A Histochemical Study of Normal and Denervated Red and White Muscles of the Rat*

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Plates 18 to 20

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ABSTRACT

The distribution and characterization of the fibers of normal and denervated red and white muscles of the albino rat are reported in this study. Histochemical procedures for succinic dehydrogenase, lipides, adenosinetriphosphatase, esterase, and glycogen were utilized to differentiate muscle fibers, and these methods facilitated the study of the distribution of fiber types within whole muscle.

Muscle fibers of the granular type (dark or red fibers) can be clearly distinguished from those with clearer sarcoplasm (light or white fibers) by methods for demonstrating succinic dehydrogenase, lipides, and esterase. The method for adenosinetriphosphatase reveals differences only under the special conditions described in the text. Additional fiber types are described in the cat's diaphragm and in the extrinsic ocular muscles of the rat. Succinic dehydrogenase and adenosinetriphosphatase activities of the soleus and biceps femoris were studied 14 days after denervation of these muscles. The histochemical findings are discussed principally in the light of current biochemical knowledge of these enzymes.

INTRODUCTION

It has long been known that skeletal muscles of many animals vary in color over a wide range from white to red (Kühne, 1865; Needham, 1926; Hines, 1927). While some of the red color may be attributed to the blood supply, Kühne showed with homogenates that the difference in hue between muscles persisted after exsanguination. Recently, Haurowitz and Hardin (1954) ascribed this remaining color to myoglobin and cytochromes within the muscle fibers.

Morphological studies of red and white muscles (Bell, 1911; Bullard, 1912–13) revealed two general categories of fibers, dark and light, which were visible in unstained preparations. For the most part, dark fibers were described as being of smaller diameter and containing more sarcoplasm and granules than the light fibers. Both faintly refractive (mitochondrial) and strongly refractive (lipide) granules were described. According to Needham (1926), Knoll, in his survey of animal muscles (1889–91), showed that most mammalian muscles are mixed, that is, that they contain both dark and light fibers. Recent investigation of skeletal muscle with the electron microscope (Edwards, Ruska, de Souza Santos, and Vallejo-Friere, 1956) has revealed variations in many aspects of cytological structure in fibers from a wide variety of species.

It has, therefore, been recognized for some time that the terms "red" and "white," as applied to whole muscle, do not imply homogeneity of the component fibers. However, there have been few methods for tissue sections by which fibers could be differentiated and their distribution studied. Denny-Brown (1929), utilizing Sudan red dyes, considered the quota of dark and light fibers in relation to physiological properties of whole muscle. More recently, Günther (1954) and Krüger and Günther (1956 *a* and 1956 *b*) have studied differences between muscle fibers, using a gold chloride method to demonstrate the sarco-

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plasmic reticulum. Histochemical methods have been little utilized to understand the differences among fibers. Also, while many changes have been followed in denervated muscles (Hines and Knowlton, 1935; Tower, 1939; Fischer and Ramsey, 1945–46; Humoller, Griswold, and McIntyre, 1950; Krüger and Günther, 1956 *a*), the characteristics of fiber types have received little attention from a histochemical point of view.

To explore the differences between fibers more fully, and to provide some clear cut methods by which types of fibers could be demonstrated, it seemed of interest to apply histochemical methods to the fibers of normal and denervated muscles. In this paper, such observations are presented, principally on normal and denervated soleus and biceps femoris muscles of the albino rat. Supplementary descriptions are included of normal diaphragm, ocular muscles, tibialis anterior, and gastrocnemius muscles from the rat, cat, and frog.

Material and Methods

Male albino rats of Wistar strain (150 to 200 gm.) were used for most of the observations of normal muscle and for all the experiments involving denervation. A few observations were made on the normal muscles of cats and frogs.

Histochemical Procedures.—The muscles were removed immediately after killing the rats with chloroform. In the case of frogs, the muscle was removed after pithing, and from cats during anaesthesia. For all procedures, each muscle was freed by blunt dissection, tied firmly to a round dowel, and then freed by cutting the tendons. This was necessary to prevent contraction and distortion.

For frozen sections and tests for succinic dehydrogenase adenosinetriphosphatase,[1] esterase, and glycogen, the muscles attached to dowels were thrust into a 95 per cent alcohol and dry ice mixture at approximately −70°C. for 10 to 20 minutes, and were then removed into the cryostat (−20°C.), where they were cut. Even with this rapid freezing, occasional ice crystals formed within some cells and produced distortion. Sections were cut at 10 μ for all procedures. Control sections, that had been frozen in test tubes and placed in the same mixture, possessed equal succinic dehydrogenase activity, indicating that the alcohol probably did not penetrate into the fibers in this period of time. For the demonstration of succinic dehydrogenase the sections were not allowed to dry, but were immersed at once in the incubating medium, and treated according to the method described by Padykula (1957). This modification, using neotetrazolium, was derived from the work of Seligman and Rutenburg (1951), Padykula (1952), and Rosa and Velardo (1954). To keep the moist sections on the slides, 0.5 per cent parlodion in ethyl acetate was used to affix the edges of a row of sections and, since this solution has an inactivating effect upon the enzyme, observations were made only on the parlodion-free central areas of the sections. These areas had activity comparable to that of untreated sections. When denervated and normal muscles were compared for succinic dehydrogenase and ATPase, sections from each were incubated on the same slide as an additional control measure.

The frozen sections for ATPase, esterase, and glycogen were air-dried and refrigerated for 1 day to a few weeks. ATPase activity, demonstrated according to the Gomori principle, was characterized by using sulfhydryl inhibitors and activators (Padykula and Herman, 1955). Non-specific esterase activity was determined by the Pearse procedure (1953) using α-naphthyl acetate as a substrate. Glycogen was localized by the periodic-acid Schiff reaction (McManus, 1946; Hotchkiss, 1948) on frozen and paraffin sections.

For demonstration of lipides, the muscles were fixed in buffered 10 per cent formalin, washed, embedded in gelatin, frozen at −30° C., and cut in the cryostat at 5 or 10μ. The sections were stained with Sudan black B for 7 minutes. Control sections were extracted with acetone for 30 to 60 minutes before staining.

Operative Procedure.—The soleus or the anterior head of the biceps femoris was denervated in one hind limb of a given animal, and the opposite side served as a control. For this operation the rats were anaesthetized with ether, and the muscle was exposed. Each of these muscles is supplied by a single nerve. When observed under a dissecting microscope, the nerve was raised with a small metal hook to separate it from its accompanying blood vessels and totally severed, and the proximal end was turned back. Some control operations were performed in which the muscle was exposed and the nerve lifted, but not cut. The rats were then put into a roomy cage and were observed to move the operated hindleg freely during the following days. Animals were sacrificed on the 14th post-operative day for all tests except lipides. Lipide changes were studied at 18 to 19 days, since Audova (1923) has shown with whole muscle that fat deposition in denervated muscle increases only slowly, and is approximately 50 per cent above normal at this time. This period is not long enough to permit reestablishment of functional nerve-muscle connection in the rabbit (Gutmann, Guttmann, Medawar, and Young, 1942).

Supplementary biochemical tests were run for succinic dehydrogenase and ATPase with homogenates of muscle using the methods described by Padykula (1957).

[1] ATPase will be used as an abbreviation for adenosinetriphosphatase.

RESULTS

Normal Muscle:

Succinic Dehydrogenase.—The muscle fibers of the rat's soleus are, with minor variations, uniformly and highly active (Fig. 1). In this red muscle, diformazan granules precipitate in delicate longitudinal rows throughout the sarcoplasm, but the greatest concentration of diformazan is found immediately under the sarcolemma. In contrast to the *soleus*, the mixed muscles, *biceps femoris* and *tibialis anterior*, contain fibers which differ strikingly in enzymatic activity (Fig. 3). This activity difference extends along the length of the fibers. In the *tibialis*, the fibers showing the greater enzyme activity are markedly smaller in diameter than those possessing less activity. In the *biceps*, however, this correlation tween size and degree of activity is not so clear cut, because some moderately large fibers show considerable activity and small fibers with low activity are occasionally seen, which may be cross-sections through the tapering ends of large pale fibers. In the more active fibers of these mixed muscles, there is a somewhat greater deposition of diformazan beneath the sarcolemma than in the central sarcoplasm, though the gradient displayed in the deposition of diformazan is not so striking as in the soleus. The larger, white fibers, on the other hand, do not show this subsarcolemmal activity, but have, instead, a relatively uniform distribution of succinic dehydrogenase activity throughout the sarcoplasm.

In a cross-section of extraocular muscle, three quite distinct types of fibers are visible (Fig. 4). The largest fibers show a moderate degree of enzyme activity, and the small fibers fall into two sharply contrasting categories, those which are highly active and those whose activity is exceedingly low. As far as we know, three types of fiber have not been previously distinguished by histochemical criteria, although Siebeck and Krüger (1955) have described two types of ocular muscle fibers on structural grounds alone. The level of enzymatic activity in the eye muscles as a whole is generally higher than that of leg muscles, for sections such as that shown in Fig. 4 show a dense precipitate after 15 to 20 minutes of incubation, whereas 30 to 45 minutes are required to give the precipitation seen in Figs. 1 and 3.

In all other muscles studied for succinic dehydrogenase (*diaphragm*, *external oblique*, and *pectoral* muscles of the rat, and *gastrocnemius* of rat, cat, and frog), two types of fibers were observed, when compared within the same muscle. The fibers with greater enzymatic activity have a smaller average diameter than those which are less active. In frog muscle the level of activity is generally lower than in the mammalian muscle.

ATPase.—The pattern of ATPase activity at pH 9.4 in the *soleus* and the *biceps femoris* is unlike that of succinic dehydrogenase. In preparations of the *soleus* of the rat, fibers vary in enzymatic activity, but within a given fiber there is uniform staining (Fig. 11). There is no constant correlation between sizes of fibers and their depth of staining. In sequential sections a given darker fiber can be seen to retain its high level of activity throughout its length. The intrafusal fibers of muscle spindles in the *soleus* are more active than the surrounding muscle fibers.

In the *biceps*, all fibers stain uniformly (Fig. 13), and no difference can be seen between the largest and smallest fibers. This is true also when the muscle sections are incubated with ATP and cysteine. If, however, the reaction is first inhibited with *p*-chloromercuribenzoate and then reactivated with cysteine or 2,3 dimercaptopropanol (BAL), the checkerboard pattern characteristic of mixed muscle is obtained (Fig. 14), with the small fibers staining more intensely.

Extraocular muscles of the rat, when studied for ATPase activity, show essentially uniform activity in different groups of fibers.

Lipides.—With Sudan black B, I bands, mitochondria, and fat droplets are demonstrated (Dempsey, Wislocki, and Singer, 1946; Baker, 1944–45). After this stain the fibers of the *soleus* appear alike in cross-section, with heavier staining occurring under the sarcolemma. However, sections of the *biceps*, *gastrocnemius*, and *pectoral* muscles of the rat have the mixed appearance, with fibers of greater and lesser sudanophilia intermingled (Fig. 5). The larger fibers are less sudanophilic. The cat's *diaphragm* was found to have an unusual structure (Fig. 6). In addition to the dark and light fibers, a distinctive third type is visible, characterized by intense dye uptake in the subsarcolemmal position, but less over-all sudanophilia in the interior of the fiber.

In longitudinal sections of the *soleus* more detail is visible (Fig. 7). Sudanophilic I bands alternate with unstained A bands. The nuclei are visible as unstained ovoids outlined by halos of

closely packed grey granules. These granules, which are most numerous under the sarcolemma, occupy much of the peripheral cytoplasm between nuclei. Rows of granules are also observed in the interior of the fiber, forming delicate streaks in the sarcoplasm. These granules fit the criteria for mitochondria, consisting partly of phospholipide, because they are most numerous where the activity of succinic dehydrogenase is greatest and they are not extracted by 30 to 60 minutes in acetone.

In longitudinal sections, with the oil immersion lens, small black neutral fat droplets are also visible within the fiber and are arranged largely in line with the I bands, as described by Bullard (1912–13). In addition, in fibers rich in fat, droplets occur at the level of the A bands. These droplets are extractable with acetone, whereas the staining of the I bands and mitochondria is unaffected by such treatment.

Non-Specific Esterase.—With the Pearse method the *soleus* was found to consist of fibers of essentially equal enzymatic acitivity, but the *biceps* contained larger, less active fibers, and smaller, more active ones (Fig. 9). The reaction product is, for the most part, deposited evenly within the fiber. The dense precipitation evident at the motor end plates is caused by acetyl cholinesterase (Couteaux, 1955).

Glycogen.—With frozen sections of muscle, far better localization of glycogen is obtained than with paraffin sections. In the latter, the stained material often accumulates at one side of the fiber, whereas with frozen sections, glycogen is more evenly dispersed within the fibers. In the *soleus,* fibers are essentially uniform, while in the *biceps,* fibers show great variation in glycogen content (Fig. 10). Although glycogen tends to be more abundant in the large fibers, a positive correlation between fiber size and glycogen content is not found consistently.

Denervated Muscle:

Succinic Dehydrogenase.—The *soleus* shows a marked decrease in activity 14 days after denervation (Fig. 2), and this decrease is most pronounced under the sarcolemma. The colorimetric measurements made of this change in homogenates show a loss of about 50 per cent activity. This confirms earlier biochemical results (Knowlton and Hines, 1934; Humoller, Griswold and McIntyre, 1951). In the biceps there is a clear cut reduction of enzyme activity in the smaller fibers, but a less marked change in the larger fibers. Thus, while the difference in size remains, there is less difference in enzymatic activity.

ATPase.—Two weeks after denervation, there appears to be a slight increase in the test for this enzyme in the *soleus* (Fig. 12) and *biceps.* When measured per unit wet weight of muscle, colorimetric tests supported this observation. The occasional fibers of the *soleus* that stained more darkly are still evident, while the fibers of the denervated *biceps* remain uniformly stained.

Lipides.—The denervated *soleus* (Fig. 8) and *biceps* show a decrease in over-all sudanophilia. The clarity and sharpness of the cross-striations is reduced in denervated fibers, because there is loss of sudanophilia of the I bands, and a development of weak staining of the A bands. There is sharply decreased staining of the perinuclear mitochondria. From Audova's (1923) work with whole muscle, an increase in neutral fat was expected, but a consistent change in fat droplets within the fiber could not be demonstrated in our preparations.

Glycogen.—In general there is a marked decrease in the glycogen of denervated *biceps* and *soleus,* although some glycogen remains in a few fibers.

DISCUSSION

Until recent years, Sudan III and IV were the main histochemical tools for differentiating the "dark" and "light" fibers of skeletal muscle (Bell, 1911; Bullard, 1912–13; Denny-Brown, 1929). With the description of differences in succinic dehydrogenase activity among the fibers of mixed muscles (Padykula, 1952; Thimann and Padykula, 1955; Wachstein and Meisel, 1955; Moore, Ruska, and Copenhaver, 1956), it became apparent that histochemical methods could add further to our understanding of this intramuscular variability. In the present study, metabolic differences among muscle fibers are revealed by the histochemical procedures for succinic dehydrogenase, lipides, ATPase, esterase, and glycogen.

It is suggested from this investigation that the smaller fibers contain more mitochondria per unit area than the larger fibers. This characteristic is reflected histochemically by greater succinic dehydrogenase activity and deeper staining with Sudan black B in the small fibers. The validity of the tetrazolium method for succinic dehydrogenase is well established, although the precise

mechanism of reduction of these salts has not yet been elucidated, other than that a flavoprotein is required (Brodie and Gots, 1951; Shelton and Schneider, 1952). Lawrie (1953) demonstrated a close correlation in the amounts of succinic dehydrogenase, cytochrome oxidase, and myoglobin present in three skeletal muscles of the horse. This suggests that the smaller fibers may also contain more myoglobin than the larger ones. In addition, Lawrie reported a sigmoidal relation between the percent of myoglobin and the Q_{O_2}, measured as cytochrome oxidase, in the muscles of a wide variety of vertebrates. Recent solubilization (Neufeld, Scott, and Stotz, 1954) and purification (Singer and Kearney, 1954; Singer, Kearney, and Bernath, 1956) of succinic dehydrogenase from beef and pork heart mitochondria show the primary enzyme to be a ferroflavoprotein. These points emphasize that the color or density differences of vertebrate fibers and muscles are caused in part by the concentration of myoglobin, cytochromes, and flavin enzymes, such as succinic dehydrogenase.

Biochemical studies provide strong evidence that mitochondria contain a fairly high percentage of lipide, largely as phospholipide (Claude, 1941; Chargaff, 1942). Sudan black B, therefore, may reveal differences in mitochondrial density by a different staining mechanism, and support the picture of mitochondrial distribution revealed by the tetrazolium method.

Matlack and Tucker (1940) reported esterase activity in muscle homogenates and the histochemical distribution of esterase appears similar to that of succinic dehydrogenase. While this suggests that esterase activity may be associated with mitochondria, it is possible that other cell particles also have this same distribution among the muscle fibers. This possibility is enhanced by the recent report of Underhay, Holt, Beaufay, and de Duve (1956) that esterase of rat liver is a true microsomal enzyme.

Glycogen appears evenly dispersed within a muscle fiber in frozen sections, and this distribution contrasts that obtained with the paraffin method in which glycogen is carried to one side of the fiber during fixation. It is interesting that Takahashi and Iwase (1955) have also demonstrated uniform cytoplasmic distribution of hepatic glycogen with the paraffin method by treatment with M/3 NaOH before fixation. While no consistent correlation appears to exist between muscle fiber size and amount of glycogen, the question cannot be considered closed. The lability of muscle glycogen (Cori, 1931; Russell and Bloom, 1955) emphasizes the need for removing the muscle from unstressed anaesthetized animals.

The ATPase demonstrated here corresponds most closely to the biochemical descriptions of myosin ATPase (Padykula and Herman, 1955). This enzyme is most active in the alkaline range; is activated by Ca^{++}; and is dependent on the —SH group for activity. It is not likely that the Mg^{++}-activated mitochondrial ATPase of Kielley and Meyerhof (1948) is producing precipitation here. That enzyme has a pH optimum of 6.8 and is inhibited by Ca^{++} (Chappell and Perry, 1953). Thus, it should have negligible activity under our conditions. Moreover, we found uniform distribution of ATPase activity in mixed muscles, while Wachstein and Meisel (1956) in demonstrating ATPase in muscle at pH 7.2 reported that some fibers stained more darkly than others. This suggests that in their work the mitochondrial ATPase was demonstrated histochemically.

Our results in mixed muscle following the reversible inhibition of ATPase (pH 9.4) find no explanation at present. The staining of biceps' fibers is inhibited by *p*-chloromercuribenzoate as expected (Singer and Barron, 1944; Padykula and Herman, 1955). However, after reactivation with cysteine or BAL, the mixed appearance is seen for the first time, with the smaller fibers staining more intensely. Several possible effects may be in operation. The mitochondrial ATPase may somehow be activated by this treatment. If diffusion of reaction products occurs within each cell, the effect may be an increase in precipitate without cytological localization. Another possibility would be inhibition or activation of the fibrillar ATPase of large or small fibers selectively.

Viewed in relation to physiological knowledge of muscle activity, the present findings raise a problem. If red muscle is more active physiologically than white (Needham, 1926; Millikan, 1939), then it is curious that the dark fibers are of smaller diameter than the pale fibers. Muscle is commonly observed to hypertrophy when increased demands for work are made upon it, and Edds (1950) has demonstrated that the average diameter of fibers does increase in compensatory hypertrophy. It would be of interest to follow the histochemical patterns in mixed muscle during

such compensatory hypertrophy after increased work load.

After nerve section, a reduction of myoglobin content, succinic dehydrogenase, and cytochrome oxidase activity occurs in 14 days (Whipple, 1926; Humoller, Griswold, and McIntyre, 1951; Humoller, Hatch, and McIntyre, 1952). It is of interest that the myosin ATPase activity demonstrated here does not decrease in denervated muscle at a time when there has been a great decline in the sudanophilia and succinic dehydrogenase activity of the mitochondria of muscle. This supports Humoller's (1951) idea that changes in energy-producing structures in denervated muscle are of prime importance in the early events leading to atrophy. However, Michelazzi, Mor, and Dianzani (1957), studying ATPase at pH 6.9 in muscle during the first 16 hours after denervation, found a fall in myofibrillar ATPase as well as a rise in sarcosomal ATPase. The discrepancy between their findings and ours may be caused by the difference in the time factor. However, their measurements were made below the optimal pH for myofibrillar ATPase. Histochemical study of mitochondrial ATPase after denervation would be of interest in the light of their findings.

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Explanation of Plates

Plate 18

Fig. 1. Normal soleus of the rat, cross-section, succinic dehydrogenase, 45 minutes incubation. Note the uniform reactivity of the fibers with marked precipitation of diformazan in the subsarcolemmal position. × 400.

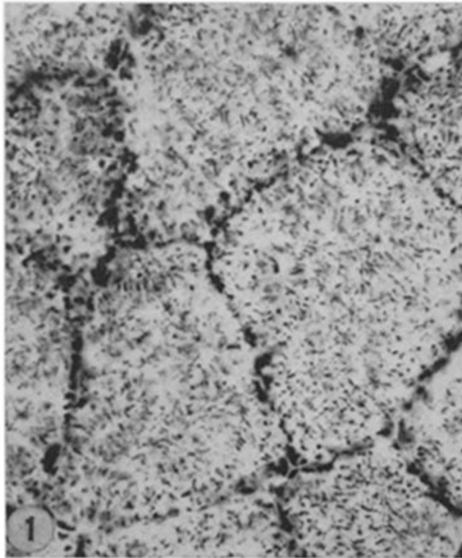
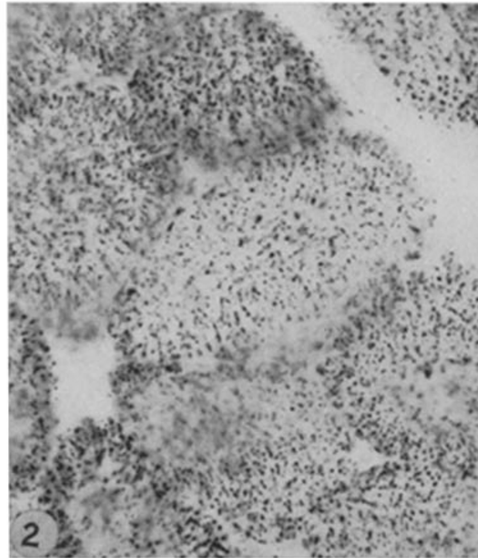
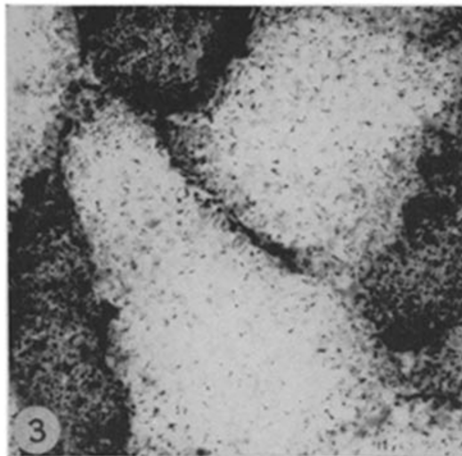
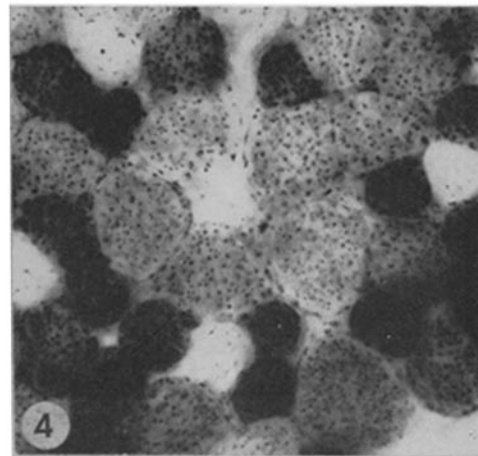
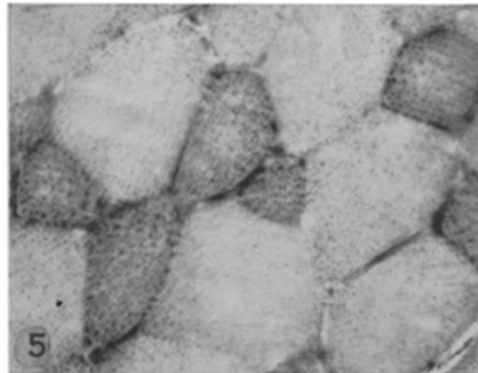
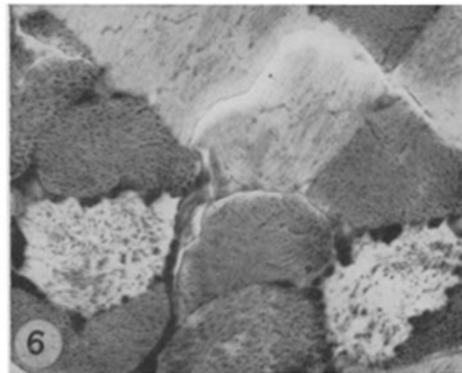
Fig. 2. Soleus of the rat denervated for 14 days, cross-section, succinic dehydrogenase, 45 minutes incubation. Compare with Fig. 1 and note the decreased dye precipitation at the periphery of the fibers. × 400.

Fig. 3. Normal tibialis anterior of the rat, cross-section, succinic dehydrogenase, 45 minutes incubation. The smaller fibers have markedly greater deposition of diformazan than the larger fibers. × 400.

Fig. 4. Normal extraocular muscle of the rat, cross-section, succinic dehydrogenase, 20 minutes incubation. Note the three types of fibers. × 400.

Fig. 5. Normal biceps femoris of the rat, cross-section, Sudan black B. Note the greater sudanophilia of the smaller fibers. × 100.

Fig. 6. Normal diaphragm of the cat, cross-section, Sudan black B. Note that there are three types of fibers. Compare with Fig. 5. × 350.

(Nachmias and Padykula: Normal and denervated rat muscles)

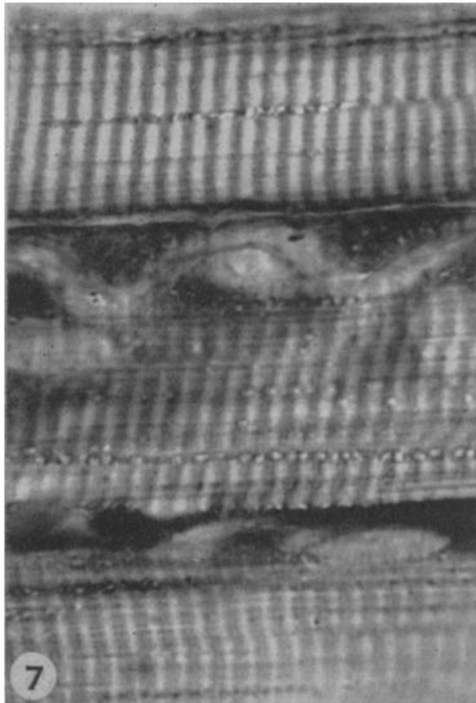
Plate 19

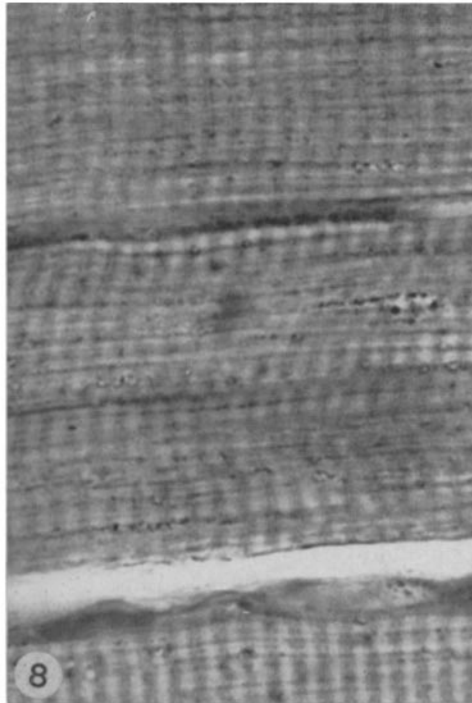
Fig. 7. Normal soleus of the rat, longitudinal section, Sudan black B. Note the unstained oval nuclei, strong perinuclear staining, and the sudanophilia of the I bands. × 800.

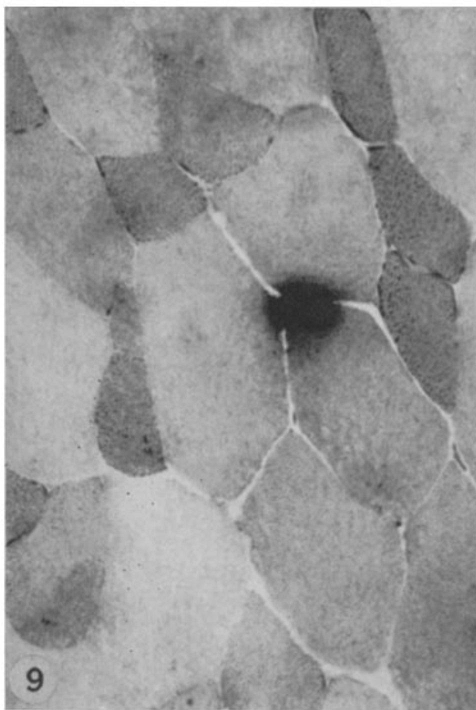
Fig. 8. Soleus of the rat, denervated for 20 days, longitudinal section, Sudan black B. There is a decrease in the perinuclear staining and in the sudanophilia of the I band. Compare with Fig. 7. × 800.

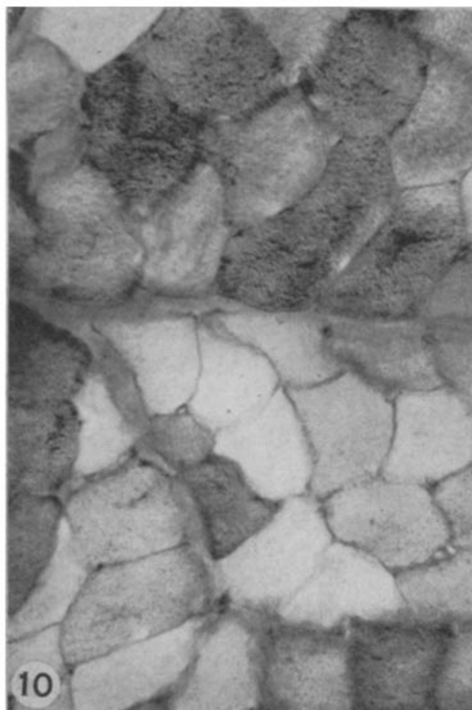
Fig. 9. Normal biceps femoris of the rat, cross-section, Pearse esterase method. Dense staining occurs at the motor end plate. Note greater esterase activity of the small fibers. Compare with Fig. 5. × 350.

Fig. 10. Normal biceps femoris of the rat, cross-section, periodic-acid Schiff method, frozen section. There is variation in glycogen deposition among fibers. × 100.









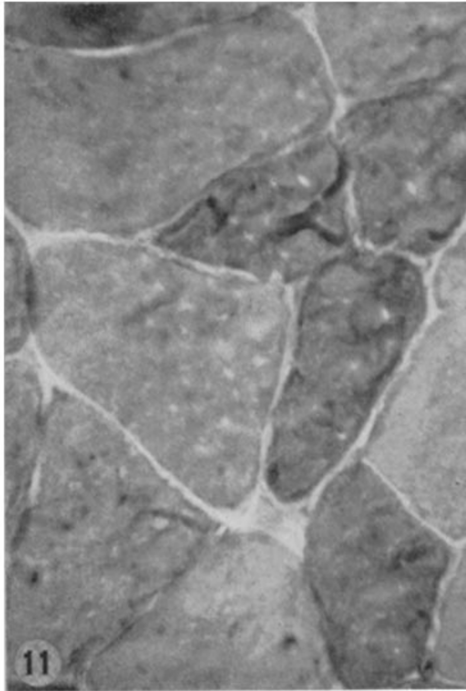
(Nachmias and Padykula: Normal and denervated rat muscles)

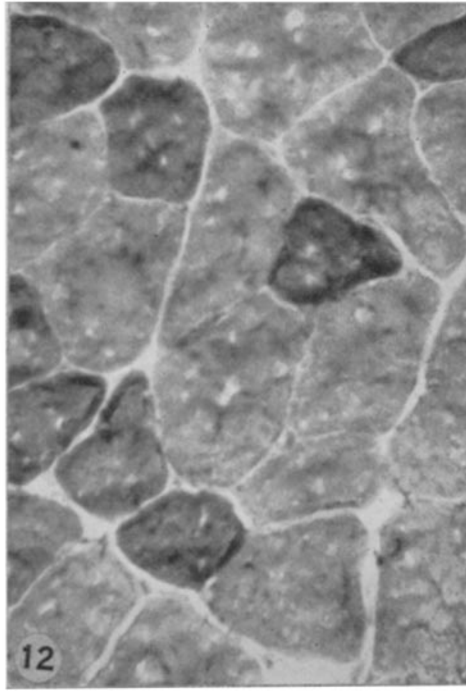
PLATE 20

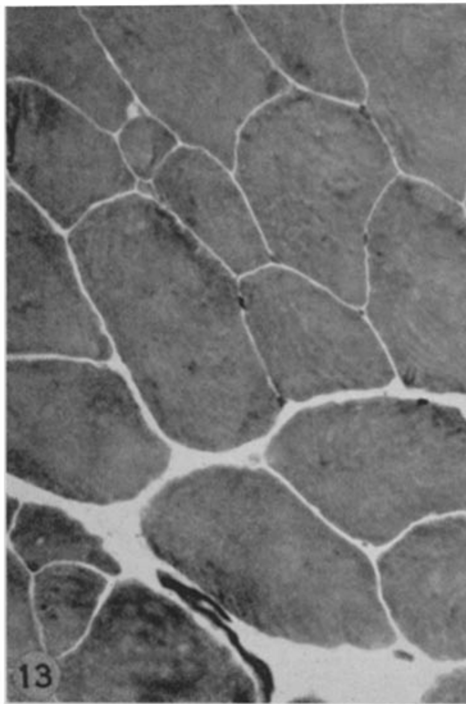
FIGS. 11 and 12. Normal soleus on the left, and soleus of the rat denervated for 14 days on the right, cross-sections, ATPase, 10 minutes incubation. These sections were incubated on the same slide. Note the difference in staining reaction of the fibers and compare with Fig. 1. In the denervated muscle, there is slight increase in intensity of staining as compared with the normal. × 440.

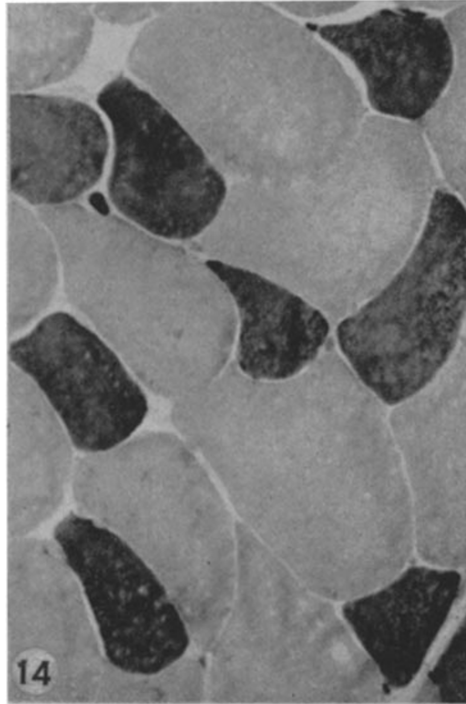
FIG. 13. Normal biceps femoris of the rat, cross-section, ATPase, 10 minutes incubation. Note the uniform staining and contrast with Figs. 9 and 5. × 350.

FIG. 14. Normal biceps femoris of the rat, cross-section. The ATPase of this section was first inactivated with para-chloromercuribenzoate in the presence of ATP (15 minutes), and subsequently the activity of the ATPase was restored with cysteine and ATP (30 minutes). Contrast with Fig. 13. × 350.









(Nachmias and Padykula: Normal and denervated rat muscles)