CHAPTER 3

A HISTOCHEMICAL STUDY OF CERTAIN ENZYMES IN THE THORACIC MUSCLES OF THE BEETLE (HELIOCOPRIS BUCEPHALUS FABR.)

The recent electron microscopic studies of Edwards and his collaborators (1954a, 1954b) brought to light certain significant cytological and biochemical differences between the flight (red) and leg (white) muscles of some insects. These differences have been found to be more pronounced in the higher orders of the insects where the flight and leg muscles differ considerably in their colouration, tracheation and the content of the sarcosomes. Such a correlation between structure and function has been observed in the earlier chapters. Among insects, the Coleoptera consist of diverse forms some of which are fast runners but are unable to fly, some good swimmers and can use their wings for flight to a limited extent and the others which are good fliers. It was therefore thought desirable to study the localization of the enzymes lipase and succinic dehydrogenase, acid and alkaline phosphatases and ATPase in the beetle, *Heliocopris bucephalus* Fabr.

Materials and Methods

Owing to the large size and being easily available in sufficient numbers, this beetle (*Heliocopris bucephalus*) was chosen as the material. The beetles were collected locally while they were flying about. They were decapitated and fixed on a wax board keeping the dorsal side up. The median incision exposed the alimentary canal and the internal musculature. The alimentary system was carefully pulled out and the fat bodies removed from the muscles. From the meso-metathoracic region only the fibrillar flight muscles were used and from the prothoracic region muscles which help in the movement of the first pair of legs were removed separately with the help of a pair of clean watchmaker's forceps. Fresh frozen sections of these muscles were taken and the following methods were employed for the study of the different enzymes and the measurement of the fibre diameter.

Lipase:

Gomori's Tween method using "Tween 80" as substrate was employed to demonstrate the localization of lipase. Fresh frozen sections cut according to the method of George and Scaria (1958a) were mounted on clean dry slides and dried in vacuum over calcium chloride for 15 minutes to ensure adhesion. Sections were fixed in cold (4^{o}C) 6% neutral formalin for 3 to 4 hours period which is found to be sufficient for lipase histochemistry (George and Iype, 1960). The fixed sections were then washed in tap water for 30 minutes and kept in the incubation mixture after rinsing them in distilled water. Boiled sections incubated along with the samples were used as control. The incubation mixture was prepared as described by Pearse (1954) and George and Scaria (1958a). After the incubation period of 8 hours which was found to be the optimum, the sections were treated with lead nitrate and ammonium sulphide respectively, exactly as described by Pearse (1954). The sections were not counterstained.

Succinic dehydrogenase:

Histochemical localization of succinic dehydrogenase was demonstrated using neotetrazolium as the hydrogen acceptor. The incubation medium was prepared as described by George and Talesara (1961a). Sections cut from fresh frozen muscles were placed on clean dry slides and dried quickly in a vacuum for 5 minutes to get the sections adhered on the slides. Sections of the flight muscles and leg muscles were mounted on the same slide and incubated in the proper medium after keeping them for 15 minutes in cold phosphate buffer at pH 7.2. Within 3 to 4 minutes the sections of the flight muscles took up a deep blue colour and light pinkish colour by those of the prothoracic muscles. The sections were then washed thoroughly in phosphate buffer and fixed in 10% neutral formalin for 1 hour and mounted in glycerine jelly after washing away the formalin. Sections similarly treated in the substrate blank medium were taken as control. The slides were examined in the microscope and photographed.

Alkaline phosphatase:

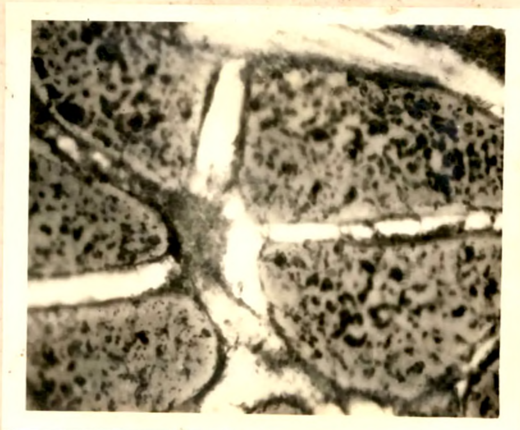
The revised method of Gomori (Peasre, 1954) using sodium glycerophospahte as substrate at pH 9.2 was adopted for the histochemical exposition of alkaline phosphatase in these muscles. Fresh frozen sections were mounted on clean and dry slides without any adhesive and dried in vacuum for 15 minutes. After fixing the sections in 10% cold (4°C) neutral formalin for 1 hour, they were washed in tap water for the same period and after rinsing in distilled water kept in the incubation mixture prepared as described by Pearse (1954) for 24 hours at 37°C. Sections kept in boiling water for 10 minutes for inactivating the enzyme prior to incubation and incubated along with the samples were used as control. Further procedure was exactly as described by Pearse (1954). Sections were not counterstained.

Acid phosphatase:

Acid phosphatase was demonstrated histochemically employing Gomori's revised method (Glick, 1949) using sodium glycerophosphate as substrate at pH 5.1. Fresh frozen sections were cut as in the case of alkaline phosphatase and incubated in the appropriate medium at 37°C. for 6 hours. The incubation mixture prepared as described by Glick (1949) was diluted to 1:2 ratio with distilled water and filtered before incubation. Sections kept in boiling water prior to incubation served as control. Rest of the procedure was as described in Chapter 1.

Adenosine triphosphatase (ATPase):

Pearse and Reis method (Pearse, 1954) using the sodium salt of the adenosine triphosphate as substrate at pH 7.4 was employed to demonstrate the ATPase activity in the muscles of the beetle. Fresh frozen sections prepared as described above were fixed in 10% neutral formalin at 4°C. for 1 hour and washed in tap water for the same period. The sections were then rinsed in distilled water and kept in the incubation medium for 3 hours at 37°C. The following controls were used. Sections kept in the medium for alkaline phosphatase, at pH 9.2 and 7.4 and sections kept in boiling water for 10 minutes prior to



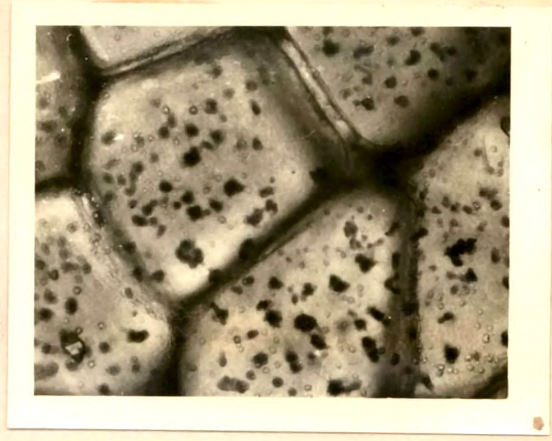


Fig. 1 100 μ

Fig. 2 50 μ

Fig. 1 and 2 - Photomicrographs of meso-metathoracic (fibrillar) and prothoracic muscles respectively treated to demonstrate lipase activity.

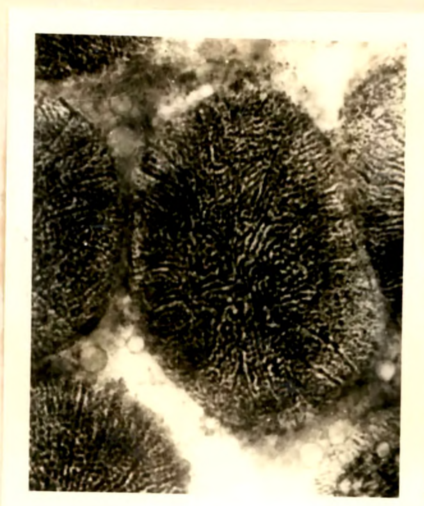


Fig. 3 100μ Fig. 4

Sections of the thoracic muscles of the beetle treated histochemically to demonstrate succinic dehydrogenase activity.
Fig.3- Meso-metathoracic fibrillar flight muscle.
Fig. 4- Prothoracic nonfibrillar muscles.

incubation and sections incubated in substrate blank medium. Sections were not counterstained.

Fibre diameter:

Fresh frozen sections of the above mentioned muscles were cut according to the method of George and Scaria (1958a) and mounted in glycerine jelly, were used for the measurement of fibre diameter. A microscope with occular eye piece and micrometer scale was used for the measurement.

Results

Lipase:

Both fibrillar flight muscles and the non-fibrillar prothoracic muscles gave a positive staining reaction for lipase. The precipitate denoting lipase activity was more in the flight muscles than that in the leg muscles, which can be confirmed from the microphotoghaphs (Figs 1 and 2).

Succinc dehydrogenase:

The dark blue granules of formazan formed were considerably more in number in the fibrillar flight muscles than that in the prothoracic leg muscles. The prothoracic muscle fibres stained pink a few granules scattered here and there in the fibres. Photomicrographs 3 and 4 represent the fibrillar and non-fibrillar thoracic muscles respectively of the beetle showing the localization of SDH activity.

Phosphatases:

All the three phosphatases tested for, namely alkaline and acid phosphatases and adenosine triphosphatase were found to be concentrated more in the fibrillar flight muscles than those in the non-fibrillar leg muscles. In the case of acid phosphatase the nuclei of the muscle fibres in both the muscles stained more, differentiating them from the surrounding sarcoplasm. In general the staining pattern of these enzymes were more or less the same except for the slight difference in the intensity of the staining for the specific enzymes tested.

Fibre diameter:

The fibrillar flight muscles were found to be considerably larger in size than the non-fibrillar muscle fibres. The fibrillar muscle fibres have an average diameter of 220 μ while the non-fibrillar muscle fibres are on an average 150 μ in diameter. Muscle fibres of both the muscles were counted from a number of beetles and the average is given as the mean value.

Discussion

The results obtained from the histochemical study of certain enzymes in the flight and leg muscles of the beetle show that the flight muscles possess all the enzymes tested for in considerably large concentration than the leg muscles. The presence of these enzymes and their concentration have been discussed and related to the higher metabolic activity of the fibrillar flight muscles which has a greater capacity to hydrolyze fat into fatty acids and oxidize fatty acids for energy during prolonged activity. Since the earlier work of

Barron and Tahmisian (1948) on the metabolism of the cockroach muscle, much work has been done on the Krebs cycle intermediates and the related enzymes. Roeder (1951), Sacktor and Bodenstein (1952), Sacktor (1958), Zebe (1956), Zebe and McShan (1957) have shown that most of the oxidizing enzymes occur in the insect muscles in more than five times the concentrations obtained in vertebrate skeletal muscles.

The presence of the phosphatases especially acid phosphatase and adenosine triphosphatase in considerably high concentrations as revealed by the histochemical study is of interest. In the case of vertebrate flight muscles it has been shown that alkaline phosphatase is more in the narrow red fibres which are also rich in lipase and oxidizing enzymes, (George, Nair and Scaria, 1958; George, Susheela and Scaria, 1958b). Acid phosphatase and adenosine triphosphatase were shown to be more in the broad white fibres which are poor in oxidizing enzymes (George and Pishawikar, 1959, 1960). In the lower vertebrates such as fishes and reptiles, the red muscles contain more of these phosphatases than those in the white muscles (Hota, 1954; Rodrigues, 1959; Ogata, 1960). In the case of the slow and fast contracting leg muscles of the American cockroach it has been shown (Chapter, 8) that all the phosphatases are concentrated more in the slow contracting red muscles of the cockroach leg which also contains a higher concentration of lipase and SDH than the fast contracting white muscles of the same part. The distribution pattern of the above mentioned enzymes in the beetle muscles also presents a parallel observation, in that the flight muscles which are fibrillar and reddish in colour possess more of the phosphatases than the pale or white leg muscle of the beetle.

In the earlier chapters it was possible to show a relationship between the lipase activity and the diameter of the muscle fibres of insects of the same family. It was true for the leg muscles of the cockroach where the slow contracting muscle has a higher lipase activity and the fibre diameter was smaller when compared with the diameter of the fast muscle (Smit, 1958) which has a lesser concentration of lipase (Chapter 7). But the fibrillar muscles of insects are an exception to this relationship as can be seen from the results obtained in this chapter as well as in chapters 8 and 10. The diameter of the fibrillar flight muscle is on an average of 220 μ while the leg muscle fibres are 150 μ. The relationship, that could be shown in the case of some insects (Chapter 1 & 2) regarding the enzyme concentration and the fibre diameter of a muscle was possible due to the structural features of the same muscles in the different species.

In insects unlike the vertebrates the structural pattern of the flight muscles differs so conspicuously that there are more than three types of muscles in insects classified on the basis of their histological features. The term fibrillar is frequently used to describe the indirect flight muscles of the members of certain insect groups, notably Coleoptera, Hymenoptera and Diptera; muscles which move the wings not by directly on the wing basis, but by the mechanical deformation of the exoskeleton of the wing bearing segment. The term originated from von Siebold's observation that these muscles could be easily fragmented into their component fibrills unlike the wing and body muscles of other insects. Among the other characteristics of the fibrillar muscle may be mentioned the well developed internalized tracheolar system of the fibre, the randomly scattered nuclei, the large diameter of the fibrils and fibres. The muscles of the leg and the rest of the body have been styled 'tubular' since their nuclei usually occupy only the core of the fibre. In this case the interfibrillar sarcoplasm is greatly reduced, the fibre is typically narrow and the tracheal supply is superficial. A further term' closely packed' has been applied to the flight muscles of Orthoptera and Lepidoptera where the radial arrangement of the fibrils found in the tubular muscle has been lost and where the nuclei are situated peripherally. Pringle (1957) and Boettiger (1960) have given a good account of these muscles with well illustrated examples. It should be mentioned that these terms are approximate rather than absolute and that structural differences in details occur between muscles generally referred to in a single category.

The peculiarities of fibrillar muscles have been correlated with the high metabolic demands made by the flight muscles of the higher insects. But Tiegs (1955) has observed that the aphids and thrips which possess fibrillar flight muscles are feeble fliers while the closely packed muscles of butterflies and locusts help them in their migratory flights. Nevertheless, in the fibrillar flight muscles of the active insects of the higher orders, the sarcosome (Mitochondria) content is very high, almost one half of the total cross sectional area of the fibre. This can be seen from figure 3 which demonstrates the SDH activity. And it is known that SDH is localized mostly in the sarcosomes of the muscles.

From observations on fresh frozen sections, for fibre diameter it also became evident that the tracheolar pattern is extensive at all depths within the fibres. The copious supply of the intracellular tracheoles may be compensating for the unusually large size of the fibrillar muscle fibres. Tiegs (1955) found that the fibrillar flight muscle fibres of certain flies appeared to be of very great size reaching 1800 μ in Rutila potina one of the species in which innervation of the fibres also was examined. It is possible however, that these giant fibres are in reality compound structures enclosed within a common membrane as the topography of the plasma membrane cannot be observed under the light microscope and he suggested that the fibres are broken up into smaller units by the large tracheal trunks and all these small units are innervated separately. If these small units are true fibres then the relation between the enzyme concentration and the size of the muscle fibre may have some relation in the fibrillar flight muscles also since the fibre diameter of the fibrillar muscle fibres ranges from 90 to 4000 μ in different insects (Tiegs 1955). Variation in fibre diameter though a general feature of the muscular tissue in vertebrates, such a wide variation is a unique character of the fibrillar muscles of insects.

The conclusion arrived at from the study of the flight and leg muscles of the different insects by Edwards and his collaborators (1954a, 1954b, 1955) suggest that though there do exist structural and functional variations in the fibrillar and non-fibrillar muscles of the insects, their physiological and biochemical differences could be attributed to their mode of function to a certain extent as far as the active insects are concerned. It was also observed that some insects belonging to the higher orders which possess flight muscles of fibrillar type are very poor fliers. Hence the suggestion by Edwards, Santos, Santos and Sawaya (1954a) that the specialization and the evolution of the fibrillar muscles can be phylogentic in origin in the higher orders of insects. But until there are substantial evidences in support of these views on the physiological and phylogenetic specialization of the firillar muscles, no definite conclusion could be arrivecd at. A detailed study of these muscles on their morphology as well as their physiological and biochemical features may throw some new light on this unique system.