Histochemical Observations on the Succinic Dehydrogenase and Cytochrome Oxidase Activity in Pigeon Breast-muscle

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With two plates (figs. 1 and 2)

SUMMARY

Histochemical and cytochemical observations were made on the exact localization and distribution pattern of succinic dehydrogenase system and cytochrome oxidase in the pigeon breast-muscle by employing slightly modified methods. Succinic dehydrogenase activity, which was not detected earlier either histochemically or biochemically in the broad white fibres, was demonstrated by using a modified incubation medium under strictly anaerobic conditions, with neotetrazolium as the hydrogen acceptor. The size, localization, and distribution pattern of the histochemically demonstrable diformazan and indophenol blue granules showed a more or less close resemblance to the mitochondrial staining in the individual red as well as white fibres. The occurrence of high oxidative metabolism in the narrow red fibres was revealed by the presence of a large number of succinoxidase-positive granules in these fibres. On the other hand, the presence of fewer, smaller granules indicated very low oxidative metabolism in the broad white fibres.

The presence of the fewer, smaller succinoxidase-positive granules in the broad white fibres nevertheless shows that these fibres too possess mitochondria where at least a certain amount of oxidative activity does take place, and that they are to be considered as analogous to the white fibres of the other vertebrate skeletal muscles. It is also suggested that these granules are to be considered as mitochondria in the general sense and that the distinction between sarcosomes and mitochondria as proposed by previous authors needs reconsideration.

INTRODUCTION

It has been shown (George & Jyoti, 1955; George & Naik, 1958a, 1958b, 1959) that the pectoralis major muscle of the pigeon consists of two distinct types of fibres, a broad, glycogen-loaded white variety with few mitochondria, and a narrow, fat-loaded, red variety having a large number of mitochondria in them. These two types of fibres existing side by side in one and the same system attracted our special attention. They have become the subject of more extensive studies with a view to understand their mutual relationship and mode of action.

In recent years the pigeon breast-muscle has been extensively used by biochemists and physiologists for studies in cell metabolism. What is known from these studies applies to the muscle as a whole and not to its separate components. Quantitative biochemical investigations on the two types of

fibres is by no means easy, since the complete isolation of these fibres without damage appears extremely difficult if not impossible. Studies on the relative distribution and localization of metabolites and enzymes in these fibres by histochemical and cytochemical methods were therefore undertaken in our laboratories. George and Scaria (1956) demonstrated the presence of a lipase in this muscle. George and Iype (1960) using improved histochemical method showed that lipase is localized in the red, fat-loaded fibres. George, Nair, and Scaria (1958) showed that alkaline phosphatase is mainly confined to the same fibres, while George and Pishawikar (1959) have shown a higher concentration of ATPase in the white, glycogen-loaded fibres.

George and Scaria (1958) in a histochemical study of dehydrogenases (succinic, malic, lactic, and glycerophosphate dehydrogenases) could not detect any of these in the broad white fibres. They suggested that these fibres in the pigeon breast-muscle are a unique system in which none of the oxidative processes concerned with the above enzymes takes place, and that these fibres therefore cannot be considered as analogous to the white fibres of the other vertebrate skeletal muscles studied. The method employed by these authors was that of Straus and others (1948), who used the colourless salt, triphenyl tetrazolium chloride, as the hydrogen acceptor. George and Talesara (1960), employing the method of Kun and Abood (1949), who used TTC under aerobic conditions, studied quantitatively the distribution pattern of succinic dehydrogenase in the different layers of the pigeon breast-muscle and concluded that the main bulk of the enzyme is restricted to the narrow red fibres. These observations on the succinic dehydrogenase system in the pigeon breast-muscle, made by histochemical and biochemical methods, were recorded under aerobic conditions. Within the limitations of the methods employed no significant enzyme activity was detected in the broad white fibres.

It is well known that in a living system the transport of hydrogen from succinate in the presence of the succinic dehydrogenase system brings about the reduction of cytochrome C. This reduced cytochrome C is in turn oxidized by the molecular oxygen in the presence of cytochrome oxidase, which is the terminal enzyme in the entire respiratory chain. It was therefore thought necessary to examine the distribution of cytochrome oxidase in the two types of fibres. The classical ‘Nadi’ reaction was employed for the histochemical demonstration of the localization of the enzyme. The muscle sections thus treated revealed the presence of a detectable concentration of cytochrome oxidase in the broad white fibres. The results thus obtained naturally questioned the validity of the earlier observations made regarding the absence of the succinic dehydrogenase activity in the broad white fibres, and also regarding the specificity of the methods employed, both histochemical and biochemical.

This stimulated us to seek a more specific and sensitive method for the detection of the succinoxidase system, so that it would be possible to detect histochemically even small concentrations of the enzymes. We were able
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to improve the usual methods. Under strictly anaerobic conditions it was possible to demonstrate succinic dehydrogenase activity in the broad white fibres, which could not be done with the ordinary methods. In this paper we report the distribution and the localization of succinic dehydrogenase and of cytochrome oxidase by improved and modified histo-chemical methods.

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 both sexes weighing between 280 to 340 g were used throughout.

Histochemical localization of the succinic dehydrogenase system

The activity of the succinic dehydrogenase system in the narrow red fibres could be demonstrated easily by the ordinary histochemical methods. But the broad white fibres in which the concentration of this enzyme is extremely low requires a more sensitive and improved method of treatment. The specificity and the sensitiveness of the method in turn would depend upon a number of factors. Several authors (Straus & others, 1948; Black & Kleiner, 1949; and Black & others, 1950) used triphenyl tetrazolium chloride as the hydrogen acceptor for the demonstration of succinic dehydrogenase in animal tissues. Later, Seligman and Rutenburg (1951), Padykula (1952), Rutenburg, Wolman, and Seligman (1953), and Whitehead and Weidman (1959), in their modified methods for the histochemical localization of succinic dehydrogenase in various tissue sections, reported that under anaerobic conditions the tetrazolium reduction was more rapid and intense, and some tissue sections which were not stained under aerobic conditions were stained under anaerobic conditions. The same observation was made by Kun and Abood (1949), Brodie and Gots (1951), Cooper (1955), and Padykula (1958) in the biochemical determination of succinic dehydrogenase. Of the various tetrazolium salts used, TTC, blue tetrazolium (BT), and neotetrazolium (NT), the latter was found to be most suitable for demonstrating succinic dehydrogenase activity histochemically. It has also been preferred by Shelton and Schneider (1952), Padykula (1952, 1958), Pearse (1954), Farber, Sternberg, and Dunlap (1956), and Casparano and Zweifach (1955). Rosa and Velardo (1954), in a modified technique, suggested the incorporation of sodium cyanide into the incubation medium 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.

The whole literature on the histochemical methods employed for the demonstration of DPN diaphorase, TPN diophorase, and the succinic dehydrogenase system has been reviewed critically in a series of papers (Sternberg, Farber, & Dunlap, 1956; Farber, Sternberg, & Dunlap, 1956a; Farber, Sternberg, & Dunlap, 1956b; Farber & Louviere, 1956; Farber &
George and Talesara—Succinic Dehydrogenase and Bueding, 1956). In a study of the histochemical localization of specific oxidative enzymes, Farber and Louviere (1956) showed that the addition of any one of a number of soluble redox dyes to the incubation medium serves as a carrier between the enzyme and the tetrazolium salt, thereby increasing the rate of staining and making it possible to demonstrate the presence of enzymes which do not react directly with tetrazolium. Nachlas and others (1957) devised a simple method by the use of new tetrazole (nitro-blue tetrazolium) for the cytochemical demonstration of succinic dehydrogenase system. Pearson (1958) used nitro-neotetrazolium chloride. We have, however, restricted ourselves to the use of neotetrazolium because of the very satisfactory results obtained with it. The TTC method of Straus and others (1948) was also tried for comparing the results obtained by our modified method which successfully demonstrated the presence of the succinic dehydrogenase system in the broad, white fibres of the pigeon breast-muscle. Our modifications were mainly in the composition and use of the incubation medium. We based our methods on those reported by Seligman and Rutenberg (1951), Padykula (1952), Rutenberg, Wolman, and Seligman (1953), Rosa and Velardo (1954), Farber and Louviere (1956), and Farber, Sternberg, and Dunlap (1956b). The composition of the incubation medium used (in a total volume of 6 ml) was as follows:

- PO₄ buffer (0.1 M, pH 7.6) 2.5 ml
- Na succinate (0.5 M neutralized to pH 7.6) 0.6 ml
- CaCl₂ (0.004 M) 0.5 ml
- AlCl₃ (0.004 M) 0.5 ml
- NaHCO₃ (0.6 M, freshly prepared) 0.3 ml
- NaCN (0.03 M, brought to pH 7.6) 0.5 ml
- Neotetrazolium (3 mg/ml, freshly prepared) 1.0 ml
- MgSO₄ (0.005 M) 0.05 ml
- Methylene blue (2 mg/ml) 0.05 ml

The incubation mixture was boiled in a small vial to remove dissolved oxygen. The vial was stoppered immediately. After cooling, the mixture was stored in a bath maintained at 37°C till the sections were ready for incubation.

For each experiment a bird was decapitated and bled. A piece of muscle 1 cm in length, consisting of the complete depth of the muscle, was immediately cut out and mounted on the stage of a carbon dioxide freezing microtome. The muscle-block was frozen hard as quickly as possible so as to avoid the formation of ice crystals and distortion of the tissue. Sections between 20 to 40 μ thick were cut and floated in ice-cold phosphate buffer for nearly 10 min in order to remove the endogenous substrates from dehydrogenase activity. The sections were then transferred quickly to the vial containing the incubation mixture. The method followed for creating the anaerobic conditions required for the experiment was that of Padykula (1952). The vial with Padykula's arrangement was placed in a bath maintained at 37°C. A gentle bubbling of nitrogen was continued during incubation. The incubation was
carried out for 15 to 60 min as desired. After incubation the sections were washed thoroughly in phosphate buffer, fixed in 10% buffered neutral formalin at 4°C for 4 to 6 h, and after washing mounted in glycerogel. The preparations were immediately examined under a microscope and photographed.

In order to verify the specificity of the succinic dehydrogenase activity, two sets of control experiments were conducted. In one the sections from cold phosphate buffer were incubated anaerobically in the absence of succinate. In the other the sections were treated with malonate. Supplementary experiments were performed on frozen sections with TTC or NT as the hydrogen acceptor, under aerobic or anaerobic conditions, without the intervention of activators or the soluble redox dye.

To rule out the possibility of any false localization of succinic dehydrogenase activity resulting from diffusion, which is liable to occur in a mixed muscle like the pigeon pectoralis where the two types of fibres exist side by side, a simple technique was followed. In each experiment a small strip of muscle from a freshly killed pigeon was cut and transferred to a 0.9% cold NaCl solution. Immediately after this the two types of fibres from the mixed muscle were isolated by teasing them out by a pair of fine watchmaker’s forceps under a stereoscopic dissection microscope and treated separately for the demonstration of succinic dehydrogenase activity by the method mentioned above.

**Histochemical localization of cytochrome oxidase**

The classical Nadi reaction, employing a mixture of a-naphthol and dimethyl-p-phenylene diamine hydrochloride, demonstrates the presence of cytochrome oxidase in cells (Moog, 1943; Borei & Bjorklund, 1953). A critical review of the merits and demerits and the specificity of the Nadi reaction for the histochemical demonstration of cytochrome oxidase has been given by Gomori (1953), Pearse (1954), Crawford and Nachlas (1958), Nachlas, Crawford, Goldstein, and Seligman (1958), and Burstone (1959). We followed a method adopted from the reports of Moog (1943) and Nachlas, Crawford, Goldstein, and Seligman (1958) for demonstrating histochemically cytochrome oxidase in fresh frozen sections of the pigeon-breast muscle. The Nadi mixture used was composed of the following reagents:

\[
\begin{align*}
\text{PO}_4 \text{ buffer (0.1 M, pH 7.4)} & \quad 3 \text{ ml} \\
a-\text{naphthol (1 mg/ml in 1% NaCl)} & \quad 5 \text{ ml} \\
\text{Dimethyl-p-phenylene diamine hydrochloride} & \quad 5 \text{ ml} \\
(1 \text{ mg/ml in 1% NaCl)} & \\
\text{Cytochrome C (3 mg/ml)} & \quad 2 \text{ ml}
\end{align*}
\]

All the reagents were freshly prepared just before use, mixed together, and filtered. Cytochrome C was omitted from the medium in the routine experiments unless required specifically. Addition to the incubation medium of a catalase and an inhibitor for dehydrogenase was not found necessary.

As mentioned in the case of the succinic dehydrogenase system, fresh
frozen sections of the muscle were placed quickly in 0.9% cold NaCl solution and then incubated in the freshly prepared Nadi mixture at room temperature for 5 to 15 min as required. Control sections were treated with sodium azide and sodium cyanide as recommended by Moog (1943). After incubation, sections were washed thoroughly in 0.9% NaCl and mounted in saturated potassium acetate. Photomicrographs were taken immediately. It was observed, however, that the sections mounted in potassium acetate and maintained at 4°C, lasted for a number of days without any visible change in colour or pattern of granules.

As mentioned in the case of succinic dehydrogenase, the two types of fibres were isolated and treated separately for cytochrome oxidase activity. In order to compare the histochemically demonstrable granular pattern of the succinic dehydrogenase system and cytochrome oxidase with mitochondrial staining, sections of the pigeon breast-muscle were stained by Janus green B and also by Altmann's aniline fuchsin method as described by Gray (1954).

Observations

Distribution of succinic dehydrogenase system

When frozen sections were incubated aerobically without the addition of activators or soluble redox dye, with TTC as hydrogen acceptor, a regular distribution pattern of the succinic dehydrogenase system was observed in the narrow red fibres. These red fibres were stained within 10 min while the broad white fibres did not show any detectable activity of succinic dehydrogenase even after incubation for 1 h (fig. 1, D). But when the sections were treated with the modified incubation medium under strictly anaerobic conditions, with NT as indicator, the localization of the enzyme in both types of fibres was observed at the cytochemical level (fig. 1, A, B). Succinic dehydrogenase activity was revealed in the red narrow fibres within 5 to 10 min as fine bluish-violet diformazan granules distributed in a most regular pattern in both transverse and longitudinal sections. After an incubation period of 15 to 30 min the presence of succinic dehydrogenase was revealed in the white fibres by lighter bluish-violet diformazan granules, which were, of course, smaller and far fewer than those of the narrow red fibres (fig. 2, A, B). A clear picture of the exact distribution of the granules was obtained in longitudinal sections, where red and white fibres were side by side.
FIG. I

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FIG. 2

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The sarcoplasm of the muscle-fibres was stained slightly pink and also contained sharp bluish-violet diformazan granules, which were arranged in a definite linear fashion between the unstained myofibrils (fig. 1, A–C). The localization of the succinic dehydrogenase system, which could not be demonstrated in the broad white fibres by ordinary methods, thus became visible (table 1).

In addition to the diformazan granules in the sections, a diffused red colour was visible in most of the narrow red fibres. This probably represented the partially reduced form of the dye (monoformazan), or the dye dissolved in the fat which is quite abundant in the narrow red fibres. The inter-fascicular fat was stained uniformly deep red and no granules were visible. Thus the sharp diformazan granules with the distinct bluish-violet colour showed the exact sites of the localization of the succinic dehydrogenase system in the two types of fibres (fig. 2, A).

**Table 1**

Histochemically demonstrable succinic dehydrogenase in the broad white fibres of the pigeon breast-muscle

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Incubation mixture in a final volume of 6 ml; pH 7.6</th>
<th>Degree of staining in 1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubation mixture</td>
<td>aerobic</td>
</tr>
<tr>
<td>1</td>
<td>Medium A, TTC + succinate + PO₄ buffer</td>
<td>O</td>
</tr>
<tr>
<td>2</td>
<td>Medium B, NT + succinate + PO₄ buffer</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Medium C, NT + succinate + PO₄ buffer, activators (Ca++, Al³⁺⁺⁺, Mg++, and HCO₃⁻⁻⁻⁻⁻) + NaCN</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>Medium D, Reagents of medium C + methylene blue</td>
<td>++</td>
</tr>
</tbody>
</table>

O = no detectable staining; ± = trace of detectable staining; +, ++, ++++, ++++ = relative degrees of staining under different conditions.

Fresh frozen sections, at different levels in the whole depth (ventral face to dorsal face) of the pigeon breast-muscle when treated for the succinic

**Fig. 2** (plate). A and B, transverse and longitudinal sections respectively of pigeon breast-muscle, showing the distribution and localization pattern of succinic dehydrogenase in the two types of fibres. A modified incubation medium was used under anaerobic conditions. The narrow red fibres show a markedly greater deposition of diformazan granules than the broad white fibres. Note the smaller diformazan granules in the broad white fibre. Incubation period 30 min.

C and D, transverse and longitudinal sections respectively of pigeon breast-muscle, showing the localization and distribution pattern of cytochrome oxidase. Note the similarity in the size, distribution, and localization pattern of indophenol blue granules with that of the diformazans (A and B). Incubation period 10 min.
dehydrogenase system, showed that in each individual narrow and broad fibre, the degree of staining for the enzyme was more or less the same in the corresponding fibres throughout the depth of the muscle. In the narrow red fibres relatively more granules were observed towards the periphery. TTC was not found suitable for the low enzyme activity in the broad white fibres because of the diffused pale red colour it formed during enzymic reduction, which did not give a sharp contrast under the microscope. On the other hand, neotetrazolium, because of the sharp blue-violet granules it formed, was found more suitable for such studies.

**Distribution of cytochrome oxidase**

The distribution pattern of cytochrome oxidase by the Nadi reaction showed a close resemblance in all respects to that of succinic dehydrogenase. Cytochrome oxidase activity was indicated by the formation of indophenol blue granules resulting from the oxidative coupling of \( \alpha \)-naphthol and di-methyl-\( p \)-phenylene diamine hydrochloride in the presence of cytochrome C, in both the narrow and the broad fibres of the pigeon breast-muscle (fig. 2, c, d). The diffusibility and the solubility in fat of the final dye, indophenol blue, was observed in the form of a uniform violet colour. This violet staining could easily be distinguished from the actual sites of the localization of cytochrome oxidase in the form of dark blue indophenol granules. The interfascicular fat was also stained deep violet. During a short incubation period without added cytochrome C no detectable colour was observed in the broad white fibres. However, blue granules were seen in these fibres when cytochrome C was introduced into the incubation medium.

The two types of fibres isolated under a dissecting microscope and separately treated histochemically for succinic dehydrogenase and cytochrome oxidase, showed the same distribution pattern of the granules as was observed in the sections.

The longitudinal and transverse sections of the pigeon breast-muscle stained for mitochondria, succinic dehydrogenase, and cytochrome oxidase all showed close similarity as regards the actual sites of staining. On careful observation it was found that the size and distribution pattern of the diformazan as well as indophenol blue granules more or less coincided with those of the mitochondria.

**DISCUSSION**

The above observations have shown that the two types of muscle-fibres in the pigeon pectoralis, when treated for succinic dehydrogenase and cytochrome oxidase, stain with different intensity. A distinct pattern of granules representing the succinoxidase system in the sarcoplasm between myofibrils more or less coincides with the pattern of the mitochondria. It can therefore be concluded that the actual sites of the localization of the succinoxidase system is in or near the mitochondria, and the histochemically demonstrable diformazan and indophenol blue granules do represent the pattern of mito-
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It is well known that succinic dehydrogenase may be demonstrated histochemically in or near the mitochondria (Goddard & Seligman, 1952, 1953; Nachlas & others, 1957; Scarpelli & Pearse, 1958). The same applies in the case of cytochrome oxidase, as the activity of the succinic dehydrogenase system is linked with the cytochrome system. The considerably less succinoxidase activity observed in the broad white fibres is obviously due to their low mitochondrial content. This contention is supported by both biochemical (Hogeboom, Schneider, & Palade, 1948; Harman, 1950; Paul & Sperling, 1952; Harman & Osborne, 1953; Chappell & Perry, 1953) and electron microscopic studies (Barnett & Palade, 1957; Sedar & Rosa, 1958), which have shown that succinic dehydrogenase is exclusively present in the mitochondria. The granules thus made visible in the broad white fibres of the pigeon breast-muscle are to be regarded as mitochondria. These have not been reported by previous workers.

The narrow red fibres possess a large volume of sarcoplasm and numerous granules of large size (fig. 1, B, C), while the broad white fibres have considerably less sarcoplasm and possess much fewer, smaller granules (fig. 1, A). Further details regarding the actual size and exact position of these smaller succinoxidase-positive granules in the broad white fibres could only be made available by using the electron microscope. The myofibrils of both the red narrow and the broad white fibres do not show any detectable positive reaction for the succinoxidase system. The absence of this system in myofibrils has been shown biochemically by Harman and Osborne (1953) in a pure suspension of myofibrils.

In fresh, unfixed, frozen sections all granules representing sites of succinoxidase activity are spherical and of about the same size in fibres of the same type (larger in the red fibres than in the white). Various authors (Harman & Osborne (1953); Kitiyakara & Harman (1953); Weinreb & Harman (1955); Harman (1955)) have distinguished two types of cytochondria, the spherical forms as sarcosomes and the large elongated ones as mitochondria. Both are said to be linearly arranged along the myofibrils in the red fibres of the pigeon breast-muscle. In our fresh frozen preparations, however, we were able to detect only spherical granules in both types of fibres: the elongated ones (Harman's mitochondria) were not found (fig. 1, A, B). A similar picture to ours (fig. 1, B) was obtained by Harman also (fig. 1, 1955). His claim of the existence of the rodlet mitochondria does not appear convincing. Evidence from electron microscopy in support of his opinion (his figs. 2 and 3) does not appear to be convincing. We are inclined to believe that the spherical granules in both the types of fibres are to be regarded as mitochondria in the general sense.

When frozen sections were incubated in Nadi mixture with added cytochrome C, an increase in cytochrome oxidase activity was observed. This suggests that the broad white fibres possess a very low concentration of cytochrome C, whereas the narrow red fibres seem to be very rich in it. In a
similar study Katchman and Shooter (1955) and Maruyama and Moriwaki (1957) have shown, in pigeon breast-muscle and in the thoracic muscle of the honey bee respectively, the dependence of succinoxidase activity on cytochrome C concentration. It seems probable that the low succinoxidase activity in the broad white fibres of the pigeon breast-muscle is associated with the higher glycogen deposition. Thus the characteristic glycogen load may be a result of poor oxygen supply, leading to anaerobic metabolism.

These observations throw some more light on the earlier observations made regarding the occurrence of oxidative enzymes in the two types of fibres. The presence in the white fibres of a few smaller granules, responding positively to tests for succinic dehydrogenase and cytochrome oxidase, indicates the existence of some sort of cytochondria in them with a very low oxidative metabolism, even less than that of the white fibres of the other vertebrate skeletal muscles. It should, however, be emphasized that the white fibres of pigeon breast-muscle are not fundamentally different from the white fibres of the other vertebrate skeletal muscles. On the contrary, it can be concluded from these studies that in such a mixed type of muscle as the pigeon pectoralis, the broad white fibres, chiefly loaded with glycogen and with very low concentrations of oxidizing enzymes, function mostly anaerobically and in all probability are meant for quick and sudden contraction for a short duration, whereas the narrow red fibres loaded with fat and with very high concentrations of the Krebs cycle enzymes, are used for sustained contraction.

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