CHAPTER 5

HISTOCHEMICAL DEMONSTRATION OF CERTAIN DPN-LINKED DEHYDROGENASES AND ALDOLASE IN THE RED AND WHITE FIBRES OF THE PIGEON BREAST MUSCLE

The presence of SDH and cytochrome oxidase in the broad white fibres, using improved histochemical methods has already been shown (Chapter 2). By employing an indirect method, certain oxidizing enzymes and a lipase were assessed quantitatively in the red and white fibres of this muscle and it was found that the broad white fibres too contain these enzymes though in an extremely low concentration (Chapter 4). The present chapter deals with a study undertaken to demonstrate the localization and distribution pattern of certain DPN-linked dehydrogenases such as malic, lactic, D-glucose, a-glycerophosphate and glutamic dehydrogenase by improved histochemical methods in the red and white fibres of the pigeon breast muscle. In addition a histochemical study of aldolase which plays an important role in glycolysis was also undertaken with a view to have a comprehensive picture of the localization and distribution pattern of this enzyme, in the two types of fibres.

Materials and Methods

The chemicals used as specific substrates for the various dehydrogenases were of the C. P. grade (Merck). Fructose 1:6 diphosphate and the cofactor DPN used were of L. Light & Co. and Nutritional Biochemicals Corporation respectively.

DPN- linked dehydrogenases

The literature on the various methods based on the use of tetrazolium salts for the histochemical demonstration of the different dehydrogenases has been recently reviewed by Pearse (1960) and also as mentioned in Chapter 2. Though nitro-blue tetrazolium with a high redox potential has been used in the most recent methods, in the present study, however, NT was used due to the nonavailability of the former.

For the histochemical demonstration of the various DPN-linked dehydrogenases the method as described by Pearse (1960) using NT as the hydrogen acceptor, was followed. NaCN was added into the incubation medium (Rosa and Velardo, 1954; and Chapter 2) as an effective blocking agent for cytochrome oxidase and also as a trapping agent for any oxaloacetic acid that may be produced during the processing of the sections. Nicotinamide was also added in the incubation medium to prevent DPN destruction. The incubation medium used for the various dehydrogenases (in a total volume of 3 ml.) was as follows:

0.1 M PO_4 buffer, pH 7.0.....1.0 ml.
0.5 M (Specific substrate, neutral salt).0.6 ml.
Neotetrazolium..... 2 mg.
DPN 6 mg./ml.....0.3 ml.
Nicotinamide (0.1 M).....0.3 ml.
$MgCl_2$ (0.05 M).....0.3 ml.
NaCN (0.03 M adjusted to pH 7).....0.5 ml.

For each set of experiments a bird was decapitated and bled. A piece of muscle 1 cm. in length, consisting of the complete depth of the pectoralis major muscle, was immediately cut out and fresh frozen sections between 20 to 40 μ thick were cut on a freezing microtome, and floated in ice cold phosphate buffer for nearly 10 minutes so as to allow sufficient time to remove the endogenous substrates, prior to testimg for the various dehydrogenases. The sections were then transferred to the cuvettes containing the incubation mixture with specific substrates as described above and incubated at 37° C. for 10 - 30 minutes as required. In order to verify the specificity of the various DPN-linked dehydrogenases, a control was run side by side omitting the specific substrate from the incubation medium. After incubation the sections were washed thoroughly in phosphate buffer, fixed in 10 % buffered neutral formalin at 4° C. for 2 hours and after washing mounted in glycerogel. The sections were immediately examined under a microscope and photographed.

Aldolase

The enzyme catalyzes the splitting of Fructose 1:6 diphosphate into 3-phosphoglyceraldehyde and a-phosphodihydroxy acetone. The only available method being that of Allen and Bourne (1943) was used for the histochemical demonstration of aldolase in the two types of fibres of the pigeon breast muscle, using Fructose 1:6 diphosphate as the substrate. Frozen sections, of the muscle strip (fixed in 80 % alcohol for 24 hours) were incubated for 2 hours at 37° C. Further enzymic breakdown of

the two triosephosphates reaction products was prevented by the presence of iodoacetate in the incubation medium and the reaction products were precipitated by a mixture of magnesium and ammonium chlorides in alkaline solution. The precipitated phosphates were revealed by their conversion to the cobalt salt and finally to the brownish black sulphide. One without the substrate was used as control.

Results and Discussion

The size, localization and distribution pattern of the various histochemically demonstrable DPN-linked dehydrogenases except for a-glycerophosphate, in the red as well as white fibres of the pigeon breast muscle, showed a more or less close resemblance to that of SDH and cytochrome oxidase already dealt with in detail (Chapter 2). The sarcoplasm of the fibres though reduced in the case of the broad white fibres, was stained slightly pink and contained sharp bluish violet diformazan granules, which were arranged in a definite linear fashion between the unstained myofibrils and thus showed to some extent, a resemblance to the localization pattern of the mitochondrial staining. It is nevertheless, interesting to note that even in the case of lactic dehydrogenase in the fresh frozen sections used in the present study as well as in the fixed sections obtained according to Novikoff and Masek (1958), the localization was intramitochondrial.

The pH of the incubation medium was adjusted to 7 so as to avoid the possibility of getting any staining reaction for 'Nothing Dehydrogenase' as the SH groups or glutathione

may reduce exogenous DPN at alkaline pH levels (Zimmermann and Pearse, 1959). Tsao (1960) recently reported the electrophoretic heterogenity of some of the dehydrogenases but by histochemical reactions no differences of this sort can be studied.

All the dehydrogenases, studied showed an uniform distribution pattern of the localization of enzyme activity in the individual fibre types throughout the depth of the muscle, since this muscle does not contain any intermediate types of fibres. Moreover the diameter of the two types of fibres is more or less constant in the different regions and shows no significant variation from the average values (George and Naik, 1959a).

All the DPN-linked dehydrogenases studied, except for a-glycerophosphate, show a higher concentration of these enzymes in the narrow red fibres whereas the presence of a few such diformazan granules in the broad white fibres suggests the possibility of a few mitochondria and hence a low level of oxidative metabolism there. These histochemical results agree with the available biochemical data (Chapter 4) on the individual red as well as white fibres of this muscle. However, it is rather surprising that lactic dehydrogenase which is a glycolytic enzyme shows a high level of activity in the narrow red fibres.

Of the various dehydrogenases studied histochemically, the reduction of NT was found to be rapid in sections treated for malic dehydrogenase, almost as much as that of SDH in the two types of fibres. Similar results were also obtained in

the biochemical study (Chapter 4) on this muscle. It should be mentioned here that George and Scaria (1958c) were unable to demonstrate the presence of D-glucose dehydrogenase histochemically in the breast muscle of the pigeon. a-glycerophosphate was found to be present in appreciable amount in the broad white fibres. It is rather difficult to say whether the enzyme is more in the broad white fibres or in the narrow red fibres because of the solubility of the enzyme which is responsible for its improper and diffused localization. Recently it has been shown that a-glycerophosphate is metabolized by two types of dehydrogenases, one a soluble DPN-linked enzyme (Sacktor and Cochran, 1957) and the other a structurally bound dehydrogenase (Chance and Sacktor, 1958; Sacktor and Cochran, 1958) not requiring a nucleotide but reacting via the cytochrome system to oxygen. As far as the second type is concerned it may not be present in the broad white fibres since these fibres are poor in cytochromes. It should be noted here that a-glycerophosphate plays an important role in the energy problems of the insect flight muscles (Sacktor and Cochran, 1958). It is the first type that is the soluble DPN-linked enzyme which is present in major quantity in both the types of fibres. The sections, however, when incubated with the substrate without any cofactor for nearly 30 minutes, revealed no visible colour in either of the fibres.

These histochemical results on DPN-linked dehydrogenases and the earlier observations (Chapters 1, 2, 3 and 4) suggesting a high oxidative metabolism in the narrow red fibres

and a low oxidative metabolism in the broad white fibres points to the fact that the broad white fibres of the pigeon breast muscle too possess mitochondria which may differ from those of the narrow fibres in shape, size or form. The broad white fibres therefore are to be considered fundamentally analogous to the white fibres of the other vertebrate skeletal muscles.

The histochemical demonstration of aldolase show a higher activity of this enzyme in the broad white fibres (Fig. 1). It is to be emphasized here that in a histochemical preparation whenever a particular enzyme activity is found in appreciable amount in both the types of fibres it becomes very difficult to assess their relative concentrations in the fibre types.

Fig. 1. Microphotograph of a transverse section of the pectoralis major muscle of the pigeon showing the distribution pattern of aldolase in the narrow and broad fibres.

The two fibre types differ markedly in the density of the granular inclusions and fat droplets which are present in the

narrow type and obliterate to some extent the true nature of the staining. This is further enhanced by the diameter of these fibres in being very small. With these differences it is clear that mere casual comparisons would not be accurate. In this particular case specially the colour produced after the final reaction, is a dull brownish one which does not give a sharp contrast. In the case of aldolase, this being a soluble glycolytic enzyme only a broad disposition was revealed and the exact intracellular localization was not found possible to obtain.

Aldolase is reported to be present in the extra protein fraction from myofibrils of the striated muscle (Perry and Zydowo, 1959) but as mentioned earlier, no definite differentiation in the localization of the enzyme in the myofibrils, sarcoplasm or mitochondria was noticed. The suggestion of some workers (Du Buy and Showacre, 1959; Showacre and Du Buy, 1959) that mitochondria in the intact cell contain all the glycolytic enzymes, does not seem to be convincing as far as the broad white fibres of the pigeon breast muscle and also the other vertebrate skeletal muscles rich in glycolytic enzymes are concerned, since the mitochondrial content of these fibres is always low as compared to the red fibres.

It has been shown that during long and sustained muscular activity, the pigeon breast muscle utilizes fat as the chief fuel for energy (George and Jyoti, 1957). Hence looking into the nature of the fuel store in the two types of fibres (George and Naik, 1958a) and their phosphorylase

activity (Dubowitz and Pearse, 1960) it could be explained that the higher aldolase activity in the quick contracting broad white fibres is an indication of the breakdown and synthesis of glycogen, in which these fibres are rich. On the other hand, the low aldolase activity, in the slow contracting narrow red fibres enriched with fat, mitochondria and the associated enzymes of the fatty acid oxidase system as well as of the Krebs cycle, is suggestive of the fact that fat forms the chief fuel for energy in preference to glycogen during sustained activity of these fibres.