CHAPTER 3

HISTOCHEMICAL STUDY OF FAT AND CERTAIN ENZYMES RELATED TO FAT METABOLISM IN THE MUSCLE OF *CIRRHINA MRIGALA*

The role of fat as the major fuel reserve for energy during long and sustained activity has been well established in the muscles of flying birds (George and Jyoti, 1955, 1957); bats (George and Jyoti, 1958); insects (Weis-Fogh, 1952; George and Bhakthan, 1960, 1963); fishes (George, 1962; Bilinski, 1963) and muscular organs like the vertebrate heart (Bing *et al*. 1954; George and Iype, 1963) and the mammalian diaphragm (Wertheimer and Ben-tor, 1952; George and Susheela, 1961). George and Jyoti (1957) have shown that at least 77% of muscle metabolism in the pigeon during sustained muscular activity is due to the oxidation of fat. Drummond and Black (1960) in their review on fats, carbohydrates and proteins in the body of fishes during migration, have pointed out that fish muscle contains little glycogen which could get easily depleted, whereas the fat store could serve as a potentially large source of energy. Alexander (1955) found a large quantity of fat in the red muscle of certain fishes. Boddeke *et al*. (1959) in a histochemical study of the muscle in a number of freshwater fishes showed the presence of large amounts of fat and glycogen in the red muscle.

Studies on the various enzymes which are of importance in fat metabolism have been comparatively few in the case of the fish muscle. In order to obtain an understanding of the role of these enzymes, an intimate knowledge of their intercellular and intracellular localization in the muscle is essential.

Dubowitz and Pearse (1960) reported a histochemical study of the oxidative enzymes in the muscles of the goldfish. Ogata and Mori (1964) showed the presence of certain oxidative enzymes in three species of fish viz. Cyprinus carpio, Harengula zunasi and Lateolabrax japonicus. Earlier observations in our laboratory on the red and white muscles of the mackerel with respect to their fat content and enzymes (lipase and succinic dehydrogenase) showed that the red muscle is adapted for an aerobic metabolism using fat as fuel and the white for an anaerobic metabolism using glycogen (George, 1962). In the present study, the histochemical localization of myoglobin, fat and some of the enzymes associated with fat metabolism has been made in the red and white muscles of the carp, Cirrhina mrigala.

MATERIAL AND METHODS

The freshwater fish, Cirrhina mrigala was used as material. The fish was killed by decapitation, skinned and pieces of the lateral line muscle were fixed for fat in Baker's calcium formol (Baker, 1946). Thin frozen sections 15 to 20 μ thick were cut for studying the localization of myoglobin and of the enzymes, lipase, succinic dehydrogenase, some of the DPN linked dehydrogenases and cholinesterases.

Myoglobin : Fresh frozen sections were fixed in formol saline and incubated in a medium prepared according to the method of Drews and Engel (1961) as modified by Chinoy (1963).

Fat : The tissue was fixed in Baker's calcium formol for 24 hours, washed in running tap water for the same time and embedded in 15% gelatin. Sections 15 to 20 μ thick were cut on a freezing microtome and stained with Sudan black B.

Lipase : Localization of lipase activity was studied using 'Tween 85' as the substrate and alizarin red S to stain the calcium soap formed (Chapter 7).

Succinic dehydrogenase : Thin frozen sections of the tissue were cut and the demonstration of enzyme activity was accomplished at room temperature (28°C) by the improved method of George and Talesara (1961) using neotetrazolium chloride as the hydrogen acceptor.

DPN-linked dehydrogenases (malic dehydrogenase, β-hydroxybutyrate dehydrogenase) : Sections were incubated at room temperature (28°C) in a medium which contained the specific substrates (sodium-L-malate, DL-β-hydroxybutyric acid sodium salt) according to the technique of Hess et al. (1958).

Cholinesterases (acetyl and butyryl) : The method employed was that of Koelle and Friedenwald (1949) as modified by Coupland and Holmes (1957) using acetyl thiocholine iodide and butyryl thiocholine iodide as substrates.

RESULTS

By the histochemical detection of the metabolite and the enzymes studied, the three regions (red, intermediate and white) in the fish muscle could be clearly distinguished.

Myoglobin demonstrated by the Benzidine peroxidase

reaction was deposited in the form of bluish green crystals. A denser localization of the pigment was seen in the red fibres (Fig. 3) whereas the intermediate and white fibres showed very little or negligible activity.

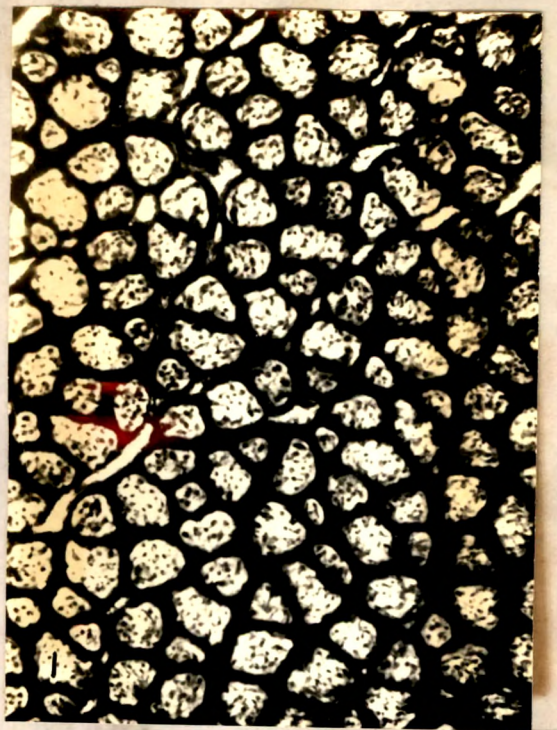
Fat staining with Sudan black B showed that a considerable amount of fat was present in the red muscle fibres (Fig. 1). However, no fat could be detected in the white fibres, although some amount was demonstrable in the fibres of the intermediate region.

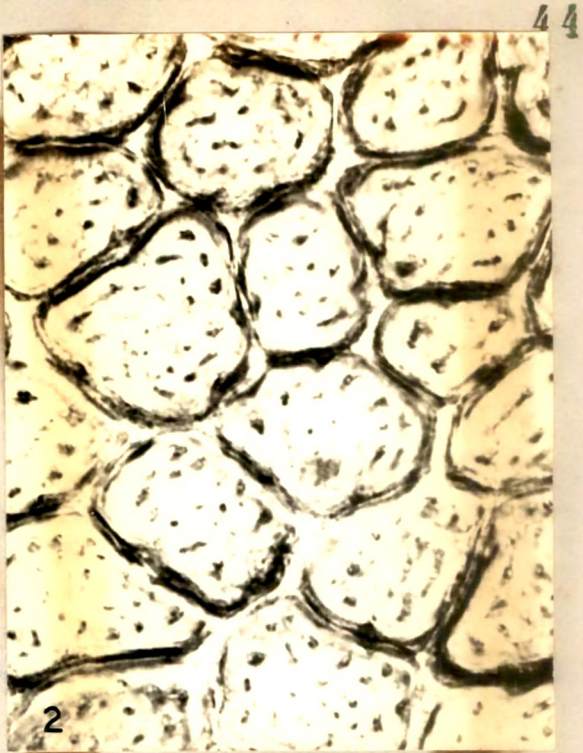
The predominant localization of lipase activity was denoted as a coloured calcium soap associated with the mitochondria which are numerous in the fibres of the red muscle (Fig. 2) and hardly evident in those of the white.

Succinic dehydrogenase activity was revealed in the form of purple granules of diformazan indicating the loci of enzyme activity which was greatest in the red fibres (Fig. 8), almost nil in the white fibres and an activity between that of the red and white fibres was found in the fibres of the intermediate region.

A very high activity of the DPN-linked enzymes was noted in the mitochondria of the red fibres (Figs. 4 & 6), particularly that of the β-hydroxybutyric dehydrogenase. The intermediate fibres showed a lower enzyme activity (Figs. 5 & 7), whereas the white fibres showed a complete negative reaction.

Acetyl and butyryl cholinesterases were present at the





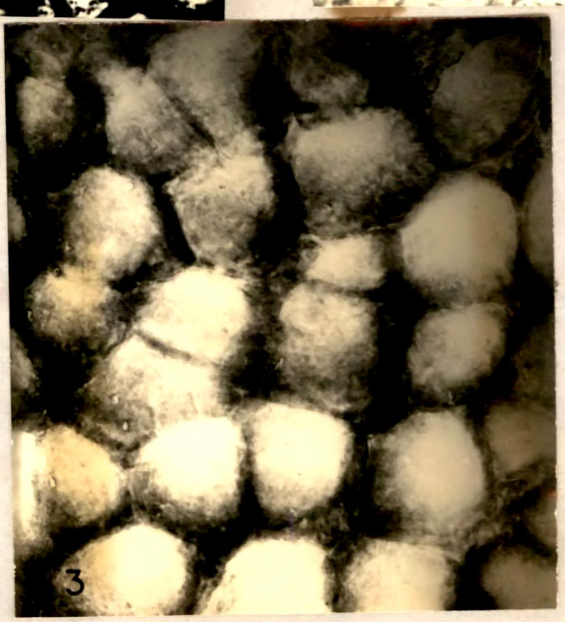
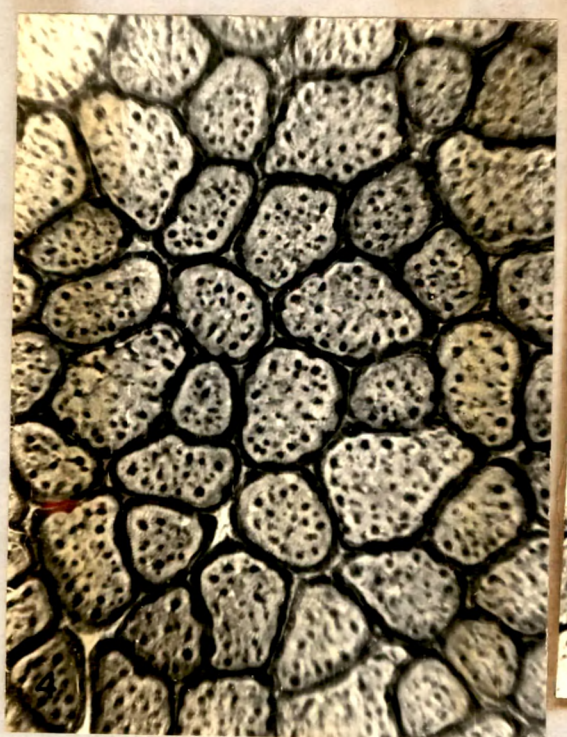


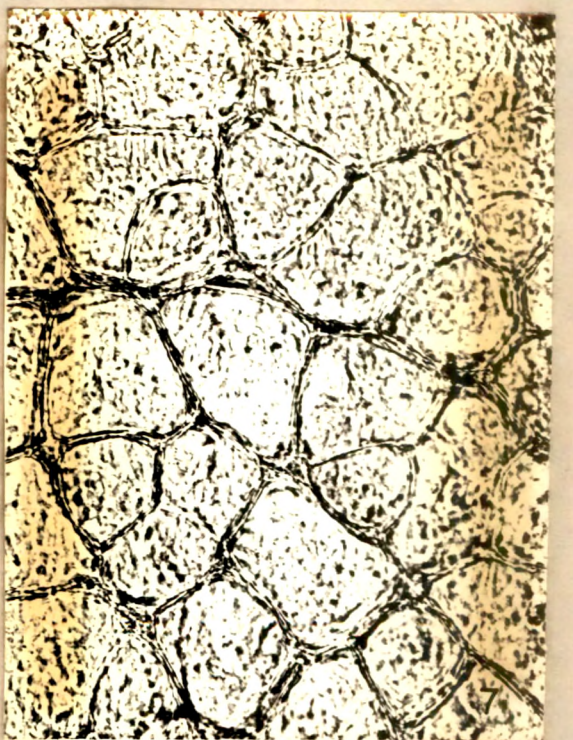
Fig. 1 Histochemical demonstration of fat in the red muscle region. X 190

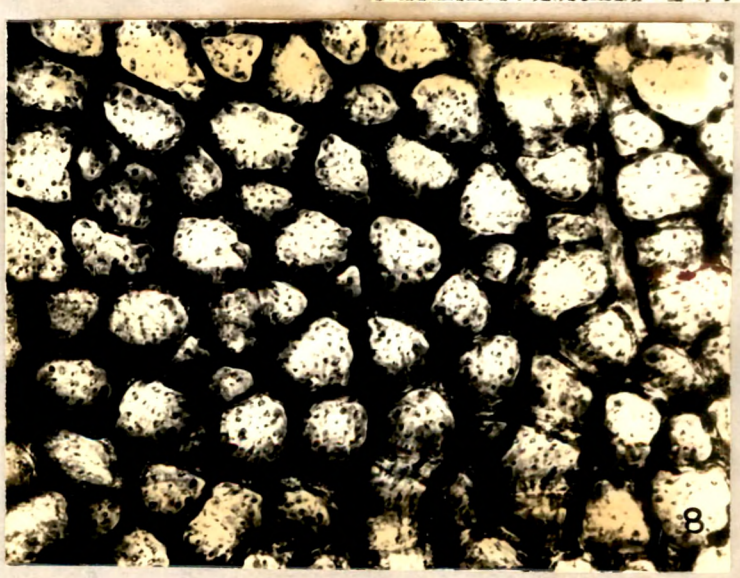
Fig. 2 Histochemical demonstration of lipase in the red muscle region. Activity is seen to be confined to the mitochondria. X 640

Fig. 3 Histochemical demonstration of myoglobin in the red muscle region. X 640



Figs. 4 & 5 Histochemical demonstration of β hydroxybutyrate dehydrogenase in the red and intermediate muscle regions respectively. The red fibres show greater activity than the intermediate ones. The enzyme activity is mainly located in the mitochondria. Fig. 4 X 300; Fig. 5 X 256





Figs. 6 & 7 Histochemical demonstration of malic dehydrogenase in the red and intermediate muscle regions. Higher activity of the enzyme is seen in the red than in the intermediate muscle region. Enzyme activity is mainly mitochondrial. X 190

Fig. 8 Histochemical demonstration of succinic dehydrogenase in the red muscle region showing the localization of the enzyme activity in the mitochondria. X 190

Figs. 9, 10 & 11 Histochemical demonstration of acetyl cholinesterase activity in the fibres of the red, intermediate and white muscle regions respectively. Figs. 9 & 11 X 400; Fig. 10 X 640

Figs. 12, 13 & 14 Histochemical demonstration of butyryl cholinesterase activity in the red, intermediate and white muscle region fibres respectively. Figs. 12 & 14 X 400; Fig. 13 X 640

Fig. 15 A portion of the white fibre from Fig. 14 enlarged to show the localization of butyryl cholinesterase. X 640

nerve endings of the red, intermediate and white fibres. The concentration of acetyl cholinesterase (Figs. 9 & 10) was found to be higher than butyryl cholinesterase (Figs. 12 & 13) in the red and intermediate fibres. Both the enzymes were present in relatively lower concentrations in the white fibres (Figs. 11, 14 & 15). The morphological nature of the neuromuscular junctions was similar in the three types of fibres, they being of the " en plaque " type as suggested by Cole (1955). The structure of the nerve ending appeared to run a short distance linearly along the periphery of the muscle fibre on either side and then turned inward obliquely to join the linear strand on the opposite side. A number of variations from this basic pattern could be observed. Some of the nerve endings showed a ramified appearance while others appeared to encircle the fibre completely.

DISCUSSION

From the results obtained, it is clear that numerous mitochondria and very high concentrations of fat and oxidative enzymes, lipase and cholinesterases are lodged in the red fibres. These fibres are therefore sites of considerably high metabolic activity. On the other hand, the fact that the white fibres in which mitochondria are extremely few and small so much so that they were not histochemically demonstrable when treated for any of the oxidative enzymes, suggests that in these fibres, there is hardly any oxidative metabolism. The inter-

mediate fibres which are intermediate with regard to the metabolite load as well as the enzyme concentrations are adapted for a limited extent of both aerobic and anaerobic metabolism.

The relatively high concentrations of myoglobin obtained in the red muscle is in conformity with the observations of Umemura (1951), Hamoir (1953) and Jebsen (1954). Matsuura and Hashimoto (1954) investigated the distribution of haemoglobin, myoglobin and cytochrome c in several fishes mainly scombroids and found the red muscle to be richer in these pigments than the white muscle. Korzhuev (1961) has postulated that the total amount of haemoglobin and myoglobin in the blood and muscles has a definite relationship with the position of the animal in the phylogenetic series. He has shown that the least amount of these pigments is typical of fish and the maximum peculiar to birds and mammals. Apart from the phylogenetic significance in the distribution of myoglobin in muscle, the importance of myoglobin in this tissue in which there is considerable amount of oxidative metabolism involving fat catabolism has been indicated by George and Talesara (1961 c). The same authors (1962 b) have shown that in the utilization of fat for energy it has first to be broken down to fatty acids and glycerol by a lipase. The red fibres of the fish muscle also contain high concentrations of fat and lipase and the various oxidative enzymes thus indicating the presence of an efficient machinery for the hydrolysis of fat and the oxidation of the fatty acids

for energy during prolonged activity. The white fibres, on the other hand, with low fat content and low lipase and oxidative enzyme activity could yield only a negligible amount of fatty acids to be oxidized by the poorly developed oxidative machinery. Recently, Bilinski (1963) has shown that the oxidation of some fatty acids takes place at a very much higher rate in the red than in the white muscles of the trout. Similar results have been obtained in three freshwater fishes of the Family Cyprinidae (Chapter 5).

Acetyl- as well as butyryl cholinesterase were found to be present at the neuromuscular junctions of the muscle fibres. Lundin (1962) demonstrated the localization of butyryl cholinesterase in muscle cells and showed its occurrence only in salt water fishes (including brackish water). He further reported that the freshwater fishes studied by him lacked the ability to hydrolyze butyryl choline. These results are at variance with those of Lundin in this respect since the freshwater fish studied by us was capable of hydrolyzing butyryl choline. Chinoy and George (1965) have shown the presence of high concentrations of acetyl cholinesterase in the red tonic fibres and butyryl cholinesterase in the white tetanic fibres in a variety of vertebrates higher than fishes. Nevertheless, in the present study, acetyl and butyryl cholinesterases were both found in higher concentrations in the red fibres than in the white, though acetyl cholinesterase was in considerably higher amounts than butyryl.

McCance et al. (1949) reported enormously high activity of butyryl cholinesterase in the mammary gland and colostrum of the bitch. Since the mammary gland is known to manifest considerable esterase activity (Montagna and Bourne, 1957), some sort of relation between the butyryl cholinesterase and the metabolism of lipids is envisaged. Gerebtzoff (1959) has postulated that "combined with the eventual participation of hepatic cholinesterase in the assimilation of food, the excretion of cholinesterase into the alimentary tract suggests that in unknown circumstances and on unknown physiological substrate, cholinesterase (and acetyl cholinesterase in the rabbit) might become one of the weapons of the hydrolytic arsenal formed by the aliesterases and the lipases". Likewise, Smith et al. (1963) observed in biopsied human muscle, that the highest activity of acetyl cholinesterase was associated with the microsomal fraction and thereby suggested that acetyl cholinesterase was primarily a microsomal enzyme. That the microsomes are the active sites of fatty acid synthesis is well established (Green, 1960). It therefore goes without saying that the function of cholinesterases outside the nervous system, is possibly related with fat metabolism.