CHAPTER 8

LIPASE AND SUCCINIC DEHYDROGENASE ACTIVITY OF THE PARTICULATE FRACTIONS OF PIGEON BREAST MUSCLE HOMOGENATE

Considerable evidence has accumulated in the recent years in support of the view that fat forms the chief fuel for energy during long and sustained muscular activity (Weis-Fogh, 1952; George and Jyoti, 1953, 1955b, 1957; Bing, 1956; and Drummond and Black, 1960). It has also been shown that the pectoralis major muscle of flying birds and other highly active muscles such as the breast muscle of the bat and the flight muscles of insects contain high concentrations of a lipase (George and Scaria, 1956, 1957; George, Susheela and Scaria, 1958b; George, Vallyathan, and Scaria, 1958). The presence of this enzyme in these muscles has also been demonstrated histochemically (George and Scaria, 1958a and 1958b). These authors (George and Scaria, 1959) further studied some of the biochemical properties of the pigeon breast muscle lipase in comparison with that of the pancreatic lipase of the same animal and observed that the muscle lipase is essentially a true lipase which hydrolyzes tributyrin rapidly. Obviously muscle lipase should play a significant role in the fat metabolism in muscle by splitting up fat into fatty acids and glycerol as the first step in the oxidation of fat for energy.

The breast muscle of birds which indulge in long and sustained muscular activity, are predominantly composed of a narrow red variety of fibres, and these with a high mitochondrial content, together with large stores of fat, high concentrations of lipase and oxidizing enzymes and myoglobin, seem well equipped for an organized oxidative metabolism. In contrast, the broad white fibres of that of a nonflying bird like the domestic fowl and the fewer such fibres present in the pigeon breast muscle are adapted for functioning anaerobically, being loaded with glycogen. These studies on the flight muscles of birds have been recently reviewed by Drummond and Black (1960).

In the light of the above findings it was thought worthwhile to determine the respective roles of the different subcellular particles in muscles in the degradation and utilization of fat as well as the biosynthesis of fat from fatty acids. With the histochemical methods available at present it is impossible to demonstrate the exact localization and distribution of lipase activity at the intracellular level as it has been found possible with certain other enzymes. Though several enzymes such as those associated with the Krebs cycle, glycolysis, oxidative phosphorylation and the fatty acid oxidase system have been studied in the cell particulate fractions of the various metabolically active tissues (Schneider and Hogeboom, 1956; and Schneider, 1959), no such studies have been made on the lipase activity in the particulate fractions of any tissue. The only work of this nature which has been recently reported is that of Dinella and Meng (1960) who have studied the lipase activity of the duodenal homogenate fractions. In the present investigation the <u>pectoralis major</u>

muscle of pigeon has been selected for the study, in an attempt to assess the lipase and SDH activities of the particulate fractions.

Material and Methods

Isolation of particulate fractions

Particulate fractions of the pigeon breast muscle homogenate were isolated by the conventional differential centrifugation method adopted from the procedures of Hogeboom, Schneider and Palade (1948), Schneider (1948), Schneider and Hogeboom (1950), Chappell and Perry (1953), and Harman and Osborne (1953). Homogenates prepared in isotonic sucrose solution have been found most suitable for the isolation of the particulates by these investigators. Apart from this, it was found that isotonic sucrose solution showed practically no effect on the lipase activity and hence it was chosen as the medium for homogenization.

In all experiments the pectoralis major muscle of laboratory pigeons (Columba livia) was used. The bird was decapitated and bled completely. Approximately 2 gm. of muscle was removed rapidly and after making multiple transverse incisions, homogenized in 0.25 M sucrose solution within 3 - 4 minutes, in a previously chilled mortar and maintained at 0° C. A portion of this muscle homogenate was preserved for the estimation of lipase and SDH activities till the various fractions were isolated from a known volume of the original homogenate. This homogenate in sucrose was made the starting point of the tissue fractionation. The subsequent experimental procedures were carried out at a temperature between $0^{\circ} - 4^{\circ}$ C. A known volume of the original homogenate was pipetted into centrifuge tubes and was centrifuged for 10 minutes at $600 \times g$. in a 'MSE Superspeed 25' refrigerated centrifuge in order to sediment the myofibrils and cell debris. The supernates (S_1) were removed and saved for further fractionation. The sediment was resuspended in .25 M sucrose solution with the help of a loose homogenizer and recentrifuged as before. The supernates (S_2) were collected and combined with (S_1) supernates. The final sediment was resuspended in a small volume of .25 M sucrose and labelled as the myofibril fraction, though this contained cell debris, some mitochondria and possibly traces of microsomes too as no attempt was made to obtain a purified preparation of myofibrils alone.

For the isolation of mitochondria the combined supernates (S_1 and S_2) were centrifuged at $7,500 \times g$. for 15 minutes. The cloudy translucent supernate (S_3) was poured off and saved for the isolation of microsomes. The residue was resuspended in .25 M sucrose and was recentrifuged as before. The supernate (S_4) was removed and combined with (S_3) supernate. The residue was suspended in .25 M sucrose and labelled as mitochondrial fraction. Microscopic examination of this suspension after staining with Janus Green B showed that the sample was a pure preparation of mitochondria free from myofibrils. The combined supernate (S_3 and S_4) saved for the isolation of microsomes was centrifuged at $40,000 \times g$. for one hour. The supernate obtained was poured in another tube and labelled as the soluble fraction whereas the residue was suspended in .25 M sucrose solution and labelled as the microsomal fraction. Having isolated the various fractions the appropriate dilutions were made and the lipase and SDH activities were determined simultaneously of the whole homogenate as well as the various fractions by the method given below.

Protein was estimated according to the micro-Kjeldahl steam distillation method (Hawk *et al*, 1954).

Succinic dehydrogenase activity

SDH activity was determined according to the procedure of Kun and Abood (1949) using TTC as the hydrogen acceptor, as mentioned in Chapter 1. The enzyme activity is expressed in µg. formazan/mg. protein/hour.

Lipase activity

Lipase activity was determined manometrically by the method adopted from Martin and Peers (1953), in a bicarbonate carbon dioxide buffer system of pH 7.4 at 37° C. using the Warburg apparatus as mentioned in Chapter 4.

Results

The percentage of yield and the specific activities of lipase and SDH of the whole homogenate as well as the various fractions of the pigeon breast muscle are shown in Table 1. The percentage of recovery of lipase was found to be more than that of SDH. The maximum yield of lipase was obtained for the soluble fraction whereas the mitochondrial and the microsomal fractions showed in each case nearly half of the yield of the soluble fraction. As mentioned earlier a purified preparation of myofibrils was not attempted and thus this preparation was contaminated with cell debris, few mitochondria and possibly traces of microsomes as is revealed by the lipase and SDH activities of this fraction. The specific lipase activity of the microsomal fraction was found to be nearly 2 and 3 times higher than that of the mitochondrial and soluble fractions respectively. SDH activity was found to be mainly confined to the mitochondrial fraction. The microsomal and the soluble fractions though occasionally revealed the presence of SDH, did not show it in any appreciable amount.

Discussion

The extensive studies on lipids and esterases made by the schools of Willstater and Rona are well known and the literature has been exhaustively reviewed by Ammon and Jarrma (1950). It is generally agreed that tributyrin is hydrolyzed readily and rapidly by a 'true lipase' (Dunkley and Smith, 1951; Martin and Peers, 1953; Sumner and Somers, 1953; George and Scaria, 1959; and Lynn and Perryman, 1960). The present study of the particulate fractions is therefore to be regarded as on 'true lipase'-a specific esterase, which hydrolyzes the triglycerides that are the most prominent lipids. Flight muscles of the birds indulging in long and sustained muscular activity show the presence of higher concentrations of intramuscular fat (George and Naik, 1960) and lipase (George and Scaria, 1956). Apart from this, birds store large amounts of fat in their adipose tissue found at many sites in the body. Migratory birds especially accumulat large amounts of fat in their adipose tissue during the premigratory periods (Odum and Perkinson, 1951; Mc Greal and Farner, 1956; King and Farner, 1956; Odum and Connell, 1956) and much of this is probably transported to sites where it is utilized during migratory flight. Though the significance of the presence of lipase in the flight muscles of birds was already suggested, the exact localization and distribution of this enzyme in cell particulates has not been attempted earlier nor has any light been thrown on the exact role of lipase in the breakdown and biosynthesis of fat to and from fatty acids in muscle at the subcellular level.

Since mitochondria are the principal locale of carrying out all of the reactions of Krebs cycle (Schneider and Potter, 1949; Paul and Sperling, 1952; Chappell and Perry, 1953; and Schneider, 1959) and of fatty acid oxidation (Schneider, 1948; Kennedy and Lehninger, 1948 and 1949), the presence of lipase in these particles may be attributed to the fact that this enzyme plays a major role in splitting neutral fats or triglycerides into fatty acids and glycerol as the first step in the oxidation of fat. The mitochondria of the pigeon breast muscle as is shown, contain an appreciable concentration of lipase and practically all the SDH. It is also known that the microsomes are the sites of lipid synthesis (Green, 1960). A high concentration of lipase and a negligible concentration of SDH in the microsomes of the pigeon breast muscle lends further support to this fact.

The enzymes involved in fatty acid oxidation are known to catalyze reversible reactions and therefore are assumed also to be responsible for fatty acid synthesis. However, it has not yet been possible to demonstrate the synthesis of fatty acids in isolated mitochondria alone which suggest the possibility that synthesis and breakdown of fat take place in different regions of the cell (Mehler, 1957; Kennedy, 1957; and Langdon, 1957). If that is the case the major role of lipase in the various fractions may vary accordingly and may even vary in their specific properties to a certain extent. Nevertheless, the possibility of esterifying fatty acids and glycerol, into triglycerides by mitochondrial lipase cannot be entirely ruled out as indicated by some workers (Stein and Shapiro, 1957; and Stein, Teitz and Shapiro, 1957).

Animal fats usually contain only small amounts of free fatty acids and it is quite natural that the newly synthesized fatty acids are immediately esterified with glycerol (formed by the breakdown of carbohydrates) to triglycerides (Kleinzeller, 1948) through the reversal of lipase action which seems to be the predominant role of lipase in the microsomes. The biosynthesis of neutral fats or triglycerides from free fatty acids has been shown by the reversal of lipase action (Borgstrom, 1953; Jedeikin and Weinhouse, 1954). Teitz and Shapiro (1956) however, found that the incorporation of labelled fatty acids into neutral fat in liver homogenate requires ATP and some heat stable cofactor and suggested the formation of 1,2-diglyceride as an intermediate in the synthesis of triglycerides. It is of interest that 1,2-diglycerides are also the primary product of the action of pancreatic lipase on triglycerides (Borgstrom, 1954; Mattson and Beck, 1955). The synthesis of neutral fats from labelled palmitate has also been reported (Stein, Teitz and Shapiro, 1957). These authors have suggested that the microsomes may bring about the initial steps while the mitochondria catalyze the final step in the conversion of fatty acids to triglycerides. Whatever may be the sequence of events in the synthetic pathway in triglyceride synthesis, the microsomal lipase in the pigeon breast muscle appears to play a prominent role in the final step of the biosynthesis of neutral fats by the reversal of the hydrolytic action. The lipase in microsomes besides synthesizing neutral fats may also play a role in splitting fat into fatty acids and glycerol. These fatty acids may be made available to the mitochondria for oxidation.

As regards the myofibril fraction, nothing can be said with certainty at present unless a purified preparation free from other cellular particulates is made. The maximum yield of lipase from the soluble fraction is probably suggestive of the fact that in the intact cell the enzyme is localized mainly in the mitochondria and microsomes and that it is either liberated or partly washed off during the isolation process as this enzyme seems to be water soluble.

Recently Dinella and Meng (1960) studied the lipase activity in the duodenal homogenate fractions and reported highest recovery of the enzyme from the soluble fraction. This fairly agrees with the results obtained in the present study also. Regarding the solubilization of enzymes and purity of the particles obtained nothing could be said with certainy.

According to Schneider (1959) himself it is not known "whether enzymes or other substances found in the soluble fractions of tissues, as prepared by our present procedures, have been lost from mitochondria or other particulate structure during their isolations or whether the enzymes found in low concentrations are true components of these fractions or contaminants". In conclusion it may be said that the mitochondrial lipase appears to be mainly responsible for splitting neutral fats into fatty acids and glycerol whereas the microsomal lipase in the esterification of fat from fatty acids and glycerol in the final step of fat synthesis. Nevertheless, the theoritical possibility of the reversible action of both the mitochondrial as well as the microsomal lipase cannot be ruled out.