CHAPTER 5

INTRACELLULAR DISTRIBUTION OF LIPASE AND SUCCINIC DEHYDROGENASE IN THE FLIGHT MUSCLE OF THE BEETLE, HELIOCOPRIS BUCEPHALUS FABR.

Investigations on the intracellular localization of enzymes in the insect muscle have not been many in comparison with those on the vertebrate muscle. The intracellular localization of cytochrome c oxidase (Watanabe and Williams, 1951) adenosine triphosphatase (Sacktor, 1953a, 1953b) lactic and glycerophosphate dehydrogenase (Zebe and McShan, 1957) has been investigated. These authors observed that most of the oxidative enzymes were found to be concentrated in the mitochondrial fraction. The enzymes in the glycolytic system and soluble dehydrogenases were found in the soluble supernatent fraction (Sacktor, 1955; Zebe and McShan, 1957). Neither the cell nuclei and myofibrils nor the microsomes and the soluble part of the cytoplasm, were studied exhaustively. It has been emphasized in the earlier chapters that those insects indulging in long and sustained muscular activity, utilize chiefly fat for energy and they possess a high concentration of the fat-splitting enzyme, lipase and the oxidizing enzyme SDH in their muscles. In the light of these observations it was thought desirable to undertake the present investigation in order to locate the intracellular sites of these enzymes in the muscle cell.

Materials and Methods

The histochemical as well as the quantitative studies on the thoracic muscles of the beetle (Chapters 3 and 4) revealed that the fibrillar flight muscles of this insect possess a lipase and SDH in large concentrations in contract to the prothoracic muscles. It was also noted that concentrations of these enzymes are considerably more in the flight muscles of this beetle than that in the flight muscles of other insects that have been studied. Moreover, this beetle being easily available in sufficient numbers after the rainy season and also the muscle being large in size, this insect was chosen as the material for the present investigation. The muscle was homogenized in a chilled mortar and the homogenate was used for differential centrifugation.

The isolation medium and the fractionation procedure:

It was found that 0.25 M sucrose solution (isotonic), as the isolation medium gave better results than the 0.88 M sucrose (hypertonic) used as the fractionation medium by Schneider and Hogeboom (1951). In the present study also the medium for homogenization and isolation of particulate fractions was 0.25 M sucrose solution which was found to have no effect on the activity of lipase. A 10% homogenate was prepared. In order to remove unbroken fibres and fragments of the cuticle, the homogenate was centrifuged for 5 minutes at low speed (about 100 x g) in a "MSE Superspeed 25" centrifuge in cold (0 - 4°C.) and the resulting clean homogenate

was practioned as follows.

After the aliquot of the homogenate was reserved, the rest of the homogenate was centrifuged for 5 minutes at 600 x g. The sediment which consisted mainly of myofibrils and cell nuclei, was washed with 0.25 M sucrose, recentrifuged and dispersed in 0.25 M sucrose so that the final volume was equal to the volume of the homogenate used, thus yielding the 10% myofibrillar and nuclear suspension. The supernatent was further centrifuged for 15 minutes at about 7000 x g. The sediment was washed with sucrose, recentrifuged, dispersed in 0.25 M sucrose and adjusted with additional sucrose to the volume of the initial homogenate. This suspension appeared on microscopical examination, to consist of particles remarkably similar to that of mitochondria in the intact fibres. The supernatent fluid was spun at 40000 x g. for 45 minutes and the brownish jelly like sediment (microsomal fraction) was dispersed and diluted with 0.25 M sucrose to the original homogenate volume. The clear supernatent obtained was also diluted to the original homogenate volume (10%) and used as the soluble cytoplasmic fraction.

Enzymes; Methods:

Lipase:

Lipase activity in the particulate fractions of the beetle flight muscle was determined manometrically adopting the method of Martin and Peera (1953) using a bicarbonate-carbon dioxide buffer system of pH 7.4 at 37°C. in a Warburg apparatus. Tributyrin (4% v/v emulsified in 0.0148 M sodium

bicarbonate solution with a drop of "Tween 80") was used as the substrate. The procedure followed was exactly as described in the previous chapters. In a total fluid volume of 3 ml in the reaction flask, contained, 1.5 ml of 0.025 M sodium bicarbonate solution and 1 ml of the enzyme material in the main chamber and 0.5 ml of the substrate in the side arm. After gassing with a mixture of 95% nitrogen and 5% carbon dioxide for 3 minutes, the flasks and manometers were equilibrated for 10 minutes in the constant temperature (37°C.) water bath of the Warburg apparatus. The substrate was tipped in and allowed 3 minutes for proper mixing of the solution in the flasks and the initial readings were noted. Readings were taken at every 15 minutes for 1 hour. The lipase activity is expressed in terms of µl carbon dioxide liberated for mg. protein of the enzyme material for 1 hour. Protein was estimated employing the micro-Kjeldahl steam distillation method (Hawk, et. al, 1954)

Succinic dehydrogenase:

The method developed by Kun and Abood (1949) using 2:3:5 Tryphenyl tetrazolium chloride (TTC) as the hydrogen acceptor, was used for the estimation of SDH activity. TTC was reduced to coloured formazan due to the emzymic reaction and the colour developed was measured on a colorimeter. The procedure has already been described in an earlier chapter (Chapter, 4). The incubation mixture contained 0.5 ml 0.1 M phosphate buffer of pH 7.4, 0.5 ml of 0.5 M sodium succinate, 1 ml freshly prepared 0.1% TTC solution and 1 ml enzyme material, thus making up a total volume of 3 ml in

the test tube. After incubating the mixture at 37°C. in a water bath for 1 hour 7 ml acetone were added in the tube. The tubes were stoppered tightly and shaken thoroughly before centrifuging at 3000 r.p.m. for 3 minutes. The clear supernatent obtained was used to read the colour on a Klett-Summerson photoelectric colorimeter at 420 mμ. SDH activity is expressed as μg formazan formed per mg. protein for 1 hour.

Results

The results obtained from the study of the distribution pattern of lipase and SDH in the particulate fractions of the beetle flight muscles, are presented in Table 1. It is evident from the results obtained that lipase is concentrated more in the soluble cytoplasmic fraction (Supernatent). The next higher value was obtained for the microsomal fraction, while mitochomdrial and the myofibrillar and nuclear fractions follow in this order, for lipase activity. Mitochondrial fraction gave the highest value for SDH activity followed by the myofibrillar and nuclear fraction, microsomal and supernatent fractions in the order of activity. The recovery of lipase activity after differential centrifugation followed by the period of enzyme assay, was nearly cent per cent (99.75%). This indicates that lipase is quite a stable enzyme and can withstand the rigors of the procedures employed to a considerable extent. The SDH recovery was only 76.1% when compared with the whole homogenate activity. It is known that freezing and thawing and the time lag due to fractionation,

reduces the activity of the oxidative enzymes (Barting and Rosenthal, 1960).

Table I. Intracellular distribution of lipase and SDH in the different particulate fractions of the beetle flight muscles. The value given is the average of 6 experiments.

Enzyme	Homoge nate.	Percentage values				
		Myofibrils & Nuclei.	Mito- chon- dria	Micro- some	Super- natent	Recovery %
Lipase	100	10.75	20.8	27.6	40.6	99.75
SDH	100	20.4	30.5	14.9	10.3	76.1

Discussion

The occurrence of lipase and SDH in high concentrations in the flight muscles of the beetle has been demonstrated and the roles of these enzymes with regard to sustained muscular activity have been discussed in chapter 4. It is known that lipase brings about the break-down of fat into fatty acids and glycerol and also the synthesis of fat from fatty acids and glycerol. This duel role of synthesis as well as degradation is a characteristic feature of most enzymes and is well established in the case of lipase (Baldwin, 1957). The fat in the muscle, before entering into metabolic mill should be hydrolized into its component fatty acids and glycerol. The occurrence of

this enzyme in all the particulate fractions especially in the supernatent is of considerable interest. The same has been discovered in the vertebrate heart (George and Iype, 1961b) and skeletal muscles (George and Talesara 1961b). This leads to the suggestion that lipase in muscle has a diffused pattern of distribution and the enzyme could be recovered mostly in the supernatent, a finding observed by DeNella and Meng (1960) also for the hog intestinal lipase.

Microsomes are known to be the sites of fat synthesis in muscle (Green, 1960). The considerably high activity found in the microsomal fraction is therefore to be expected in a muscle which contains considerable amount of fat. It is a possibility that the microsomes synthesize fat in large amounts in the insect muscle because of the absence of a well developed circulatory system as found in the vertebrates, for the transport of the fuel reserve to the muscles. The role of fat bodies of insects in synthesizing fat, in this connection however, cannot be ignored. But the reserve fat in the insect muscle is so high that even the intercellular spaces in the muscle, are filled with fat globules. A considerable part of this fat must have been synthesized in the muscle itself by the microsomes and kept as reserve fuel for energy.

The mitochondrion is the store house of most of the oxidative enzymes (Green, 1954) which are arranged inside its insoluble frame work. This makes it difficult for the enzymes to diffuse out, especially SDH and cytochrome oxidase. The fat content of the insect sarcosomes (Mitochondria) is shown

to be quite high (29%) (Watanabe and Williams, 1951). The lipase present in the mitochondrial fraction could be for the hydrolysis of this fat for oxidation. But the requirement of ene-rgy in insect flight muscle during activity is so high, more than 50 times the normal requirement (Weis-Fogh, 1952) that extra fat from outside the mitochondria has to be supplied as fattyacids to the mitochondria. This would mean that the lipase in the microsomal fractions would also hydrolyze fat for the supply of fatty acids to the mitochondria. Fatty acid oxidation has bee-n shown to take place in the mitochondria. (Kennedy and Lehninger, 1949, Green, 1954, 56). Therfore the lipase in a soluble form in the flight muscles of the beetle could also play a similar role in the utilization of fat in this muscle. It can be suggested therefore that the diffusibility of lipase helps the mitochondria to obtain more fatty acids for oxidation.

The myofibrillar fraction also contains a good percentage of lipase. So far only a few enzymes such as ATPase and aldolase are shown to be present in myofibrils (Chappell and Perry, 1953; Perry, 1959). The soluble nature of lipase must have contributed to a certain extent to the activity obtained in this fraction. It may also be possible that all the mitochondria and the microsomes could not be separated completely since it was found that there is a good amount of SDH activity in the myofibrillar fraction. This fraction contains the nuclei also. Therefore the activity observed in this fraction need not be entirely from the myofibrils. However, this does

not rule out the possibility of the myofibrils possessing some lipase.

The concentrations of the oxidative enzymes in the insect muscles has been investigated in the whole homogenate (Barron and Tahmisian, 1948) Roeder, 1951) as well as in the different particulates, especially mitochondria (Watanabe and Williams, 1951, 1953; Sacktor, 1953; Sacktor and Cochren, 1958; Chance and Sacktor, 1958; Zebe and McShan, 1957). These authors observed that all the oxidative enzymes which are significantly related to the oxidation of Krebs cycle and other intermediate substrates are exclusively found in the mitochondria of the insect flight muscles. The SDH activity in the mitochondrial fraction, was the highest among all the particulate fractions. It has been shown that the minute tracheoles are directly connected with mitochondria in the flight muscles of insects (Edwards and Ruska, 1955).Therefore it is possible to visualize the immediate and complete oxidation of the substrates in the flight muscles. Zebe and McShan (1957) have suggested that the insect flight muscles are adapted for the aerobic metabolism due to the negligible amount of lactic dehydrogenase present in the flight muscles of insects. They also suggested that the absence of glycolysis in the flight muscles of insects can be correlated with the large amount of glycerophosphate dehydrogenase found in the soluble form in the supernatent as well as the DPN-linked particle bound glycerophosphate dehydrogenase. Glycerophosphate was oxidized at a higher rate (10 times) than any of the Krebs

cycle intermediates (Sacktor, 1958). There was not much difference in the concentration of this enzyme in insects which utilize fat and carbohydrates for energy (Zebe and McShan, 1957). It is also known that SDH and cytochrome oxidase activities in the flight muscles of insects are considerably higher when compared with the activities of these enzymes in the vertebrate skeletal muscles (Roeder, 1951; Watanabe and Williams, 1953, Zebe, 1956). The higher concentration of SDH in the flight muscle mitochondrial fraction should be to assist the fatty acid oxidation. SDH level has been shown to be more in sites where fatty acid oxidation takes place (George and Iype, 1960). Further, the oxidation of fatty acids is completely an aerobic process and is well supported by the tracheal system of respiration.

The comparatively lesser concentration of SDH in the microsomal fraction can be attributed to the synthesis of neutral fats in the microsomes. George and Iype (1960) have suggested that the occurrence of less SDH and more lipase in an organ may be regarded as meant for the sythesis of fat while more SDH and less lipase for the oxidation of fat. In this respect the mitochondria in the beetle flight muscles can be considered as the site of fatty acid oxidation while the microsomes for the sythesis of fat.

The SDH level obtained in the myofibrillar and nuclear fraction can mostly be attributed to the mitochondria and some microsomes remaining unseparated. A pure preparation

of myofibrils seems to be the most delicate step in the study of tissue fractionation. The data so far available on SDH in tissue particulates show that this enzyme is a mitochondrial enzyme (Schneider, 1958) and the presence of this in the supernatent and other particulates may be due to the breaking down of mitochondria into smaller particles which are obtained in the microsomal fraction as well as in the supernatent.

The literature on the biochemical studies on cell particulates has been recently reviewed by Schneider and Hogeboom (1956). They have concluded that cellular fractions are the results of the integrated action of mitochondria with other cell particulates. The important role of mitochondria in large numbers in the flight muscles of insects, which are metabolically most active among the skeletal muscles so far studied, therefore needs no special emphasis.