CHAPTER 4

A QUANTITATIVE STUDY OF THE DISTRIBUTION PATTERN OF CERTAIN OXIDIZING ENZYMES AND A LIPASE IN THE RED AND WHITE FIBRES OF THE PIGEON BREAST MUSCLE

That certain structural and biochemical differences exist in the red and white muscles of vertebrates in general, are known from the works of a number of investigators including Needham (1926), Denny-Brown (1929), Paul and Sperling (1952), Lawrie (1952, 1953a and 1953b), Edward et al (1956), Nachmias and Padykula (1958), Tonzetich and Kare (1960), Ogata (1960) and many others. These differences have been studied by employing histochemical or biochemical methods mostly on the mixed types of muscles or the red and white muscle portions taken separately either from different muscles of the same animal or from different animals but not on the fibres isolated from a mixed muscle. It has hitherto been a common practice to carry out all biochemical determinations on the vertebrate skeletal muscles without taking into consideration the constituent fibre types and the ratio of their distribution in a particular part of the muscle from where the material for study has been taken. It was also observed that in every such piece of muscle its cellular components not only differ in their diameter, metabolite store, and various other constituents but also differ markedly in the ratio of their distribution pattern in the different regions of the same muscle (George and Naik, 1957a and 1959a; George,

Susheela and Scaria, 1958a).

Some histochemical and biochemical studies have been made by George and his school with a view to have a comprehensive picture of the biochemical and biophysical potentialities of the red and white fibres of the pigeon breast muscle in particular and other mixed types of vertebrate skeletal muscles in general. It has been shown that the distribution pattern of SDH (Chapter 1) and other constituents such as iron and protein (Chapter 3) vary in the different layers of the pigeon breast muscle. It was suggested that the results cannot be obtained constant in a muscle of this type or any other muscle unless the ratio of the distribution pattern of the different types of fibres is taken into consideration.

Recently Ogata (1960) showed the differences in some labile constituents and some enzymatic activities between the red muscle taken from the central part of M. soleus and the white muscle taken from the posterior edge of M. adductor magnus. The results obtained by the author do throw some light on the general nature and behaviour of the two types of muscles taken separately , but in a mixed muscle like the pigeon pectoralis it is worthwhile investigating quantitatively by some suitable methods the various constituents in the individual fibre types.

In a histochemical study of the various DPN-linked dehydrogenases (Chapter 5) and SDH and cytochrome oxidase (Chapter 2) employing more sensitive and modified methods,

it has been possible to demonstrate the localization of these enzymes in the broad white fibres of the pigeon breast muscle previous attempts to accomplish this (George and Scaria 1958c) having failed. By this it became evident that these fibres too possess mitochondria and that they are to be considered fundamentally analogous to the white fibres of the other vertebrate skeletal muscles contrary to what was suggested by George and Scaria (1958c). To put these biochemical observations to further test the present investigation on the activities of the various enzymes such as cytochrome oxidase, succinoxidase, malic oxidase, lactic dehydrogenase and lipase has been carried out in the different layers of the pigeon breast muscle employing the indirect method of assessment without isolating the fibres (Chapters 1 and 3) and based on the distribution pattern of these fibre types as worked out by George and Naik (1959a).

Material and Methods

In order to ensure that only uniformly well developed pectoralis major muscle was used, well fed and fully grown, normal laboratory pigeons (Columba livia) of either sex weighing between 300 to 350 gm. were used.

Details of the theoritical basis of the method employed have already been given (Chapters 1 and 3).

In each experiment a bird was decapitated and the blood completely drained off. A muscle strip of nearly 2 square cm. cut along the direction of the fibres was removed

from the middle of the pectoralis major muscle with its entire thickness. Similarly another piece with exactly the same topography was quickly cut from the muscle of the other side. One of this was used for the assay while the other for recording the distribution of the broad and narrow fibres in the different layers. Slices of desired thickness were cut immediately on a freezing microtome and the fibre count of the narrow as well as the broad was taken according to the procedure cited earlier (Chapter 1). A 1 to 2 % homogenate out of each muscle slice representing a particular depth was immediately prepared in ice-cold glass redistilled water. The same ateps in the procedure were followed for all the enzymes studied. In the case of lipase special care was taken to remove the traces of blood completely.

Oxidizing enzymes

Oxidizing enzymes such as cytochrome oxidase, succinoxidase, malic oxidase and lactic dehydrogenase were assayed at 37° C. with the conventional Warburg manometric techniques through oxygen uptake. In each case the Warburg flask contained 3 ml. of the total reaction mixture in the main compartment and 0.2 ml. 6 N KOH and a roll of filter paper in the centre well. The gas phase used was air and after temperature equilibration in the bath for 10 minutes, the levels were adjusted and the oxygen uptake was recorded at 10 minutes intervals for 30 minutes.

The enzyme activity is expressed in microlitres of oxygen consumed per mg. of dry muscle per hour. The dry weight of the homogenate was determined on 1.0 ml. duplicate samples dried to constant weight at approximately 100° C. Glass redistilled water was used for preparing all the reagents used. 0.2 to 0.4 ml. of approximately 1 % homogenate was used in each case for the enzyme assay. Endogenous oxygen uptake for this small amount of tissue used was found to be almost negligible. All the chemicals used in the assay were of routine C. P. grade (Merck) except DPN and cytochrome c which were of the Sigma Chemical Co.

Cytochrome oxidase

The activity of this enzyme was measured by the manometric method of Schneider and Potter (1943). The principle on which the assay depends is that a non-enzymatic reduction of cytochrome c by ascorbic acid is used to serve as the substrate for cytochrome oxidase. The correction for the oxygen uptake resulting from spontaneous auto-oxidation of ascorbate was made as suggested by the authors.

Succinoxidase system

Succinic dehydrogenase, the Slator factor and the cytochrome oxidase system constitute the succinoxidase system. The manometric technique described by Schneider and Potter (1943) was used for determining the enzyme activity.

Malic oxidase system

Malic dehydrogenase and the associated hydrogen carriers,

DPN, cytochrome reductase and the cytochrome oxidase system constitute the 'malic oxidase' system. The overall activity of this complex of enzymes was determined according to the manometric technique of Potter (1946). Glutamate was added to remove oxalacetate by means of the transmination reaction (Straub, 1941) and nicotinamide was added to inhibit DPN destruction (Mann and Quastel, 1941).

Lactic dehydrogenase

This enzyme activity was determined manometrically through oxygen uptake by the method adopted from the reports of Straub (1940), Vestling and Augusta (1950) and Hunter and Hunter (1957). The reaction flask contained;

0.1 M PO_4 buffer, pH 7.6.....0.8 ml.

3.5×10^{-4} M cytochrome c.....0.5 ml.

0.5 M DL-lactate.....0.8 ml.

1 % DPN.....0.3 ml.

Muscle homogenate.....0.4 ml.

Glutamate was also included in some experiments for removing pyruvic acid but no significant increase in the oxygen uptake was observed.

Lipase activity

Lipase activity of the homogenate 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. The reaction flask contained 1.5 ml. 0.025 M bicarbonate solution and 1.0 ml. homogenate in the main chamber and 0.5 ml. 4 % (v/v)

tributyrin in 0.0148M bicarbonate solution (emulsified by shaking with a drop of 'Tween' 80) in the side arm. The flasks and the manometers were gassed with a mixture of 5 % carbon dioxide and 95 % nitrogen for 3 minutes. The substarte was tipped in after equilibration for 10 minutes and after equilibration of another 3 minutes the levels were adjusted and the readings taken at regular intervals of 15 minutes for 1 hour. The lipase activity is expressed as µl CO_2/mg. dry muscle/hour.

Results

The activity of the various enzymes studied and the number of broad and narrow fibres per square mm. in the different layers of the pigeon breast muscle is shown in Table 1. The results so obtained clearly indicate that with

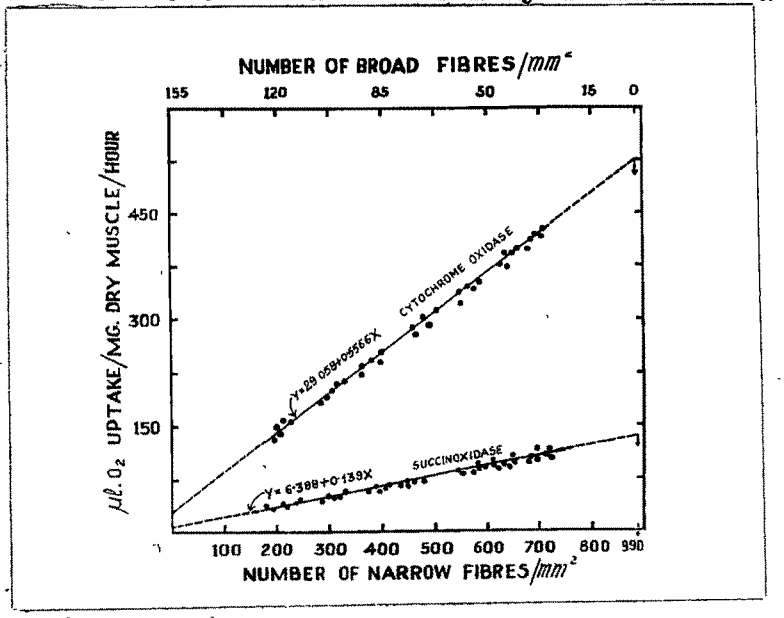

Fig. 1. Graph showing the relation between the number of narrow and broad fibres per square mm. and cytochrome oxidase and succinoxidase activity as obtained at different depths of the pectoralis major muscle of the pigeon.

the variation in the ratio of the distribution of the broad and narrow fibres, the concentrations of the various enzymes studied also vary accordingly. The activity of these enzymes was found to be minimum, in the 0.5 mm.-thickness of the most superficial layers where the broad fibres are maximum, whereas it was maximum in the layer taken from the interior of the muscle. Thus a definite pattern in the distribution of the two types of fibres and their associated constituents is observed in this muscle.

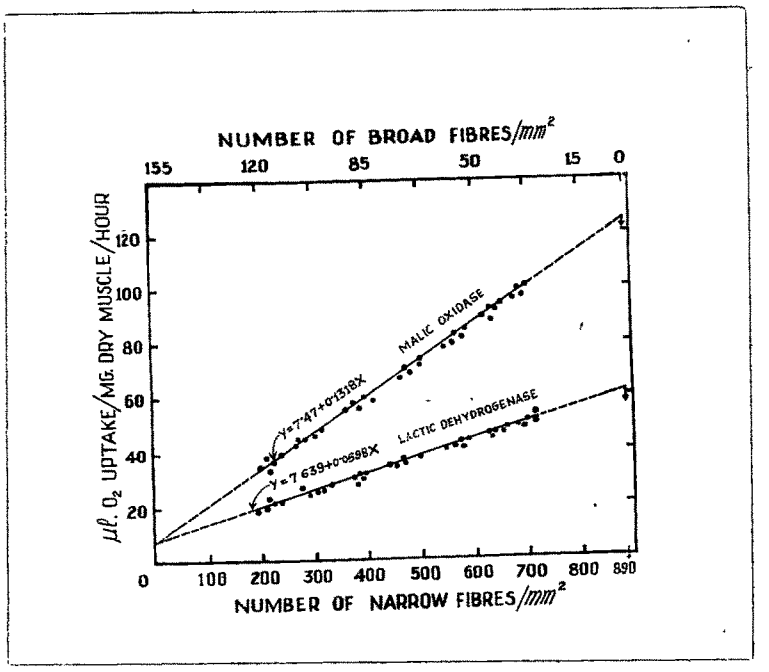

Fig. 2. Graph showing the relation between the number of narrow and broad fibres per square mm. and malic oxidase and lactic dehydrogenase activity as obtained at different depths of the pectoralis major muscle of the pigeon.

Figs. 1 - 3 are a graphic representation of the ratio of narrow and broad fibres against the activity of the various enzymes as obtained from different layers of the muscle. As

already described in detail (Chapters 1 and 3) the use of the regression line obtained in each case, was made for deriving the enzyme activity in the individual red and white fibres, by extending this line on either sides as indicated by the broken line (Figs. 1 - 3). The activities of the various enzymes so derived in the individual red and white fibres are shown in Table 2. It should, however, be noted that the values derived by this indirect application of the method may not be absolute since the freezing and thawing of the muscle piece while cutting, though for a very short period, might destroy some amount of the enzyme. Nevertheless, the results obtained do show the relative enzyme concentrations in the two types of fibres of this muscle.

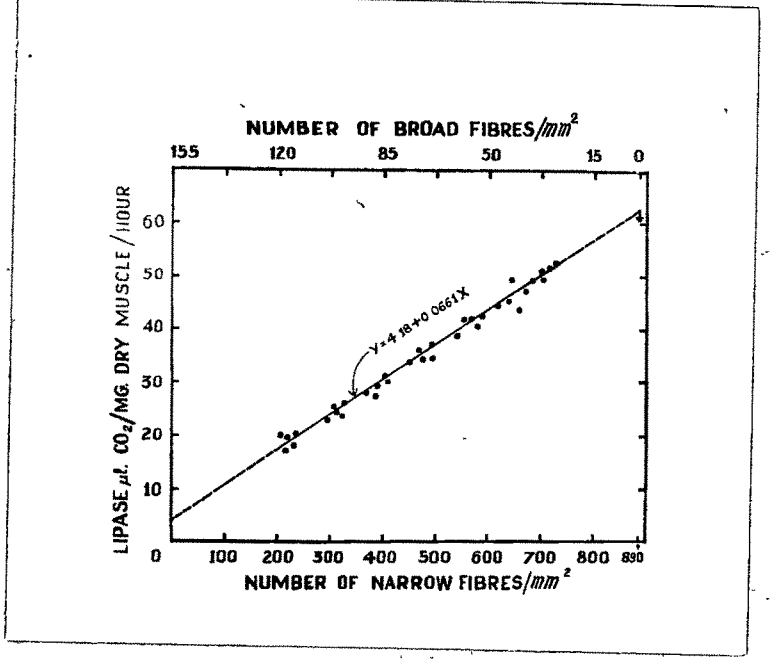

Fig. 3. Graph showing the relation between the number of narrow and broad fibres per square mm. and lipase activity as obtained at different depths of the pectoralis major muscle of the pigeon.

From the results obtained (Figs. 1-3 and Tables 1-2) it could be easily noticed that the major bulk of all the enzymes studied is confined to the narrow red fibres of this muscle though the broad white fibres too possess all these enzymes, but in extremely low concentrations as was shown by histochemical methods (Chapters 1 and 5).

Discussion

While studying the SDH, cytochrome oxidase (Chapter 2) and various DPN-linked dehydrogenases (Chapter 5) histochemically by using improved methods, the demonstration of detectable concentrations of the various oxidizing enzymes in the broad white fibres was achieved which was not accomplished earlier. It was therefore suggested that the broad white fibres too possess mitochondria and that these fibres are to be considered fundamentally analogous to the white fibres of the other vertebrate skeletal muscles. The present biochemical determinations of the various oxidizing enzymes and a lipase in the broad white fibres (Table 2) lend further support to the contention that these fibres are not devoid of the above mentioned enzymes.

A higher concentration of the oxidizing enzymes and a lipase in the narrow red fibres, suggest the presence of a well organized 'cyclophorase' system including specially the enzymes of the Krebs cycle and fat utilizing system whereas the broad white fibres are poor in these contents. George and Naik (1958a and 1958b) have shown that the broad white fibres are glycogen-loaded and poor in fat inclusions

and mitochondria, whereas the narrow red fibres are fat-loaded and have a high mitochondrial content and are poor in glycogen. Based on these observations it could be said with a fair amount of certainty that the oxidation of fatty acids in the pigeon breast muscle takes place mostly if not entirely in the narrow red fibres. With a high fat content and its associated enzymes it is more or less certain that fat forms the chief fuel for energy in preference to glycogen during long and sustained contractions of the narrow red fibres of the pigeon breast muscle and the same appears to be the case in the flight muscles of birds indulging in such prolonged muscular activity as in migratory flights. The activities of the various enzymes and the amounts of the constituents assessed might appear to be very low in the broad white fibres as compared to the narrow red fibres, but these values for the broad white fibres are quite comparable to those obtained for the white fibres of other vertebrate skeletal muscles.

In a mixed muscle like the pigeon pectoralis and other vertebrate skeletal muscles in general, the distribution in a definite fashion of the fibre types with their associated constituents within, cannot be considered as without any significance. The manner of distribution of the various fibre types in different muscles should reflect the extent of their mechanical and physiological efficiency. In most of the biochemical studies hitherto made this heterogenity of the muscle has not been taken into consideration at all while making comparative studies which obviously would not reveal

the true story. In a study of the SDH activity in the pectoralis major muscle of a few representative types of birds and a bat (Chapter 7) it was not possible to explain satisfactorily certain points having a bearing on the extent of enzyme activity and the fibre diameter, body weight and muscle weight. This was however, overcome when the ratio of the distribution of the fibre types of a particular muscle under camparison was taken into consideration (Chapter 7).

In the pigeon breast muscle as has been observed, the broad white fibres are concentrated more towards the periphery in a single fasciculus and this number was found to be increased to the maximum in the fasciculi situated in the superficial and the deepest layers. Thus the number of the broad fibres is the least in the fasciculi situated in the interior of the muscle as shown in Table 1. This is sufficient evidence to demontrate by experimental data that variations in the different metabolites, enzymes and other cellular constituents do exist and are considerably higher in the different regions of this muscle. It should be noted, however, that this variation is not random but follows a definite distribution pattern (Table 1) and not a unique case in the pectoralis major of the pigeon but may occur in other muscles too as was seen in the pectoralis major muscle of the bat (George and Naik, 1957b).

The results obtained for the different layers of the pigeon breast muscle indicate that the metabolically most active centres lie somewhere in the middle of the dorso-ventral axis where the number of the narrow fibres is the highest and

that of the broad fibres the least. Hence the general belief that the muscle fibres towards the periphery of the muscle are more active than those in the interior and, due to higher activity increase in diameter cannot be accepted as a universal rule and at least in the case of the pectoralis major muscle of the pigeon this does not hold good. As far as the broad fibres are concerned this seems to be true only in the sense that these fibres, being concentrated towards the periphery of the muscle, and having more of structural protein are mechanically efficient in exerting a vigorous pull.

Thus the variation in the fibre types accompanied by variations in their metabolite load in a definite pattern may well be associated with the mechanical and the physiological efficiency of the different fibres and ultimately of the muscle as a whole.