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Histochemical studies on the red, white and intermediate muscle fibers of some skeletal muscles. Ⅲ. Histochemical demonstration of oxidative enzymes, phosphorylase and glycogen in respiratory muscle fibers

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Histochemical studies on the red, white and intermediate muscle fibers of some skeletal muscles. Ⅲ. Histochemical demonstration of oxidative enzymes, phosphorylase and glycogen in respiratory muscle fibers*

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Abstract

1) Three different types of muscle fibers were clearly distinguished in the intercostal muscles and the diaphragm of human, cat and rat by histochemical demonstration of oxidative enzymes, phosphorylase and glycogen. 2) The intercostal muscles and diaphragm each presented dissimilar patterns of the muscle fibers. The diaphragm did not show any definite correlation between the diameter and the histochemical reaction of muscle fibers but its red fibers indicated a more intense uptake of Nitro-BT formazan deposits in the subsarcolemmal region. In this connection, the relationship between the motor innervation and histochemical evaluation of the diaphragm was described. 3) Phosphorylase and glycogen showed reciprocal reactions to the oxidative enzyme activity. They were generally high in large fibers but low in small fibers, and moderate in intermediate fibers.

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**HISTOCHEMICAL STUDIES ON THE RED, WHITE AND
INTERMEDIATE MUSCLE FIBERS OF SOME
SKELETAL MUSCLES**
**III. HISTOCHEMICAL DEMONSTRATION OF OXIDATIVE
ENZYMES, PHOSPHORYLASE AND GLYCOGEN IN
RESPIRATORY MUSCLE FIBERS**

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Recent histochemical studies have revealed the existence of some specific type of fibers in mammalian striated muscles other than the red and the white muscle fibers. OGATA^{1, 2, 3} was first to report that the limb muscles of the vertebrate are composed of three types of fibers, which can be distinguished from each other by their activity of oxidative enzymes, i. e., the red fibers, which are rather small in diameter, having a higher enzyme activity, the white fibers of large diameter with a low enzyme activity and the so-called 'medium fibers' or 'intermediate fibers' which are moderate both in size and enzyme activity.

On the other hand, two different opinions are offered for the physiological function of intercostal muscles^{4, 5}, i. e., slow type muscles such as soleus and fast muscle as tibialis anterior. As far as the motor innervation of diaphragm is concerned, the opinions are also divided. Some^{6, 7} consider the lower intercostal nerves also to innervate the diaphragm muscle and the others^{8, 9} regard the fibers from the intercostal nerves as purely the sensory ones. Generally, it is assumed that ordinary respiratory muscles, which are endowed with dual character of automaticity of the heart and voluntary character of the skeletal muscle, may indicate certain specific histochemical features different from other skeletal muscles.

In the previous communication¹⁰, the author described the intercostal muscles of cat to be composed of three types of fibers, each differing in respective succinic dehydrogenase reaction, and dealt with respiratory participation of individual intercostal muscle fibers in the respiratory movement.

The present paper describes about a more detailed histochemical study on oxidative enzymes, phosphorylase and glycogen in ordinary respiratory muscles of human, cat and rat, and the obvious existence of three types of muscle fibers, along with histochemical peculiarities of the diaphragmatic fibers.

MATERIALS AND METHODS

Internal and external intercostal muscles and diaphragm of healthy adult cats and rats served as materials. The human intercostal muscles were obtained at the thoracotomy. The fresh muscles were removed after anesthetizing the animals with pentobarbital sodium, and then kept in a -30°C deep freezer. Some of the blocks were frozen in acetone dry ice mixture at -70°C , and were immediately cut into serial sections of $10\sim 15\ \mu$ in thickness in a cryostat at -20°C . For the histochemical demonstration of oxidative enzymes, the sections were dried at room temperature and incubated for 30 minutes at 37°C in respective substrate solutions. As an electron acceptor, nitro-blue tetrazolium (BT) was used, and the following dehydrogenases were demonstrated according to the methods described by PEARSE¹¹: 1) succinic dehydrogenase, 2) malic dehydrogenase, 3) glutamic dehydrogenase, 4) lactic dehydrogenase, 5) β -hydroxybutyric dehydrogenase, 6) isocitric dehydrogenase, 7) NAD-diaphorase, and 8) NADP-diaphorase.

For the demonstration of phosphorylase and glycogen, the TAKEUCHI¹² method and the MCMANUS periodic acid Schiff technique¹¹ were employed.

RESULTS

Oxidative enzymes: In transverse sections of the intercostal muscles and diaphragm of cat and rat, three different types of muscle fibers were clearly distinguished by the intensity of oxidative enzyme activities. In the intercostal muscle, the red fiber of small size showed a higher oxidative enzyme activity, the white fiber of large diameter a lower activity, and the third type of muscle fiber of intermediate diameter a moderate enzyme activity (Figs. 1 and 2). The fibers of the diaphragm showed almost identical pattern as that of the intercostal muscle. However, in this muscle some of large fibers indicated an intense oxidative enzyme reaction, and yet some of smaller ones a very weak reaction (Figs. 3 and 4). Consequently, in the diaphragm there could be observed no close relationship between the size and histochemical reaction of the fibers in opposition to that in the intercostal muscle. The small red fibers are usually characterized by their intense uptake of Nitro-BT formazan deposit in the subsarcolemmal region but the red fibers of diaphragm exhibited a far more intense subsarcolemmal enzyme activity than that of the intercostal muscles. That is, in the red fiber of the diaphragm, there were many distinct, striated accumulations of large diformazan particles beneath the sarcolemma, while in that of the intercostal muscle, a single layer of the diformazan was observed continuous beneath the sarcolemma (Figs. 4 and 5). The intensity of enzyme reactions

in the three types of fibers were classified into minus to positive 4 (– to +4), indicating negative, trace, very weak, weak, moderately intense, and very intense reactions (Histochemical results are summarized in Table 1).

Table 1 Activities of Oxidative Enzymes, Phosphorylase and Glycogen in Intercostal Muscles and Diaphragm of Cat.

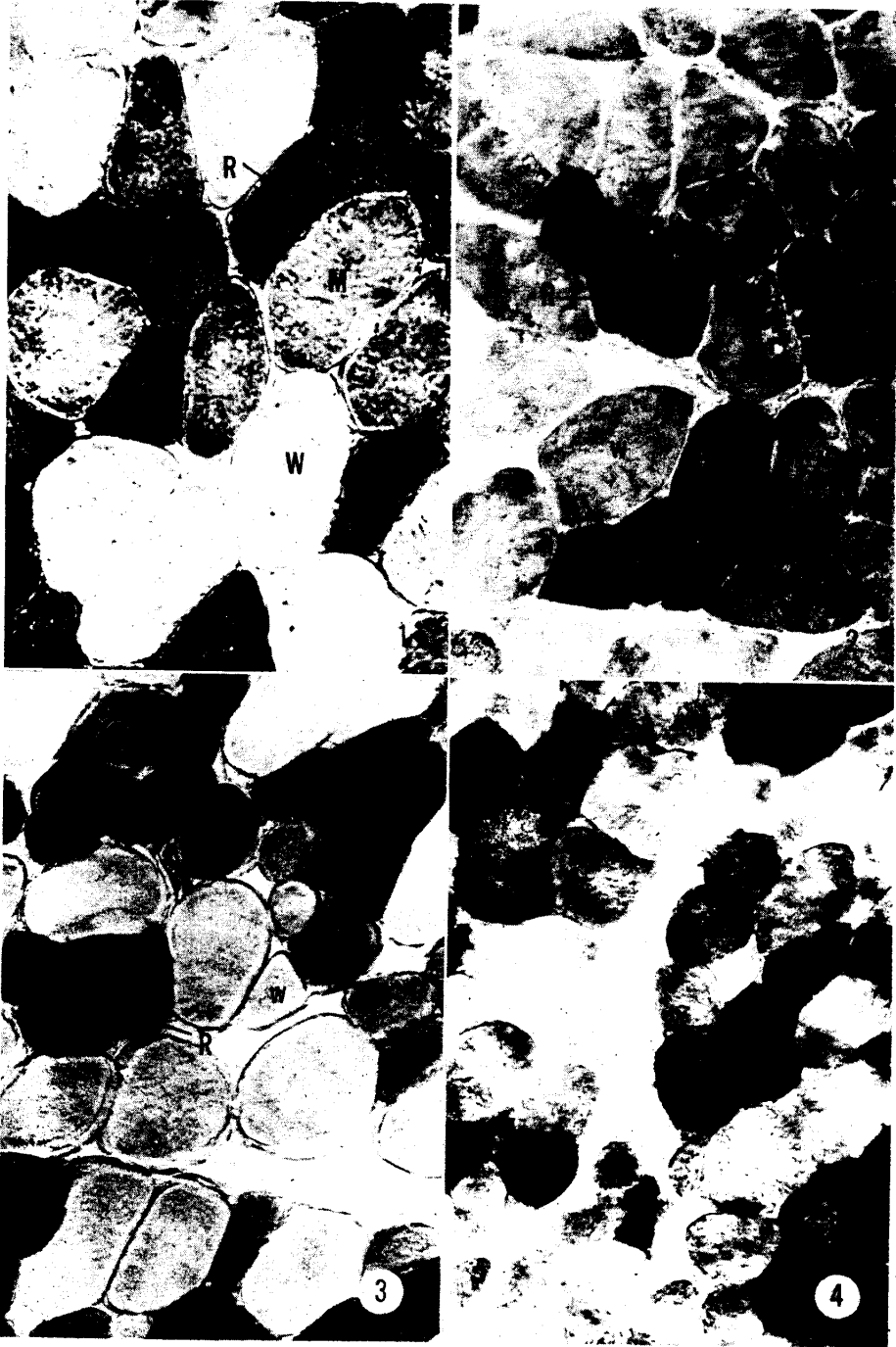
Muscle fibers Enzymes	Intercostal muscle			Diaphragm		
	Red	Intermed.	White	Red	Intermed.	White
SD	subsarcolemma	+3		subsarcolemma	+4	
	cytoplasm	+3	+2	cytoplasm	+2	+2
MD	subsarcolemma	+2		subsarcolemma	+3	
	cytoplasm	+2	+1	cytoplasm	+2	+2
GD	subsarcolemma	+1		subsarcolemma	+2	
	cytoplasm	+1	±	cytoplasm	+1	+1
LD	subsarcolemma	+3		subsarcolemma	+4	
	cytoplasm	+2	+2	cytoplasm	+2	+2
β-HD	subsarcolemma	+1		subsarcolemma	+3	
	cytoplasm	+1	±	cytoplasm	+2	+2
ID	subsarcolemma	+1		subsarcolemma	+2	
	cytoplasm	+1	±	cytoplasm	+1	+1
NAD-D	subsarcolemma	+3		subsarcolemma	+4	
	cytoplasm	+3	+2	cytoplasm	+3	+3
NADP-D	subsarcolemma	+3		subsarcolemma	+4	
	cytoplasm	+3	+2	cytoplasm	+3	+3
P-ase	+1	+2	+3	same as intercostal muscle		
G-gen	+1	+1~+3	+2~+3	same as intercostal muscle		

SD: succinic dehydrogenase
 MD: malic dehydrogenase
 GD: glutamic dehydrogenase
 LD: lactic dehydrogenase
 β-HD: β-hydroxybutyric dehydrogenase
 ID: isocitric dehydrogenase

NAD-D: NAD-diaphorase
 NADP-D: NADP-diaphorase
 P-ase: phosphorylase
 G-gen: glycogen

The distribution and histochemical profiles of these three types of fibers presented almost identical pattern in the different regions of cat diaphragm (crura, sternal and costal). Even the peripheral part of the diaphragm did not resemble the intercostal muscles.

Phosphorylase: In the case of phosphorylase, three fiber types could also be distinguished in both respiratory muscles. However, the phosphorylase reaction of muscle fibers indicated reciprocal results to the oxidative enzyme content. That is, phosphorylase was more intensely active in large white fibers showing the deep blue iodine reaction, while it was slightly active or negative in the small red fibers. The intermediate fibers showed the phosphorylase activity intermediate between the red and the white, as indicated by the faint blue iodine



reaction (Fig. 6). In serial sections prepared from the same part of the muscle and stained with respective enzymes, those fibers that showed a moderate activity with oxidative enzymes were also moderately active for phosphorylase.

Glycogen: Glycogen stainings showed the results almost similar to those in the case of phosphorylase, namely, the large fibers were usually stained intensely and the small fibers faintly (Fig. 7). However, the fibers of intermediate size did not show such a regular reaction: some of them reacted with PAS staining and others not.

Human intercostal muscle: The human intercostal muscles are also composed of three types of fibers, but the difference in the enzyme activity and the diameter is not so marked as cat intercostal muscles (Fig. 8).

DISCUSSION

Since OGATA's study^{1,2,3}, several workers have pointed out the existence of three types of muscle fibers in mammalian striated muscles. STEIN and PADIKULA¹³ have made a careful study on the distribution of succinic dehydrogenase in the medial head of the gastrocnemius of the rat. They describe three fiber types, A, B and C, differing in the amount and distribution of the diformazan deposit. These A, B and C fibers correspond to our white, intermediate and red muscle fibers respectively. SMITH¹⁴ has also reported similar findings in vastus lateralis of the rabbit, and suggests that the Nitro-BT technique is an accurate one for insoluble mitochondrial enzymes.

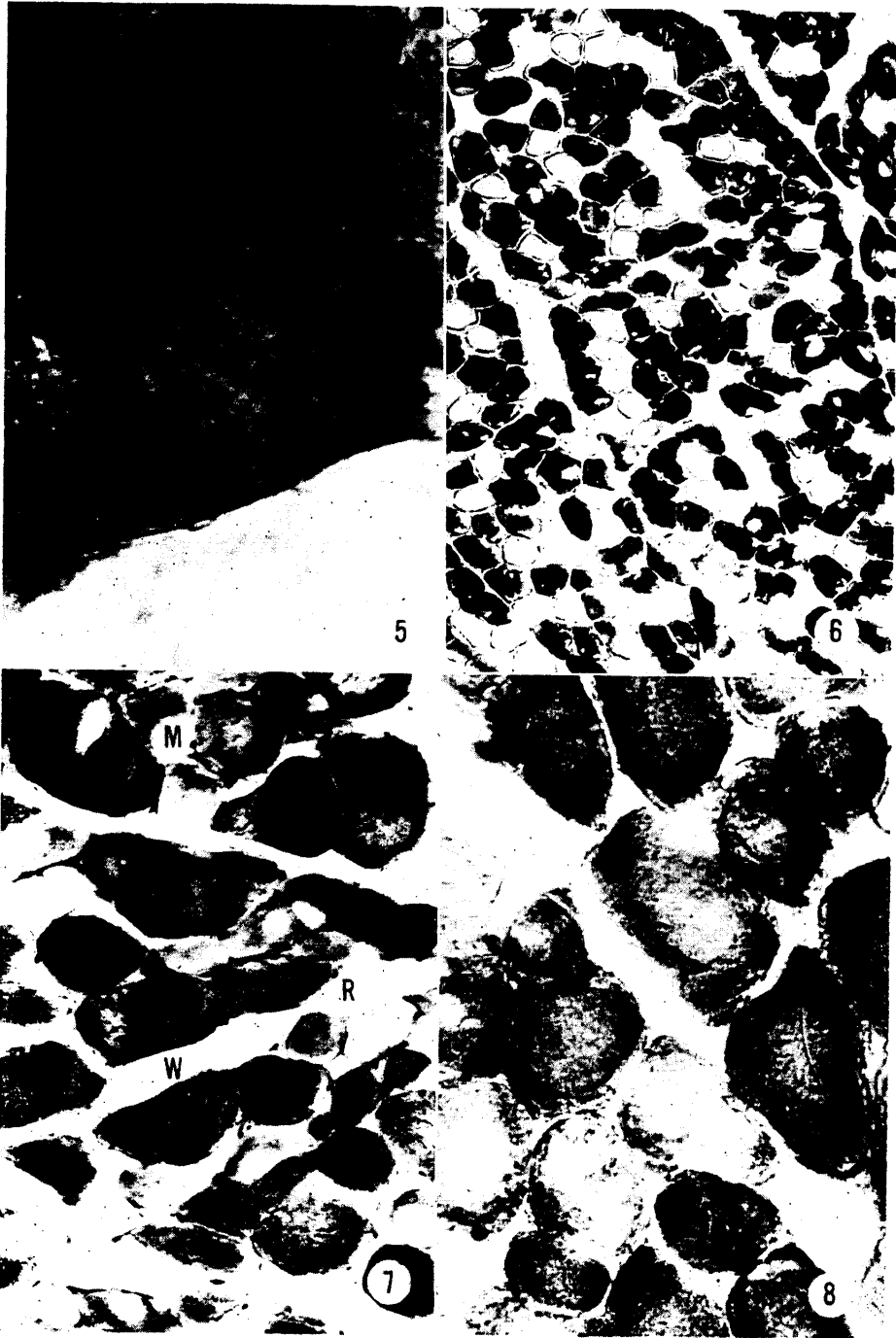
In the present study, it was clearly demonstrated that both the intercostal muscle and the diaphragm show respectively such fibers that can be divided into three types. However, the diaphragm does not show any definite correlation between the diameter and histochemical reaction. In other words, this muscle shows a greater extent in diameters in each type of fibers in contrast to that in the intercostal muscles. Especially, this tendency is more prominent in the white fibers (Fig. 9). Although GEORGE and SASHEELA¹⁵ in their histochemical study observed two types of fibers, the white broad and the red narrow ones in rat

Fig. 1 Intercostalis externus of cat, cross-section, succinic dehydrogenase. Note three types of fibers: the small red fiber (R) with a higher dehydrogenase activity, the large fiber (W) with a lower activity and the intermediate fiber (M), being intermediate in size with a moderate activity. $\times 350$

Fig. 2 Intercostalis externus of cat, cross-section, NAD-diaphorase. Note three types of fibers. R: red fiber M: intermediate fiber W: white fiber $\times 250$

Fig. 3 Diaphragm of cat, cross-section, succinic dehydrogenase. This muscle does not show any definite correlation between diameter and histochemical reaction of fibers. R: larger red fiber with a high enzyme activity. W: smaller white fiber with a low activity. $\times 250$

Fig. 4 Diaphragm of cat, cross-section, lactic dehydrogenase. Note three types of fibers. $\times 250$



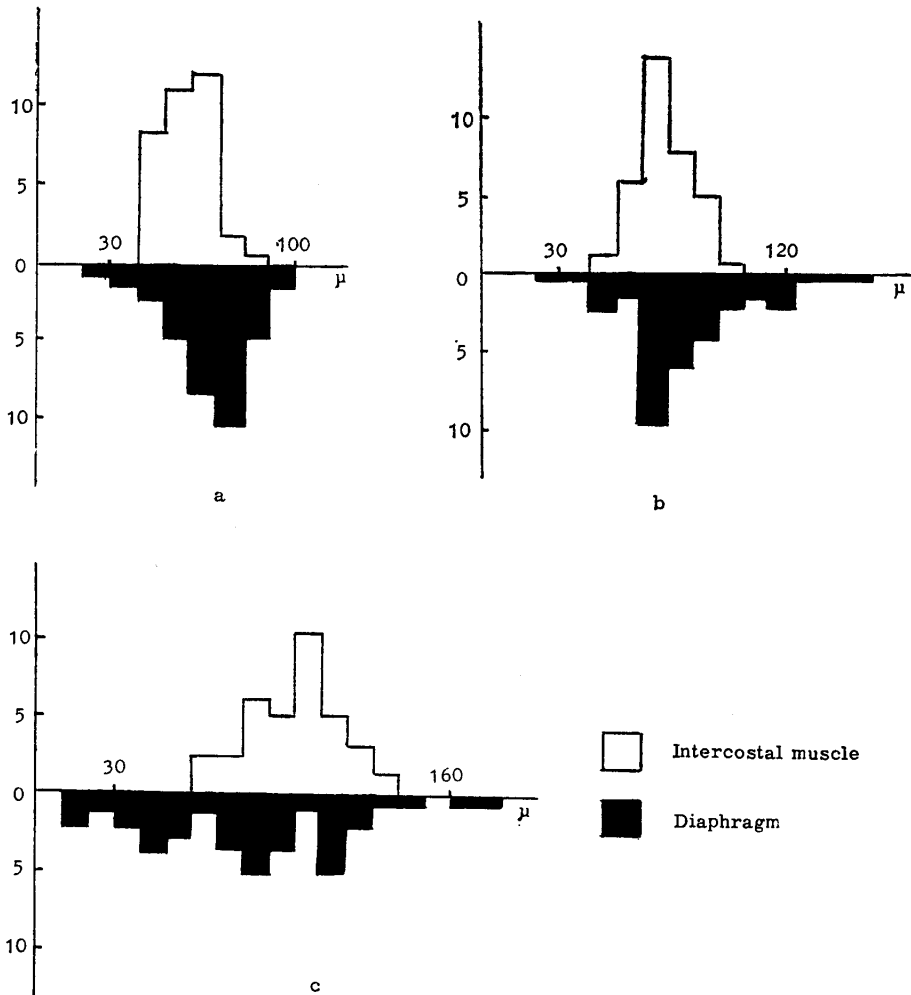


Fig. 9 Histograms of the diameter of diaphragmatic and intercostal muscle fibers. Ordinate indicates the number of fibers and abscissa indicates the diameter of fibers in microns.
 a: red muscle fiber b: intermediate muscle fiber c: white muscle fiber

- Fig. 5 Diaphragm of cat, cross-section, succinic dehydrogenase. Note intense diformazan deposit in the subsarcolemal space (F). $\times 1,200$
- Fig. 6 Intercostalis externus of cat, cross-section, phosphorylase. Three types of fibers are clearly distinguishable. $\times 165$
- Fig. 7 Intercostalis externus of cat, cross-section, glycogen (PAS). Note three types of fibers (R, W, M.). $\times 330$
- Fig. 8 Intercostalis externus of man, cross-section, succinic dehydrogenase. The difference of enzyme activities in each type of fibers is not so prominent as cat intercostal muscle $\times 360$

diaphragm, they also state that the distribution pattern of these two types of fibers appears to be at random. Consequently, the fact that the diameter of the fibers shows a considerable variation in each of these three types suggests that the individual diaphragm fibers may have some variability in metabolism, and carry on the metabolism and function different from other skeletal muscles. Such a possibility is also substantiated by the fact that the red fibers of the diaphragm possess a denser sarcosome and stronger subsarcolemmal enzyme activities as never observed in other skeletal muscles. Therefore, these results suggest that the continuous, powerful contraction of the diaphragm, like cardiac muscle, may reflect a high concentration of mitochondria.

Regarding the motor innervation of diaphragm, as described in the introductory remarks, ELLENBERGER and BAUM⁶, FELIX⁷, and others after careful macroscopic and histological examinations, conclude that lower intercostal nerves also innervate diaphragm muscle. LAST¹⁶ considers the crura, derivatives of the prevertebral rectus, being supplied by the lower intercostal nerves, not by the phrenic. However, STRAUS⁸, BOTHA⁹, and others regard the fibers from the intercostal nerves as purely sensory. If these nerve fibers are not purely sensory but serve to innervate the diaphragm motorially, it is considered that the crura pillars or the peripheral part of the diaphragm perhaps presents a similar pattern as that of the intercostal muscles. However, in the present study no regional difference in the fiber pattern was observed, i. e., the crura, the central and the peripheral parts of the diaphragm respectively indicated a typical pattern of the "diaphragmatic muscle".

With respect to the phosphorylase activity, three types of fibers were also distinguished in both respiratory muscles. Recently, DUBOWITZ and PEARSE¹⁷ maintain that a reciprocal relationship exists between phosphorylase and co-enzyme-linked oxidative enzymes in striated muscles, i. e., a high concentration of oxidative enzymes and a low concentration of phosphorylase in small fibers and *vice versa* in large fibers. They assume a possibility that some skeletal muscle fibers metabolize preferentially noncarbohydrate substances, perhaps fatty acids. More recently HESS and PEARSE¹⁸ suggest the existence of two different enzymatic pathways for glycogen synthesis and breakdown in skeletal muscle fibers: one is the phosphorylase and branching enzyme pathway and another the uridine diphosphate glucose (UDPG-) glycogen transglucosylase pathway. They demonstrated that the former is strongly active in fibers of large diameter, conversely the latter in small fibers. In general, newly formed polysaccharides from glucose-1-phosphate (G-1-P) show varying tones of color to the staining with iodine reactions. According to SWANSON¹⁹, the chain length of linear polymers of glucose unit appears to be the main factor that influences the hue of the iodine complex. SASAKI and TAKEUCHI²⁰, using the human gluteus muscle, state that

histochemically, newly formed polysaccharides are stained blue in smaller fibers and violet or red purple in larger ones. In the present study of the respiratory muscles the large white fibers were stained deep blue, the fibers of intermediate size faint blue, and the small red fibers slightly active or negative with iodine reaction.

The glycogen content was generally high in large fibers but low in small fibers, and quite variable in intermediate fibers. Usually glycogen and phosphorylase reactions do not show such a definite stainability as in the case of mitochondrial enzymes in the different types of muscle fibers. This problem is further complicated by the fact that the water-soluble glycogen, as several workers have already pointed out, may easily be shifted during fixation and thus accurate localization is difficult to achieve.

The present investigation on respiratory muscles has revealed the difference in the metabolic system according to three types of muscle fibers. The fiber type of intermediate size seems to have the rate of metabolism between the red and the white muscle fibers. The difference in the fiber pattern between intercostal muscles and diaphragm significantly suggests the functional differences of the two muscles in their respiratory actions.

SUMMARY

1) Three different types of muscle fibers were clearly distinguished in the intercostal muscles and the diaphragm of human, cat and rat by histochemical demonstration of oxidative enzymes, phosphorylase and glycogen.

2) The intercostal muscles and diaphragm each presented dissimilar patterns of the muscle fibers. The diaphragm did not show any definite correlation between the diameter and the histochemical reaction of muscle fibers but its red fibers indicated a more intense uptake of Nitro-BT formazan deposits in the subsarcolemmal region. In this connection, the relationship between the motor innervation and histochemical evaluation of the diaphragm was described.

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