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A histochemical study of the red and white muscle fibers Part III. Activity of the diphosphopyridine nucleotide diaphorase and triphosphopyridine nucleotide diaphorase in muscle fibers*

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Abstract

From the histochemical study of DPN and TPN diaphorase on the striated muscles of the cats, the following results were obtained. 1. *M. gastrocnemius*, which belongs to the white muscle by naked eye, consists of three types of muscle fibers distinguished by the DPN diaphorase staining: namely, the small muscle fibers, i. e., the red muscle fibers show a moderate activity, being stained pink, while the large muscle fibers, i. e., the white muscle fibers show a low activity, being stained faint pink. The. third type of muscle fibers: namely, the medium fibers are stained pale pink and show the enzymatic activity intermediate between the red and white muscle fibers. 2. *M. soleus*, belonging to the red muscle by naked eye, consists of three types of fibers distinguished by the DPN-diaphorase staining, i. e., the red muscle fibers are stained pink, medium fibers pale pink, and a few white muscle fibers faint pink. The diameters of these three types of muscle fibers in *M. soleus* are almost the same. 3. From the staining pattern of TPN-diaphorase in *M. gastrocnemius* and *M. soleus*, the three types of muscle fibers can be distinguished by TPN-diaphorase activity, namely, the red muscle fibers show a high TPN-diaphorase activity, being stained purple, while, the white muscle fibers a low activity, being stained pale pink. The medium fibers are stained pink and show a moderate enzymatic activity intermediate between the red and white muscle fibers. 4. The TPN-diaphorase activity is higher than the DPN-diaphorase activity in the striated muscle, but it is less active than the TPN-diaphorase activity in the kidney. However, the activity of DPN-diaphorase in the striated muscle is quite lower than that of the kidney.

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**A HISTOCHEMICAL STUDY OF THE RED AND
WHITE MUSCLE FIBERS**

**PART III ACTIVITY OF THE DIPHOSPHOPYRIDINE
NUCLEOTIDE DIAPHORASE AND TRIPHOSPHO-
PYRIDINE NUCLEOTIDE DIAPHORASE
IN MUSCLE FIBERS**

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In earlier papers of this series^{6,7}, it has been demonstrated that the red muscle fibers show a high activity in both succinic dehydrogenase and cytochrome oxidase, the medium fibers a moderate activity and the white muscle fibers a low activity. In this paper the author intends to show that the histochemical stainings for diphosphopyridine nucleotide (DPN) diaphorase and triphosphopyridine nucleotide (TPN) diaphorase, which can be demonstrated histochemically by the methods of FARBER et al. (1956),^{1,2,3,4,9} differ in each fibers, in that reaction is strongest in red muscle fibers, weakest in white muscle fibers and moderate in medium fibers.

MATERIALS AND METHODS

For the histochemical demonstration of DPN-diaphorase and TPN-diaphorase, the methods established by FARBER et al¹. were applied in this study. As the materials, the striated muscles and the kidneys of healthy adult cats of both sexes were used. The striated muscles, *M. gastrocnemius* & *M. soleus*, were removed immediately after killing the animal by a blow on the head. Without fixation, the fresh tissues were frozen and sectioned about 30 μ thick. Sections were floated on the buffer solutions, phosphate buffer 0.1 mol., pH 7.4 for the demonstration of DPN-diaphorase and veronal buffer 0.1 mol., pH 7.4 for TPN-diaphorase, cooled at 0° C. Then, within 3 minutes, they were transferred into the cold incubating medium contained in 50 ml. glass-stoppered Erlenmeyer flasks. Different incubating media were employed, each made up to contain substrates for the particular enzyme system under investigation together with appropriate

coenzymes and cofactors. The flasks were flushed briefly with N₂ and then incubated without shaking in a water bath at 37.5° for three hours. After incubation, the sections were washed successively in the following solutions: (1) physiological saline solutions containing 0.6% acetic acid (two washings), (2) 10% formalin, and (3) distilled water, and were then mounted on slides with glycerin.

The composition of the various incubating media was as follows:

A. For demonstraing DPN-diaphorase:

1. Using malate as substrate:

Na L-malt (0.5 M).....	0.3 ml.
DPN (0.45%)	0.2 ml.
Na L-glutamate (0.5 M)	0.5 ml.
NaCN (0.03 M in PO ₄ buffer)	0.5 ml.
Blue tetrazolium (1 mg./ml.)	0.7 ml.
Phosphate buffer (0.1 M, pH 7.4).....	0.7 ml.
H ₂ O to	3.0 ml.

2. Using lactate as substrate:

The medium was the same as A(1) with K L-(+) lactae (0.5 M) substituted for malate.

As the control, the incubating media without DPN solution but with, distilled water in its place were used.

B. For demonstrating TPN-diaphorase:

1. Using isocitrate or citrate as substrate:

Na D-isocitrate (0.5 M)	
or	
Na citrate (0.5 M)	0.3 ml.
TPN (0.2%)	0.1 ml.
MnCl ₂ (0.005 M)	0.3 ml.
L-Cysteine (0.1 M)	0.2 ml.
Veronal buffer (0.05 M, pH 7.4)	1.0 ml.
Blue tetrazolium (1 mg./ml.).....	0.7 ml.
H ₂ O to	3.0 ml.

2. Using malate as substrate (for malic decarboxylase):

Na L-malate (0.5 M) was used in place of isocitrate or citrate. As the control, the incubating media without TPN solution but with distilled water in its place were used.

OBSERVATIONS AND RESULTS

DPN-diaphorase: — Generally, the DPN-diaphorase activity in the striated muscle is rather weak, as compared with the activity in the kidney of the same animal. When malate or lactate is used as substrate, no difference is found in the localization of DPN-diaphorase in the sections, though the reaction appears stronger in malate than in lactate. The sections, incubated in the medium containing no DPN, show no reaction, remaining unstained.

M. gastrocnemius, which belongs to the white muscle by naked eye, is composed of three types of muscle fibers which can be distinguished from each other by the DPN-diaphorase staining as in the findings of the succinic dehydrogenase staining and cytochrome oxidase staining reported in the previous papers^{6,7}. As shown in Fig. 1. the large fibers (W), namely, white muscle fibers show the low activity of the DPN-diaphorase, being stained faint pink, while the small fibers (R), namely, red muscle fibers show a moderate activity, being stained pink. The medium fibers (M) are stained pale pink and show the DPN diaphorase activity intermediate between those of the red and white muscle fibers.

M. soleus, which belongs to the red muscle by naked eye, is composed of the fibers showing a moderate activity of DPN-diaphorase. Close observation proves that there are a few muscle fibers showing a low enzymatic activity though they are almost the same with those showing a moderate activity in size. And these fibers seem to be white muscle fibers. Among the muscle fibers showing a moderate activity, two types of fibers can be distinguished by the DPN-diaphorase activity, namely, comparatively strong and weak ones. The former may be constituted of red fibers and the latter of medium fibers. And the diameter of these three types of muscle fibers in *M. soleus* is almost the same, though those in *M. gastrocnemius* are quite different.

TPN-diaphorase: — Regardless of using the media containing citrate, isocitrate or malate, almost the same staining patterns are observed. In the absence of TPN, no staining occurred. The activity of TPN-diaphorase in the striated muscle is fairly stronger than that of DPN-diaphorase, but it is observed that the activity of TPN-diaphorase in the striated muscle is weaker than that of the kidney.

M. gastrocnemius is composed of three types of muscle fibers which can be distinguished from each other by the TPN-diaphorase staining as shown in Fig. 3. The white muscle fibers (W) show the low activity of TPN-diaphorase, being stained faint pink, while the red muscle

fibers (R) show a high activity, being stained purple. The medium fibers (M) are stained pink and show a moderate TPN diaphorase activity, which are intermediate between those of the red and white muscle fibers.

M. soleus is composed of the fibers showing a high activity of TPN diaphorase. Careful observation proves that there are a few muscle fibers showing a low enzymatic activity as in the findings of the succinic dehydrogenase, cytochrome oxidase and DPN-diaphorase. Among those of highly active fibers, two types of fibers can be distinguished by the TPN-diaphorase activity, namely, comparatively strong and weak ones. The former may be constituted of red muscle fibers and the latter of medium fibers.

DISCUSSION

In the previous papers^{6,7}, it was shown that the red muscle fibers showed a high succinoxidase activity and cytochrome oxidase activity, while the white muscle fibers a low activity. Furthermore, there exists a third type of muscle fibers, namely, medium fibers, which show a moderate succinoxidase and cytochrome oxidase activity intermediate between those of the red and white muscle fibers. It was further shown that the difference of three types of fibers in the succinic dehydrogenase reaction was due to the difference in the number and enzymatic activity of mitochondria in each muscle fibers.

It is well known that the mitochondria contain many enzymes of tricarboxylic acid cycle. According to *Green*⁵, the mitochondria contain a complex electron transfer system, by which electrons removed from substrate molecules oxidatively are transferred ultimately to molecular oxygen through a series of reversibly oxidized and reduced intermediary carriers. And one of these electron transfer systems is the succinic dehydrogenase system and others the DPN- and TPN-linked dehydrogenase systems. The histochemical methods of DPN and TPN-diaphorase were first established by Farber et al. (1956)^{1,2,3,4,9}. From their detailed study, they concluded that the tetrazolium salts were reduced not by dehydrogenases or by DPNH and TPNH, but by DPN and TPN-diaphorase by their histochemical methods of DPN and TPN-diaphorase stainings. Namely, they described that when malate or lactate was used as substrate in the DPN-diaphorase staining, no difference was observed in the localization of diformazan in the kidney sections, because tetrazolium salts were not reduced directly by malic or lactic dehydrogenase, but by DPN-diaphorase. Also, in the TPN-diaphorase staining, the localization of difor-

mazan in the kidney sections was almost the same, when the different substrates were used, because tetrazolium salts were not reduced directly by dehydrogenases, but by TPN-diaphorase. In this study, it has been found that the almost identical staining patterns are observed irrespective of the combination of various substrates employed in the staining of DPN and TPN-diaphorase in the muscle fibers as the results of the study of Farber et al⁹. in the kidney.

As described above, the histochemical methods of DPN and TPN-diaphorase also distinguish those three types of muscle fibers. Namely, the red muscle fibers show higher DPN and TPN-diaphorase activities, the medium fibers moderate activities and the white muscle fibers lower activities. From the series of present studies, it may be concluded that the TCA cycle is operating suboptimally in the white muscle fibers, as shown by the findings of low activity of succinoxidase, cytochrome oxidase, and DPN and TPN-diaphorase. But in the red muscle fibers, the simultaneous occurrence of high activities of all four enzymes permits the speculation that the TCA cycle may be important in their metabolic pathways.

It is well known that the red muscle fibers are tonic and slow contracting fibers and participate in postural adjustment, while the white muscle fibers are rapid contracting fibers and are involved in phasic movement. The red muscle fibers may be considered to produce energy constantly for the sustained function of postural adjustment. Therefore, it is thought that they may be rich in mitochondria and have high enzymatic activities of TCA cycle, and supply abundant energy. But the white muscle fibers need not continuously produce energy, because they participate only in phasic movement. In addition, it is probably not suitable for rapid and strong contraction of these fibers to contain much mitochondria in their structures. Therefore, they may be considered to possess fewer mitochondria and have lower enzymatic activities of TCA cycle. The medium fibers, which were observed in many kinds of animals as described in the previous paper⁶, seem to have the function and the rate of metabolism intermediate between the red and white muscle fibers.

SUMMARY AND CONCLUSIONS

From the histochemical study of DPN and TPN diaphorase on the striated muscles of the cats, the following results were obtained.

1. *M. gastrocnemius*, which belongs to the white muscle by naked

eye, consists of three types of muscle fibers distinguished by the DPN diaphorase staining: namely, the small muscle fibers, i. e., the red muscle fibers show a moderate activity, being stained pink, while the large muscle fibers, i. e., the white muscle fibers show a low activity, being stained faint pink. The third type of muscle fibers: namely, the medium fibers are stained pale pink and show the enzymatic activity intermediate between the red and white muscle fibers.

2. *M. soleus*, belonging to the red muscle by naked eye, consists of three types of fibers distinguished by the DPN-diaphorase staining, i. e., the red muscle fibers are stained pink, medium fibers pale pink, and a few white muscle fibers faint pink. The diameters of these three types of muscle fibers in *M. soleus* are almost the same.

3. From the staining pattern of TPN-diaphorase in *M. gastrocnemius* and *M. soleus*, the three types of muscle fibers can be distinguished by TPN-diaphorase activity, namely, the red muscle fibers show a high TPN-diaphorase activity, being stained purple, while, the white muscle fibers a low activity, being stained pale pink. The medium fibers are stained pink and show a moderate enzymatic activity intermediate between the red and white muscle fibers.

4. The TPN-diaphorase activity is higher than the DPN-diaphorase activity in the striated muscle, but it is less active than the TPN-diaphorase activity in the kidney. However, the activity of DPN-diaphorase in the striated muscle is quite lower than that of the kidney.

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EXPLANATION OF PLATES

- Fig. 1. Gastrocnemius of the cat, cross-section, DPN-diaphorase. Note the three types of muscle fibers, namely, the red muscle fibers (R) show a moderate DPN-diaphorase activity, while the white muscle fibers (W) show a low activity. The medium fibers (M) show the activity intermediate between the red and white muscle fibers. $\times 100$.
- Fig. 2. Soleus of the cat, cross-section, DPN-diaphorase. Note the two types of muscle fibers. $\times 200$.
- Fig. 3. Gastrocnemius of the cat, cross-section, TPN-diaphorase. Note the three types of muscle fibers. $\times 100$.
- Fig. 4. Soleus of the cat, cross-section, TPN-diaphorase. Note the two types of muscle fibers. $\times 200$.

