CHAPTER 2

HISTOCHEMICAL DEMONSTRATION OF GLYCOGEN AND CERTAIN GLYCOLYTIC AND PENTOSE CYCLE ENZYMES IN THE MUSCLE OF CIRRHINA MRIGALA

The existence of a heterogeneity in the cellular organization of a muscle with respect to its structural and biochemical characteristics is now well established from the studies on the pigeon pectoralis (Denny-Brown, 1929; George and Naik, 1959) and the rat skeletal muscle (Stein and Padykula, 1961).

In the pigeon pectoralis, George and Naik (1959) distinguished two distinct types of fibres : the narrow red fat-loaded variety associated with an oxidative metabolism and the broad white glycogen-loaded ones associated with a glycolytic metabolism. The presence of a higher concentration of glycogen in the white fibres than in the red was also indicated by the quantitative studies of Aleksakhina (1953), Gaspar (1957) and by the histochemical studies of Ogata (1960) in the muscles of the rabbit. Nevertheless, studies on fish muscle (Boddeke et al. 1959) have shown that the red muscle fibres contained more glycogen than the white. Similar results were obtained by Hess and Pearse (1961) in the red and white fibres of the mammalian skeletal muscle, Chinoy and George (1964) in the abdominal muscles of the pigeon and fowl and by Nene and George (1965) in certain appendicular muscles of the pigeon and fowl.

In view of these conflicting data, a study of the localization of glycogen and some of the glycolytic enzymes in fish muscle would be of interest. The information available with

respect to the glycolytic enzymes in fish muscle is mostly from biochemical studies. Studies on the glycolytic enzymes in fish tissues (MacLeod, 1960), together with those of Martin and Tarr (1960) on phosphoglucomutase, Cordier and Cordier (1957) on phosphorylase of fish muscle indicate that all the machinery for converting glycogen rapidly to lactic acid is present in the skeletal muscle of fishes.

As regards the synthesis of glycogen in the muscle, it has been suggested that the enzyme, uridine diphosphoglucose (UDPG) transglucosylase or glycogen synthetase is mainly responsible (Leloir et al. 1959; Villar-Palasi and Larner, 1958; and Hawk and Brown, 1959). The occurrence of this enzyme in muscles of different origin has been confirmed by Traut (1962), however, its presence in fish muscle has not as yet been established. It was therefore thought desirable to locate it in fish muscle.

Some of the other enzymes of importance in glycogen metabolism are the pentose cycle dehydrogenases. It has not been found possible to histochemically demonstrate these enzymes in fish muscle (Ogata, 1964). The present histochemical study of the distribution pattern of glycogen in relation with some of the glycolytic enzymes and the pentose cycle dehydrogenases in fish muscle, was therefore carried out.

MATERIAL AND METHODS

The fresh water fish, Cirrhina mrigala used as the experimental material in the present study were kept in spacious open air tanks and maintained on a ration of vitamin supplanted

wheat pellets. The fish was killed by decapitation and pieces of the lateral line muscle were taken for the various histochemical investigations. Thin frozen sections of about 15 to 20 μ thickness were used for the demonstration of enzyme activity.

Glycogen : Thin strips of the tissue were fixed in cold alcoholic picro-formol for 24 hours and embedded in paraffin wax of melting point 58 to 60°C. Sections 10 to 12 μ thick were cut and stained for glycogen by the periodic acid Schiff's reagent (Pearse, 1960).

Phosphorylase : For the demonstration of this enzyme, the incubation medium employed was according to the method of Eränkö and Palkama, using glucose-1-phosphate as the substrate. The sections after incubation were removed and transferred to Grams iodine solution for a few minutes, and mounted in iodine-glycerol.

Uridine diphosphoglucose (UDPG) glycogen synthetase : For demonstrating this enzyme the technique of Takeuchi and Glenner (1960) was employed. Glucose-6-phosphate was not added to the incubation medium.

Pentose cycle dehydrogenases (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) : For the detection of these two dehydrogenases, the sections were incubated at room temperature (28°C) in a medium which contained the specific substrates (glucose-6-phosphate barium salt and 6-phosphogluconic acid barium salt) according to the technique of Hess et al. (1958). It was found of advantage to include ethylene diamine tetracetic

acid (EDTA) 1mM in both the cases (Karnovsky and Himmelhoch, 1961).

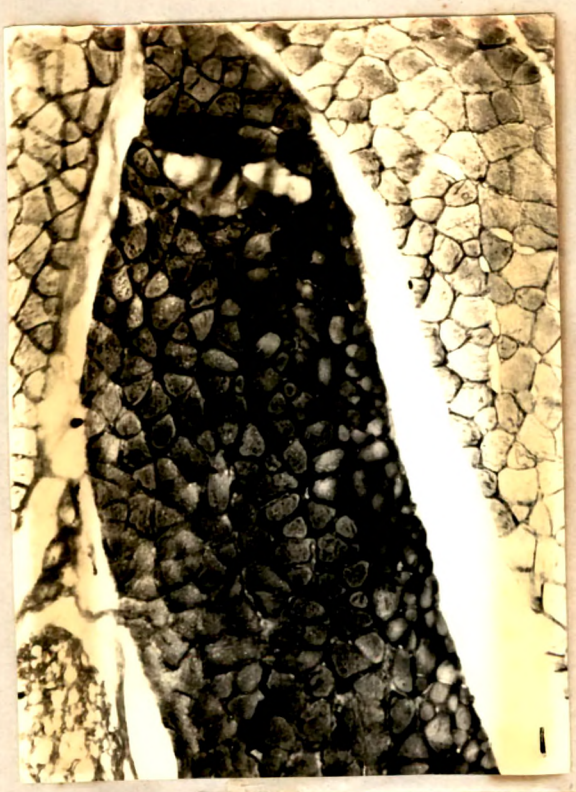
RESULTS

The muscle along the lateral line of the fish comprises of a dorsal strip of red muscle consisting of narrow red fibres, a ventral strip of white muscle having broad white fibres and an intermediate region of white fibres intermediate in size between those of the other two strips.

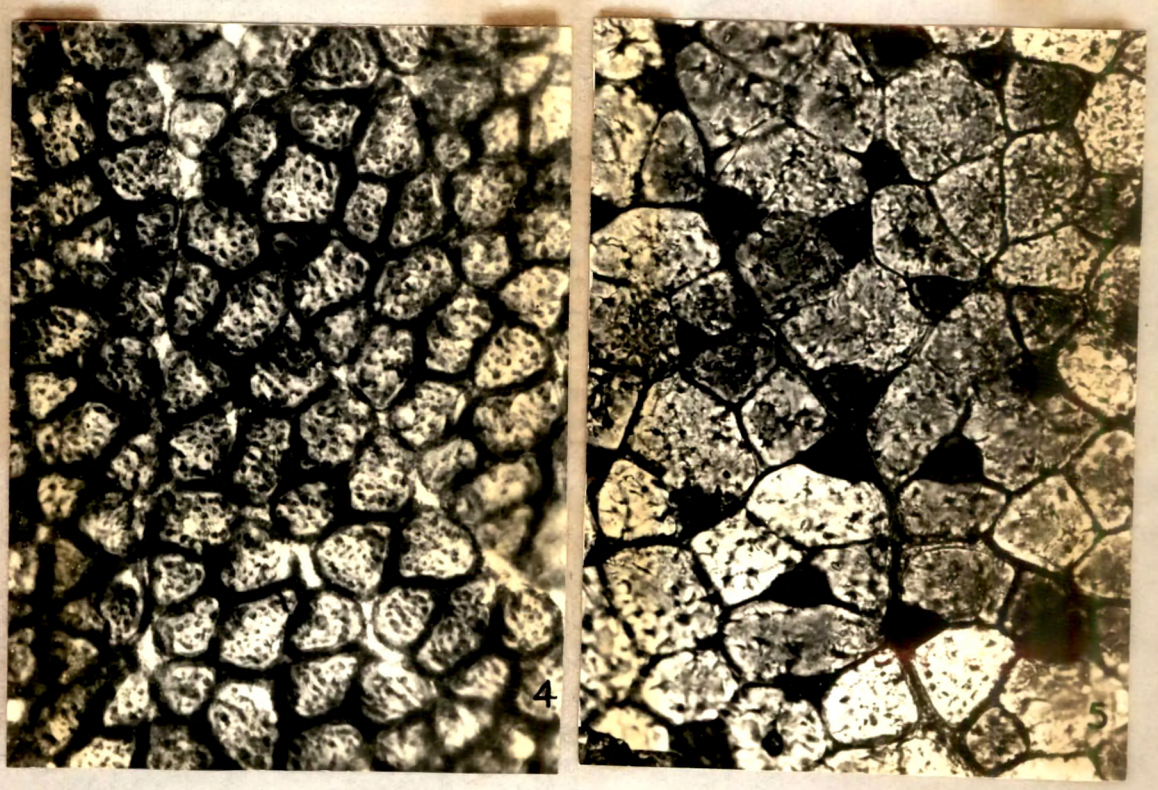
Sections stained to demonstrate glycogen gave a strong positive reaction in the red fibres (Fig. 1), a moderate reaction in the intermediate fibres (Fig. 2) and hardly any reaction at all in the white fibres (Fig. 3).

In the red muscle, the fibres reacted strongly for phosphorylase activity (Fig. 4), whereas in the white muscle, the fibres of smaller diameter gave a positive reaction and the larger ones negative. The narrow red fibres of the red muscle took up a magenta (purple) colour, whereas in the white muscle, the narrow white fibres stained blue and the broad white fibres appeared yellow. The fibres of the intermediate region (Fig. 5) displayed a staining reaction somewhat similar to that obtained in the fibres of the white muscle region.

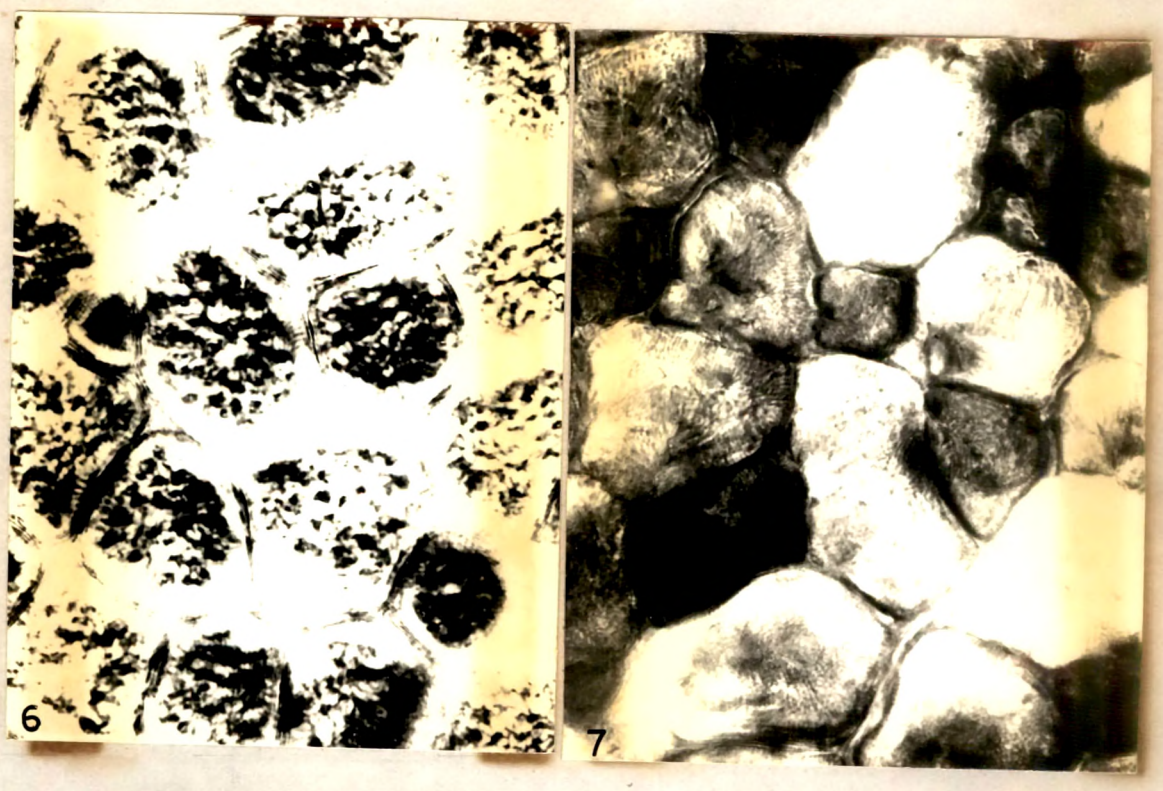
The red fibres were found to stain most intensively for UDPG glycogen synthetase activity (Fig. 6), whereas the white fibres gave the weakest reaction for this enzyme. In the intermediate region only the fibres of smaller diameter were stained (Fig. 7). The enzyme activity was seen to be localized mainly in the sarcoplasmic reticulum of the red fibres.



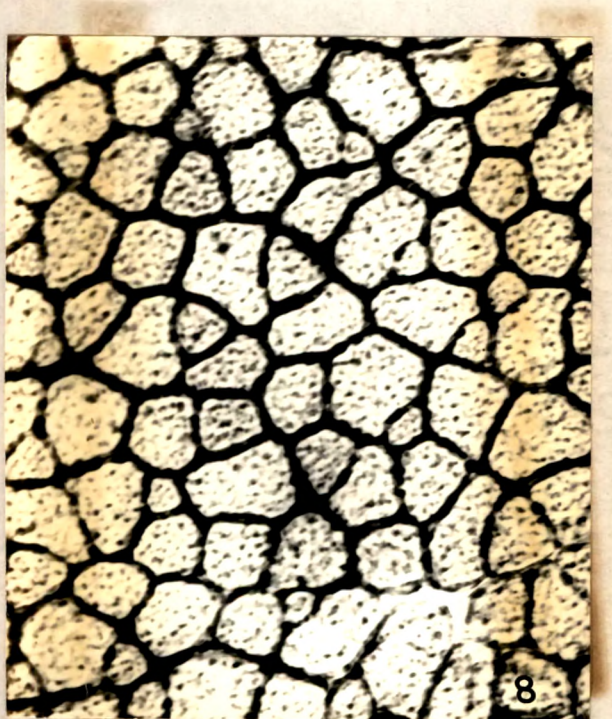
Figs. 1, 2 & 3 Histochemical demonstration of glycogen in the fibres of the red, intermediate and white muscle regions respectively. Note the highest intensity of staining is seen in the red fibres, least in the white and medium in the intermediate. Magnification in all cases X 190.



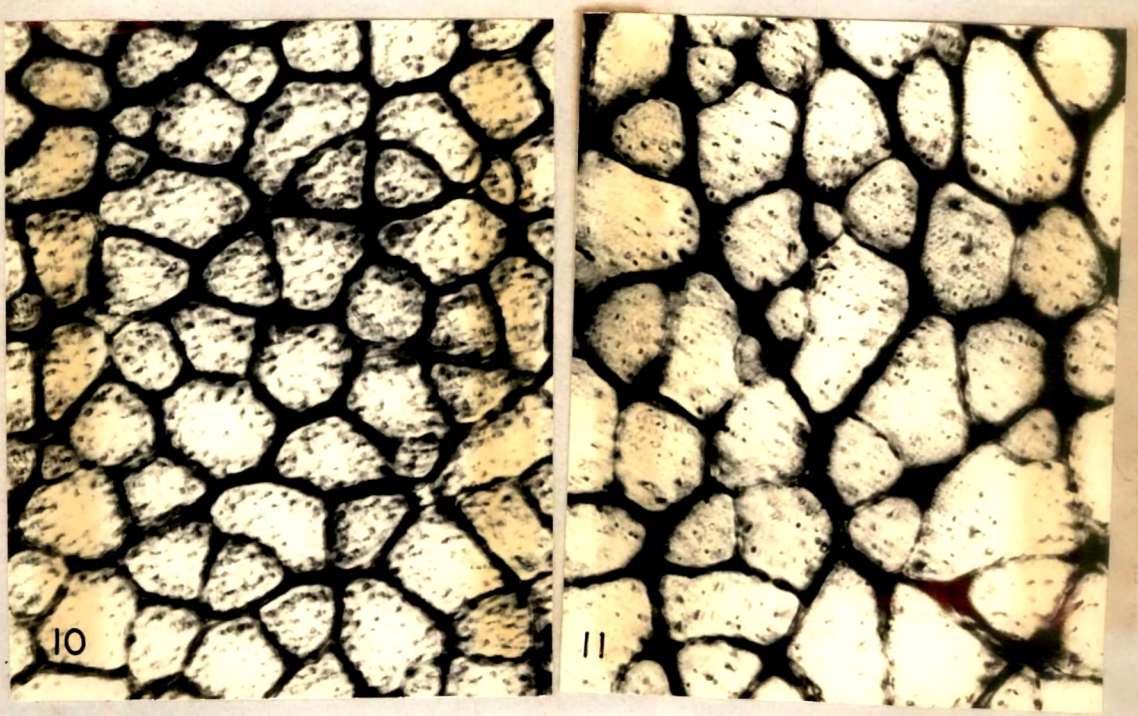
Figs. 4 & 5 Histochemical demonstration of phosphorylase activity in the red and intermediate muscle regions respectively. Highest activity of the enzyme is seen in the red fibres. The intermediate fibres show a mosaic appearance. Fibres of smaller diameter show a much higher activity than the other fibres. X 256.



Figs. 6 & 7 Histochemical demonstration of UDPG synthetase activity in the red and intermediate muscle regions respectively. The red fibres show a higher positive reaction than the intermediate ones. The enzyme activity is seen to be localized mainly in the sarcoplasmic reticulum. X 640.



Figs. 8 & 9 Histochemical demonstration of glucose-6-phosphate dehydrogenase activity in the red and intermediate muscle regions respectively. Marked activity of the enzyme is seen in the mitochondria of the red fibres. X 256.



Figs. 10 & 11 Histochemical demonstration of 6-phosphogluconic dehydrogenase activity in the red and intermediate muscle regions respectively. Enzyme activity is greater in the mitochondria of the red fibres than in those of the intermediate region X256.

The pentose cycle enzymes (G-6-P and 6-PG dehydrogenases) hitherto not detected in fish muscle were demonstrated in the fibres of the red and the intermediate regions (Figs. 8-11). The histochemical demonstration of these enzymes was accomplished by the incorporation of EDTA into the incubation medium in order to bind the contaminating metallic ions which hindered the reaction.

DISCUSSION

The results obtained suggest that glycogen as well as the glycolytic enzymes studied are concentrated in the fibres of the red muscle region. It is known that in these fibres there is a high rate of fatty acid oxidation (Chapter 5). It is possible that with increased fatty acid oxidation, glycogen is spared. It is also possible that the glycerol moiety resulting from the hydrolysis of fat by lipase action is converted to glucose which in turn is converted to glycogen. The occurrence of a high concentration of glycogen in the red fibres may thus be explained. The low concentration of glycogen in the white fibres could be ascribed to the continuous utilization of glycogen for the tetanic contractions of the white muscle. Similarly Rall et al. (1956) obtained high concentrations of glycogen in the heart muscle which is capable of sustained activity.

Phosphorylase is known to occupy a strategic location in the glycolytic sequence of reactions since it is the initial catalytic force in the chain of chemical events that leads to the phosphorylative degradation and utilization of glycogen

(Stetten and Stetten, 1960). Sasako and Takeuchi (1963) have shown that the polysaccharides formed by amylophosphorylase activity stain blue with iodine while those formed by combined amylophosphorylase and branching enzyme activity stain purple or violet. The phosphorylase activity obtained in the red fibres could therefore be attributed to the combined action of amylophosphorylase and the branching enzyme activities. The broad white fibres were found to be devoid of the enzyme activity as well as glycogen, whereas the activity in the narrow white fibres was the result of amylophosphorylase action. These results are in agreement with those of Dubowitz and Pearse (1960) who found that in cold blooded vertebrates, like the toad and goldfish, fibres which showed high oxidative activity, also showed high phosphorylase activity. Ogata and Mori (1963) also demonstrated a strong phosphorylase reaction in the red muscle fibres and weak in the white muscle fibres of fish muscle. On the other hand, in the pigeon pectoralis, phosphorylase is confined to the quick contracting white fibres which are well adapted for a glycolytic metabolism. This has been demonstrated histochemically by Dubowitz and Pearse (1960) and quantitatively by Vallyathan and George (1963). An increase in the phosphorylase activity is known to be associated with increased glycogenolysis (Cahill et al. 1957). A relation between the glycogen levels and phosphorylase activity was also established by the studies of Leonard and Wimsatt (1959), Palasi and Larner (1960) and Lyon and Porter (1963).

The other enzyme of fundamental importance in glycogen metabolism is UDPG glycogen synthetase. Andersson-Cedergren and Muscatello (1963) reported that the UDPG glycogen synthetase activity was localized in the sarcotubular fraction while phosphorylase activity was present in the soluble fraction of the muscle. The physiological importance of UDPG glycogen synthetase in the synthesis of glycogen has been stressed by Schmid et al. (1959), who observed that myopathic muscles were able to synthesize glycogen in the absence of phosphorylase through the UDPG pathway. Millonig and Porter (1961) and Porter and Bruni (1959) suggested that the endoplasmic reticulum is involved in glycogen turnover. The localization of this enzyme in the sarcoplasmic reticulum of the red fibres indicates its participation in the synthesis of glycogen (Andersson-Cedergren and Muscatello, 1963). The level of UDPG glycogen synthetase activity in the red muscle fibres was also found histochemically to be more or less proportional to their glycogen content (Hess and Pearse, 1961; Nene and George, 1965). This is in agreement with the results of Villar-Palasi and Larner (1960) who obtained quantitative data to establish a relationship between the glycogen content of the tissue and the concentration of the enzymes of the glycogen cycle. From the observations made in the present study, it can be concluded that high concentrations of glycogen, phosphorylase and UDPG glycogen synthetase are maintained in the red fibres of the fish muscle. Here is a system of fibres which is capable of synthesizing glycogen and metabolizing glycogen as

well as fat. This system differs from the broad white fibres of the pigeon breast muscle in that the latter are incapable of metabolizing any appreciable amount of fat.

The dehydrogenases of the pentose cycle, which function as an alternate glucose pathway were found to be present in high concentrations in the red fibres. The reason why Ogata (1964) could not demonstrate these two enzymes in fish muscle might possibly be due to the absence of EDTA in his incubation medium. In quantitative studies of fish muscle too, the components of the pentose cycle could not be assessed (Jones and Burt, 1960, 1961). Nevertheless, Hoskin (1959) stressed the major importance of the cycle (the hexosemonophosphate shunt) in certain fish tissues including muscle. Hochachka and Hayes (1962) showed in trout muscle a predominance of the Embden-Meyerhof pathway in warm acclimated (15°C) fish, and an activation of the pentose phosphate cycle in conjunction with a higher rate of fat synthesis during cold acclimation (4°C). It is well known that the reduced NADP (TPN) formed via the pentose cycle is used in the reductive steps of fatty acid and steroid synthesis (Siperstein, 1958). From the above results it is apparent that in fish muscle a significant proportion of carbohydrate break-down passes through the pentose cycle, the end products of which may finally be utilized in fatty acid synthesis.