CHAPTER 3

HISTOCHEMISTRY OF MUSCLE LIPASE

The occurrence of lipase in the skeletal and cardiac muscles of vertebrates was reported in the earlier chapters. The present study was undertaken to demonstrate the presence of the enzyme in the muscle by histochemical methods.

A variety of techniques are available for the histochemical demonstration of esterases in tissues . The differences in these methods are largely due to substrates. The choice of substrates is important, because of the specificity of the substrates exhibited by many esterases which enables one to distinguish one esterase from another. Since there is a certain degree of overlapping of substrate specificity between the nonspecific esterases and lipase the choice of a suitable water soluble substrate for lipase presented great difficulties in the past. In such cases advantage was taken of the observation that certain chemicals inhibit one or other of the enzymes while the remaining is either unaffected or activated. A thorough discussion on the merits and demerits of the various methods is available in recent literature on the subject (Pearse,1954; Gomori,1953; Zacks and Welsh, 1953). Gomori(1945) devised a method for lipase in which he used the "Tweens" as substrate. Later he pointed out that the saturated "Tweens" are general substrates for lipase- esterase and recommended for true lipase "Tween 80" which contain unsaturated fatty acid. This "Tween" method for lipase which has undergone several modifications at the hands

of Gomori himself and many others form the basis for the present study. The principle of this method is that the fatty acids liberated as a result of the enzymic activity of lipase is converted to insoluble calcium soap by the calcium ions present in the incubation medium. The calcium soap thus formed is subsequently converted to lead soap and ultimately to lead sulphide by treatment with lead nitrate and ammonium sulphide respectively. On microscopic examination of the sections the lead sulphide is seen as brownish black deposits at the sites of enzymic activity.

Material and Methods

Transverse sections of the pectoralis major muscle of the kite (Milvus migrans), the green parakeet (Psittacula kramari), the brown dove (Streptopelia senegalensis), the bat (Hipposideros speoris) and the breast and heart muscle of the blue rock pigeon (Columba livia) were studied. The pancreas of the pigeon was used for comparison and confirmation of the results.

The incubation medium contained 2 ml. 10% $CaCl_2$, 2 ml. 5% "Tween 80" (Atlas), 5 ml. bicarbonate buffer (Sneath, 1951) of pH 8.4 and 40 ml. distilled water. The mixture was incubated overnight at 40°C to precipitate the free fatty acids, filtered through No. 1 Whatman filter paper to remove the precipitate and a crystal of thymol was added as preservative .

Preparation of the Sections

A critical review of the various methods which have been in use for enzyme studies is presented by Pearse (1954).

Gomori originally used paraffin sections of acetone fixed tissues. Later literature shows the use of frozen sections of formalin fixed tissues by various authors (Mark, 1950; Seligman et al, 1951). In the present studies acetone fixing and subsequent paraffin embedding yielded negative results which was due either to the denaturation of the enzyme by acetone or its inactivation by heat at 58°C which was the melting point of the paraffin wax used. In order to verify this, parallel experiments were run with sections of the pancreas; the results were negative in either case. It was also observed that even with the use of paraffin wax of low melting point the results were found to be negative and was therefore concluded that the inactivation of lipase was largely due to its denaturation by acetone. This is in agreement with Mark (1950) who observed that acetone fixation destroys a large amount of lipase. This view is further substantiated by the observation that the acetone powder of the pigeon breast muscle is almost completely inactive, with respect to lipolytic activity (Chapter 5).

Fixation of tissues in buffered or neutral formalin in cold has been in use for histochemical study of enzymes. Seligman et al found that under the above conditions an appreciable amount of enzyme is left undestroyed. In pancreas at least 75% of lipase was retained . Following the recommendation by Pearse (1954) " As a starting point for further research on the "Tween" methods a standard 16-hour fixation in 6% neutral formalin at 4° should be used", I used 6% cold

neutral formalin as fixative. But instead of cutting frozen sections of tissues after formalin fixation what I did was to cut fresh frozen sections of tissues and to fix them in formalin after mounting on slides. This procedure is more advantageous in that the time taken to remove the formalin after fixation is considerably reduced.

In histochemistry the preparation of the sections is of utmost importance for obtaining good results. I could do no better than quote Pearse (1954), " The effect of fixation on enzymes continues to be of increasing importance as histo - chemical techniques for demonstrating them increase in numbers. Theoretically the best way of showing enzyme activity by histo - chemical means would appear to be by the use of fresh frozen sections of tissue blocks. There are formidable objections to both in practice, however, especially to the former . Even with the cold knife method it is technically impossible to obtain good fresh frozen section of the majority of tissues, and even if these are obtained or if thin tissue blocks are substituted there remains a further objection, Particularly if incubation with the substrate is prolonged I have observed that loss of enzyme and of other protein and non-protein materials into the incubating medium is much greater than in fixed material. This leads not only to false localization of enzyme (due to diffusion in the section) but also to widespread deposition on the section of the products of enzymic activity in the medium and to general filthiness of the sections due to partial disso - lution of its components." My experience with the freezing microtome was that it is very difficult to mount good thin

sections on the slide without transferring them from the knife into water. This procedure is not desirable because much of the soluble enzymes are likely to be lost in water during this process. But a very simple and quick method which does not need a cold room or elaborate instruments, as described below, was found to overcome this difficulty and yield highly satisfactory results. Tissues from animals are cut out immediately after killing and placed over ice in the deep freeze of a refrigerator. The tissues freeze hard in less than half an hour. Without removing them from the deep freeze they are trimmed and thin sections cut with the aid if a cold sharp razor blade. If the blade is completely dry, the sections do not stick to it. The sections thus cut are transferred to a slide over albumin, where due to the melting of the ice the sections get spread out. One can be certain that by this method no enzyme will be lost. Perhaps the only serious draw - back in this method is that it will be difficult to determine exactly the thickness of the sections and to get sections of uniform thickness throughout. But this method could be employed admirably for qualitative histochemical work on enzymes. The sections thus prepared may be fixed in cold formalin. However this precaution does not guarantee exact localization of the enzyme since the possibility of diffusion of the products of enzymic activity still remains. It was observed that in the case of the muscle lipase, satisfactory results could be obtained only with sections 40 - 80μ thick. Opaqueness of the sections due to ammonium sulphide can be removed by keeping

the sections for a few seconds in a Coplin jar of 70% alcohol mixed with a few drops of H_2O_2 . Incidentally it may be mentioned that the above method of cutting sections was conveniently used for manometric studies of tissue metabolism and to develop a new technique for the analysis of tissue components by paper chromatography (George, Pishawikar and Scaria, 1958).

Procedure

Sections of tissues from animals which were bled to death were cut as described above and mounted on albuminised slides allowed to dry at room temperature till adhesion was complete. They were then fixed for 16 hours in cold formalin (4°C) neutralised with phosphate, washed in running water filtered through cotton for 1 hour, rinsed with distilled water and incubated for 8-12 hours. The rest of the procedure was essentially the same as described by Pearse (1954). The sections were not counterstained.

Control

The enzyme was incubated by keeping the slides in boiling water for about 10 minutes.

Results and Discussion

Except in the case of controls all the preparations showed brownish black precipitates indicating lipase activity. Naturally the greatest activity is manifested in the sections of pancreas (Fig 1) by a thick deposition of the precipitate.

Fig. 1 - Photomicrograph of a section of the pancreas of pigeon showing disposition of lipase

The heart muscle (Fig 2) comes next in the order of activity. This is in conformity with the results obtained in quantitative determinations which showed that the heart muscle contain a higher concentration of the lipase than the breast muscle.

Fig.2 - Photomicrograph of a section of the heart of pigeon showing disposition of lipase

The distribution of lipase in the pectoralis major muscle of the four birds is interesting. Fig. 3 shows the cross-section of a part of the breast muscle of pigeon in which the two types of fibres, a narrow and a broad variety as described by George and Naik (1958a and '58b) are clearly discernible. The dark

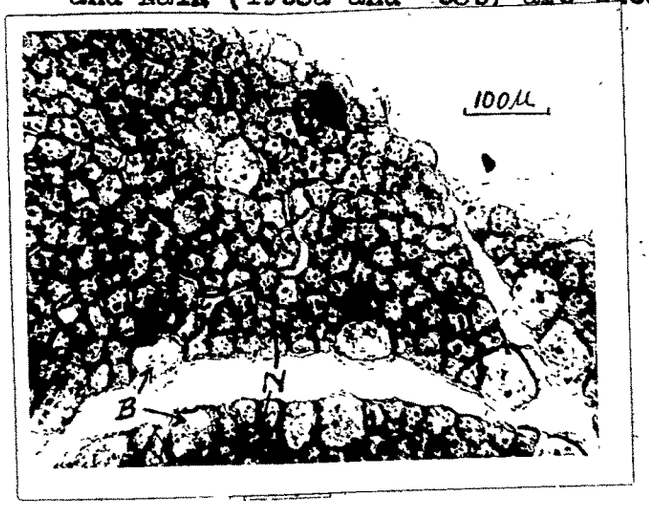

Fig.3

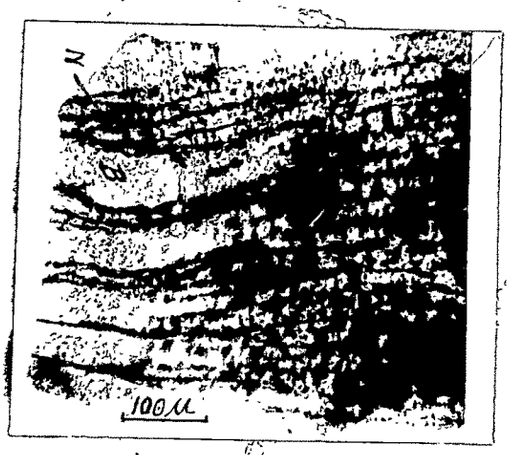

Fig.4

Photomicrographs of (Fig.3) a transverse and (Fig.4) an oblique section of the pectoralis major muscle of the pigeon showing the disposition of lipase. B, broad fibres; N, Narrow fibres

spots in the photograph represent the brownish black precipitate of lead sulphide. It can be seen that the precipitate is abundant in the fat loaded narrow fibres, where as the glycogen loaded broad fibres contain very little. Control sections do not show any precipitate. It is therefore inferred that in the pigeon breast muscle the lipase occurs mostly in the narrow fibres. However, the occurrence of a few grains of the precipitate in the broad fibres is not convincing evidence that they are formed in the same fibres; study of a large number of sections indicates that these granules in the broad fibres are the result of diffusion of products of hydrolysis from the narrow fibres. Fig.4, showing an oblique section of the muscle giving the longitudinal aspect of the two types of fibres, shows large areas of broad fibres devoid of these precipitates. This supports the view that lipase is confined, by and large, to the narrow fibres, and very little, if any, occurs in the broad fibres. In both kite and parakeet the pectoralis major consists of fibres which are more or less uniform with regard to their fat content (Naik, Ph.D. thesis) and it is seen that the lipase also enjoys a uniform distribution throughout the fasciculus (Figs. 5 and 6). On the other hand in the dove the pectoralis major consists of two distinct types of fibres, a broad white variety with clear cytoplasm and a narrow red variety with lot of lipoid inclusions, a condition very similar to that in the pigeon. In the case of the dove too as, can be seen from the photograph (Fig.7) only the narrow fibres contain the precipitate in abundance.

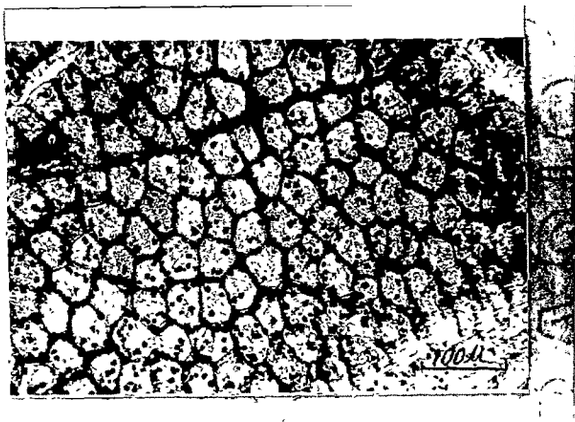

Fig.5

Fig.6

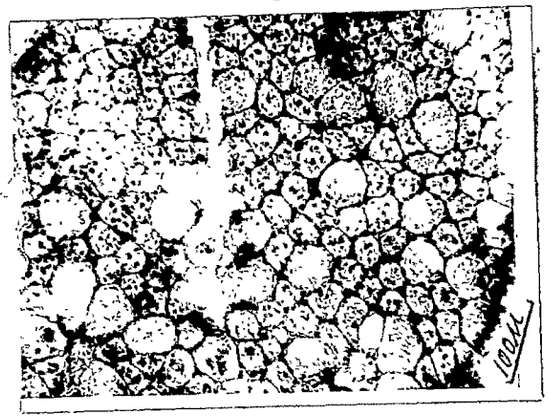

Fig.7

Fig.8

Photomicrographs showing the disposition of lipase in the pectoralis major muscle of (Fig.5) kite (Fig.6) parakeet (Fig.7) dove and (Fig.8) bat.

The few grains of the precipitate found scattered here and there in the broad fibres, without doubt, are due to the diffusion of the products of hydrolysis from the narrow fibres, as could be seen from an examination of a number of sections. Fig. 8 presents the photomicrograph of a transverse section of the pectoralis major muscle of the bat in which two types of fibres, a broad and a narrow variety, are distinguishable. Lipase is confined to the narrow fibres just as

in the case of the pigeon breast muscle. In pigeon it has been shown that the two types of fibres are distinctly different in their biochemical nature, the narrow fat loaded ones having a large number of mitochondria in them and the broad glycogen loaded ones having few or none. Since in the bat the lipase is confined only to the narrow fibres it appears possible that here also a difference in the fuel content with the accompanying adaptive features such as the content of myoglobin and mitochondria too exists in the broad and narrow fibres just as in the case of the pigeon. This proposition made by me earlier was proved to be correct by later work (George and Naik, 1957b)

Another important fact that has emerged from the present study is regarding the nature of the fat splitting enzyme in the muscle. Mention was made in the beginning about the confu - sion in nomenclature of the fat splitting enzymes. Since the substrate used in the present study is one specific for true lipase and the results obtained are comparable to that of pancreas, it confirms the view that the muscle enzyme is a true lipase and not an esterase.