig. 6) at all the concentrations tried activated slightly.

Sodium taurocholate: (Fig. 7) inhibited the enzyme considerably; at all concentrations, the maximum inhibition of 60% was at a concentration 0.05 M. Concentrations from 0.0025 to 0.01 M were tried.

Thiol reagent: Alkylating agent - (Fig. 8) Iodoacetate was found to be inhibitory at all the concentrations from 0.0025 to 0.01 M. At 0.01 M concentration the inhibitation was 20%.

Oxidizing agent: Potassium ferricyanide (Fig.9) Inhibited the enzyme nearly 10% at 0.001 M and no further inhibition in the other concentrations from 0.001 M to 0.01 M.

Sodium azide: (Fig. 10) was slightly inhibitory at higher concentrations from 0.01 to 0.1 M.

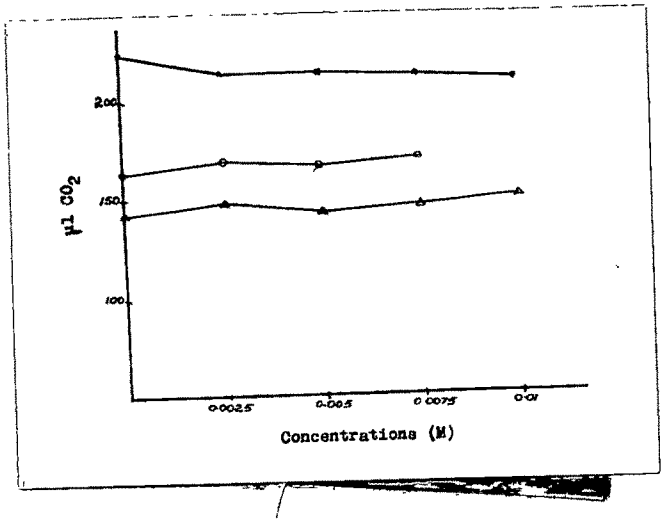

Fig. 1a

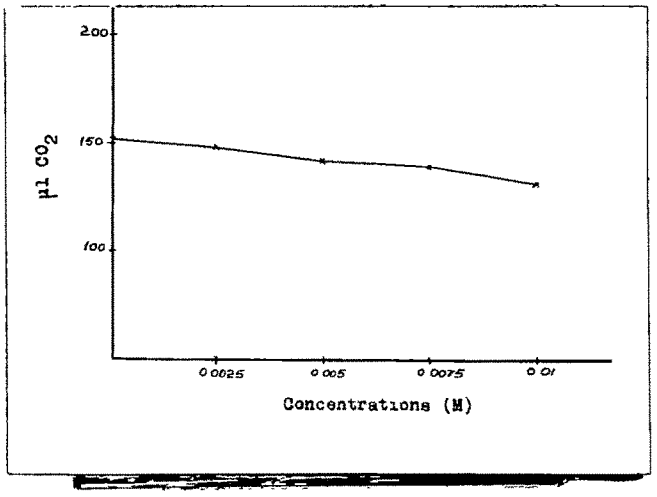

Fig. 1b

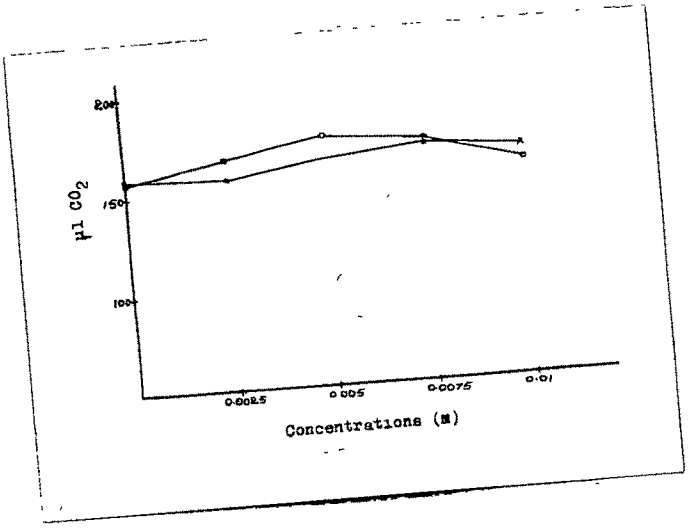

Fig. 2a

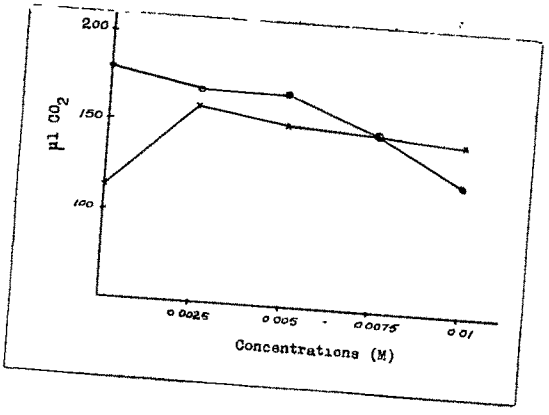

Fig. 2b

Fig. 1a, b and 2a, b - Effect of inorganic salts on the cockroach muscle lipase. $CaCl_2$ (Fig. 1a). x, NaCl; o, KCl (Fig. 1b); NH_4Cl (Fig. 2a); $MgCl_2$ (Fig. 2b).

Fig. 3 Fig. 4

Fig. 3 and 4 - Effect of intermediate metabolites on the cockroach muscle lipase. Fig. 3 (x, succinate; o, pyruvate; , lactate), Fig. 4 (citrate).

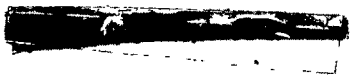

Fig. 5 Fig. 6

Fig. 5 and 6 - Effect of amino acids and ATP on the cockroach muscle lipase. Fig. 5 (o, Histidine; x, cysteine), Fig. 6, ATP, x

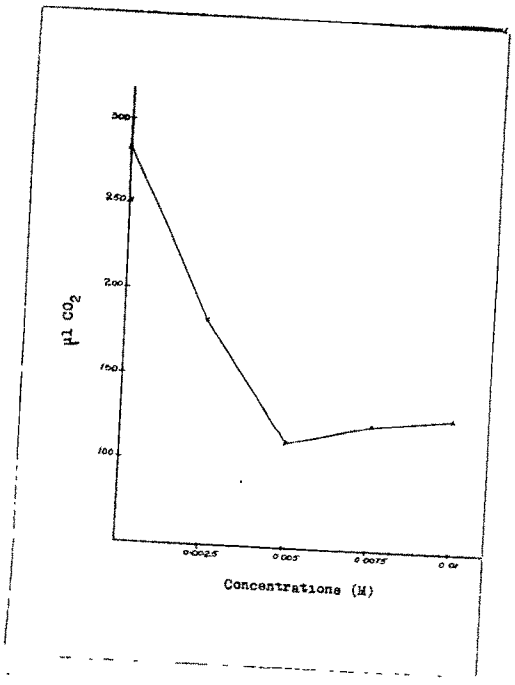

Fig. 7

Effect of sodium taurocholate on the cockroach muscle lipase.

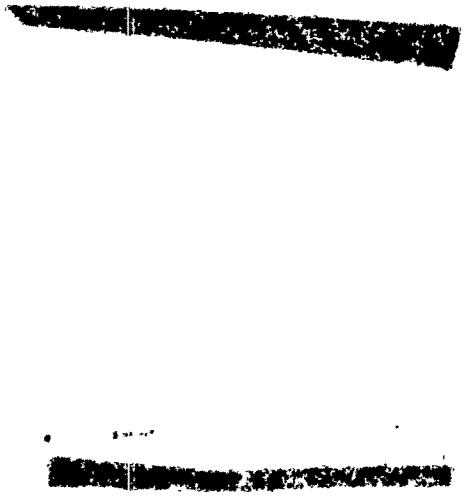

Fig. 8

Effect of iodoacetate on the cockroach muscle lipase.

Fig. 9

Effect of potassium ferricyanide on the cockroach muscle lipase.

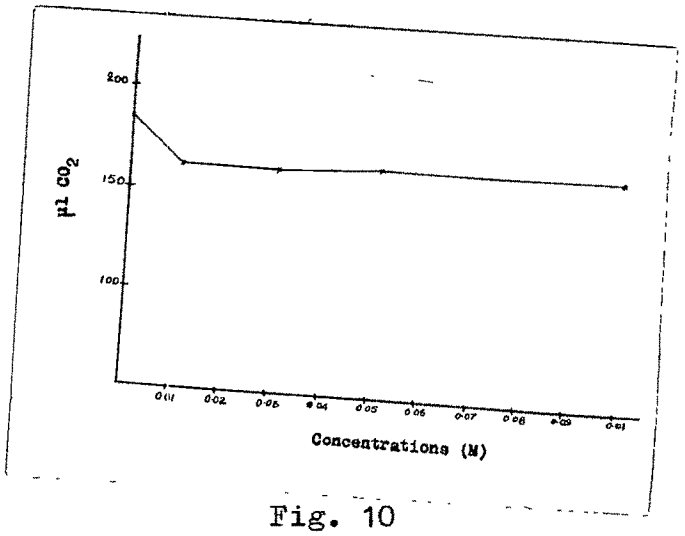

Fig. 10

Effect of sodium azide on cockroach muscle lipase.

Fig. 11

Fig. 11 - Effect of PCMB and $HgCl_2$ on the cockroach muscle lipase. o, PCMB; x, $HgCl_2$.

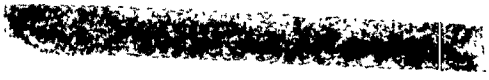

Fig.12

Fig. 12 - Effect of $ZnSO_4$ on the cockroach muscle lipase.

Glutathione and Cysteine: (Fig. 11) Glutathione activated the enzyme at all concentrations from 0.0025 M to 0.01 M. Cystiene activated at a higher rate than glutathione, the maximum being 30% at 0.0025 M concentration.

Mercaptide forming substances: (Fig. 12) PCMB (P-chloro mercuri benzoate) was highly inhibitory at all concentrations. Complete inhibition was obtained at 0.005 M to 0.01 M.

Mercuric chloride: (Fig. 12) was a marked inhibitor at all concentrations.

Zinc sulphate: (Fig. 13) Inhibited highly at all the concentrations tried from 0.01 M to 0.05 M.

Maximum inhibition of 90% was obtained at the highest concentration.

Effect of pre-incubating the enzyme first with PCMB and then with glutathione.

1.5 ml of the enzyme solution was incubated with 0.5 ml of 6 x 10^{-3}M PCMB for 15 minutes at 37°C. To another 1.5 ml of the enzyme solution was added 0.5 ml of distilled water (Tube 1) and incubated for the same period at 37°C. This was used as the control. To 1 ml of the former was added 1 ml of distilled water (Tube 2) and to the remaining 1 ml, 1 ml 1.44 x 10^{-3}M glutathione (Tube 3). In tube 1 was added 2 ml of distilled water and all the three tubes were incubated simultaneously for a further period of 15 minutes. 1 ml of each of these preparations was added to separate reaction flasks and the activity decided as usual. The final

concentration of glutathione and PCMB in the flasks was 2.4×10^{-4} and 2.5×10^{-4} M respectively (Table 1).

Table 1

Effect of pre-incubating cockroach muscle 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}) + glutathione (2.4×10^{-4}) (3)
230.55	78.1	236.98

The enzyme was inhibited by about 66% by PCMB. The inhibition was completely reversed by glutathione and activited slightly.

Discussion

Much work has been done on lipases and esterases in different materials. The recent review by Ammon and Jaarma (1950) on the subject haa been very valuable. It is believed that the hydrolysis of tributyrin is effected by a true lipase (Martin and Peers, 1953; George and Scaria, 1959; George and Eapen, 1960). These authors observed that in the manometric system the pancreatic, muscle and adipose tissue lipases hydrolyze tributyrin readily. The non-hydrolysis of olive oil by lipases from different sources in a manometric system has been atributed to the predominence of water in the system

(Martin and Peers, 1953). Moreover lipases from various sources differ from each other with regard to their solubility, specificity and kinetic properties (Desnulle, 1951; Martin and Peers, 1953; George and Scaria, 1959). Recent studies in certain biochemical properties of lipases from the pigeon breast muscle, pigeon pancreatic and adipose tissue (George and Scaria, 1959; George and Eapen, 1960; Scaria, 1958) and the intestinal lipase (DiNella and Meng, 1960) suggest that every enzyme is adapted for maxium activity in its own respective physiological environment where it occures. The results obtained in the present study also tend to support the above contention.

The pigeon pancreatic lipase was activated by sodium taurocholate (Scaria, 1958) while the pigeon breast muscle and the adipose tissue lipases were inhibited considerably (George and Scaria, 1959; George and Eapen, 1960). It has been known for since long that the addition of bile salts increase the rate of hydrolysis of fat by pancreatic lipase. The activation of pancreatic lipase by sodium taurocholate is due to the emulsification of the fatty substrate. A duel role of activation and emulsification for bile salts also has been suggested but the evidence available whilst not conclusive, suggests that the essential role of bile salts is to emulsify the substrate rather than to act as lipase activators (Wills,1955). In the present study also sodium taurocholate was highly inhibitory for the insect muscle lipase. The Krebs cycle intermediates and lactate activated

the pigeon breast muscle lipase while inhibited the pancreatic and adipose tissue lipases (Scaria,1958; George and Scaria, 1959: George and Eapen, 1960). Bile salts occur in considerably high concentrations in the physiological environment of the pancreatic lipase while the Krebs cycle intermediates occur in the breast muscle. For the insect muscle lipase the intermediate metabolites were mostly activators while slight inhibition was also seen. ATP activated the enzyme as in the case of the pigeon breast muscle lipase. Added cations were all inhibitory except calcium, which in lower concentrations activated slightly. Lipases from the alimentary canal of the carabid beetles and the long-horned grasshoppers were inhibited by magnesium chloride, sodium fluoride, strychnine and by quinine. The lipase of cockroach midgut is relatively insensitive to quinine and strychnine but inhibited by the inorganic salts (Schlottke, 1937). The partially purified lipase of Bombyx larvae was similar to the pancreatic lipase in respect to its activators and inhibitors but has a pH optimum of 9.8 (Yamafugi and Yonozawa, 1935) which is somewhat higher than that of the pancreatic lipase (Weinstein and Wynne, 1936). The lipase of the wax moth (Galleria) appears to be similar to that of the cockroach in certain aspects and was inhibited by sodium fluoride but not by quinine. The wax moth lipase differs from the beef pancreatic lipase in its ability to hydrolize wax (Mankiewiez, 1949). The lipase detected in the housefly larva (Berrick, 1958) was found to hydrolize tributyrin at a higher rate than the other triglycerides tried. Apart from these investigations little is known

about the lipases in insects.

Pancreatic lipases and esterases are classified under sulphydryl enzymes (Barron, 1951). Potassium ferricyanide and sodium azide inhibited the enzyme. Enzyme inhibition by these oxidizing agents is assumed to be due to the oxidation of -SH groups to disulphide ones (Barron, 1951). Iodoacetate, an alkylating agent inhibited the enzyme. Pigeon pancreatic and breast muscle lipases also were inhibited(Scaria, 1958; George and Scaria, 1959) by these oxidizing and alkylating agents while the adipose tissue lipase was activated by iodoacetate (George and Eapen, 1960). In the case of the pancreatic and breast muscle lipases of pigeon, it has been suggested that the inhibition by these agents may be due to their combination with the reactive $-NH_2$ groups and not with the -SH groups.

Ions of heavy metals such as Hg, Cu and Zn are said to inactivate the -SH enzymes by combining with the reactive -SH groups, forming mercaptide compounds. Pigeon pancreatic lipase has been shown to be activated considerably by Hg in low concentrations while Hg inhibited the breast muscle lipase. Wills (1960) observed that Hg and Cu were highly inhibitory, even in low concentrations, for the pancreatic lipase. He suggested that the inhibitory action of these heavy metals was due to the affinity for combining with the -SH groups. Inhibition of the cockroach muscle lipase by $HgCl_2$, $ZnSO_4$ and PCMB, appears to be due to their

reactivity towards -SH groups. The reversal of the 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 metals or by reducing the oxidized -SH groups (Barron, 1951). This reaction was also observed by George and Eapen (1960) in the pigeon adipose tissue lipase and by Wills (1960) in the pancreatic lipase. According to George and Scaria (1959) the inhibition of pigeon breast muscle lipase by PCMB is not due to the reaction with the -SH groups but is due to the binding of the reactive $-NH_2$groups of the protein. This possibility can be ruled out for the insect muscle lipase, due to the fact that PCMB inhibition was totally reversed and activated further by glutathione. PCMB is said to the most powerful inhibiting agent for -SH groups and the most specific when accompanied by reactivation (Barron, 1951). PCMB inhibited the cockroach muscle enzyme completely at concentrations 0.005 M to 0.01 M.

Glutathione and cysteine, the two reducing agents tried, activated the enzyme. Histidine and L- methionine were inhibitory in all the concentrations tried. It is possible that the activation effects observed with cysteine and glutathione may be explained on the basis of the removal of the inhibitory metals. Glutathione activated the pigeon pancreatic and adipose tissue lipases (Scaria, 1958; George and Eapen, 1960) while cysteine activated the pigeon breast muscle lipase (George and Scaria, 1959).

From the results of the present investigations it is evident that the cockroach muscle lipase appears to be an -SH enzyme or one which requires -SH or -S-S groups for its activity. Except the pigeon pancreatic and pigeon breast muscle lipases, all the lipases hitherto studied seem to possess -SH groups. Further, the cockroach muscle lipase, in all essential features, appears to be a lipase similar to the pancreatic lipase in general.