CYTOLOGICAL STUDIES OF FIBER TYPES IN SKELETAL MUSCLE

A Comparative Study of the Mammalian Diaphragm

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ABSTRACT

A comparative investigation of the mammalian diaphragm has revealed a correlation between certain cytological aspects of red and white muscle fibers and functional activity. This skeletal muscle presents the advantage of a similar and constant function among the mammals, but its functional activity varies in a quantitative manner. Both the rate of breathing (and hence the rate of contraction of the diaphragm) and metabolic activity are known to be inversely related to body size; and this study has demonstrated a relationship between cytological characteristics of the diaphragm and body size of the animal. Small fibers rich in mitochondria (red fibers) are characteristic of small mammals, which have high metabolic activity and fast breathing rates; and large fibers with relatively low mitochondrial content predominate in large mammals, which have lower metabolic activity and slower breathing rates. In mammals with body size intermediate between these two groups (including the laboratory rat), the diaphragm consists of varying mixtures of fiber types. In general, the mitochondrial content of diaphragm fibers is inversely related to body size. It appears, then, that the red fiber reflects a high degree of metabolic activity or a relatively high rate of contraction within the range exhibited by this muscle.

INTRODUCTION

Differences in skeletal muscle fibers comprising the diaphragm of the cat, albino rat, and wild rat have been reported (Bullard, 1919; Kuschinsky et al., 1956; Krüger and Günther, 1956; Ogata, 1958; Nachmias and padykula, 1958; George and Susheela, 1961; Padykula and Gauthier, 1963). Three different types of fibers can be distinguished in the diaphragm of the cat (Ogata, 1958) and of the albino rat (Padykula and Gauthier, 1963; Padykula and Gautheir, unpublished). These are small red fibers, large white fibers, and fibers with intermediate characteristics. Although the diaphragm is a mixed muscle in these species, the present study demonstrates that in a very small mammal, such as the shrew, or a very large mammal, such as the cow, this muscle is essentially homogeneous.

The functional significance of the heterogeneity of skeletal muscle fibers is, for the most part, unknown; but, in the diaphragm, it is possible to relate cytological differences to differences in functional activity. This muscle of respiration clearly has a continual and similar function in all species, but its activity among species varies in a

quantitative manner. The rate of breathing of mammals is known to be inversely related to body size. The respiratory rate of a mouse, for example, is 109 breaths per minute, while that of man is only 16 breaths per minute (Crosfill and Widdicombe, 1961). Metabolic rate is likewise inversely related to body size (Benedict, 1938; Krebs, 1950; Zeuthen, 1955). It has been suggested, furthermore, that variation in metabolic rate with body size involves the striated musculature in particular (Krebs, 1950). It might be expected, therefore, that the appearance of the diaphragm muscle fibers in mammals of different size would reflect quantitative differences in either the rate of breathing (and hence frequency of contraction of the diaphragm) or the general metabolism of the animal.

In order to investigate the relationship of cytochemical and ultrastructural features to muscle function, the diaphragm was studied in 36 different species of varying body size, using the albino rat as a standard for comparison. Our results indicate that a definite correlation exists between the fiber composition of the diaphragm and the body size of the animal. Although the diaphragm is considered to be a slow muscle, its rate of contraction (as judged by rate of breathing) varies significantly with body size; this variation appears to be reflected both in the cytological characteristics of the muscle fibers and in the relative proportions of fiber types comprising the diaphragm in all the species examined.

MATERIAL AND METHODS

Preparation of Tissue

Adult mammals representing 36 different species (Table I) were studied. Certain of the animals were weighed, but, when this was not possible, body weights represent estimates based on various published reports. In most cases, the animals were killed with chloroform. The diaphragm was exposed, and tied strips of muscle about 2 cm in length (depending on the size of the animal) were isolated midway between origin and insertion. Whenever possible, the specimens were taken from the right costal region. They were immediately frozen or fixed in formalin or osmium tetroxide. These procedures have been described in detail in an earlier paper (Padykula and Gauthier, 1963). Many of the small desert rodents were killed in the field, and the diaphragms were fixed in formalin at that time. Diaphragms of cow, steer, and pig were obtained from freshly slaughtered animals and fixed or frozen at the abattoir. Human diaphragm was obtained at autopsy.[1] Whenever possible, strips of diaphragm muscle were tied to a splint before fixing in order to prevent contraction. In those specimens which could not be tied, contracture was frequently observed.

Because it is known that the histochemical pattern varies in different parts of the diaphragm (George and Susheela, 1961), our examination was limited, wherever possible, to the right costal region in all species. In some species (albino rat, wild rat, Ord's kangaroo rat, great basin kangaroo rat, pale kangaroo mouse, deer mouse, and albino mouse), muscles of the hind limb (gracilis or semitendinosus) were prepared in a similar manner for comparison with the diaphragm.

Cytochemical Procedures for Demonstrating Mitochondria

LIPIDS: Muscle fixed in 10% neutral buffered formalin was washed, embedded in gelatin, and sectioned on a freezing microtome. Sections (5 to 10 μ) were stained with Sudan black and mounted in glycerogel. Control sections were extracted with acetone before staining.

MITOCHONDRIAL ENZYMES: Isolated strips of muscle were frozen in dry ice and ethanol at −70°C, and sections (5 to 10 μ) were cut in a cryostat. Succinic dehydrogenase activity was localized by the method of Nachlas et al. (1957), and mitochondrial ATPase was demonstrated at pH 7.2 under conditions described by Padykula and Gauthier (1963).

Electron Microscopy

Thin strips of muscle were fixed in 1% osmium tetroxide buffered to pH 7.5 with veronal-acetate, rapidly dehydrated, and embedded in Epon. Ultrathin sections were cut on a Porter-Blum or a Huxley ultramicrotome, stained with lead according to procedures of Karnovsky (1961) or Reynolds (1963), and then examined with an R.C.A. model EMU-3F microscope or a Siemens Elmiskop I. Thicker sections (about 2.0 μ) were stained with toluidine blue and examined with the light microscope.

Measurement of Fiber Area and Diameter

Images of transverse sections of Sudan black stained material were projected to achieve an enlargement suitable for measurement. The outlines of at least one-hundred muscle fibers from a given animal were traced, and planimetric measurements were made directly on the tracings. Measurements were then converted to obtain the actual cross-sec-

[1] Human material was obtained through the courtesy of Dr. Richard Lindquist, Department of Pathology, Peter Bent Brigham Hospital, Boston, Massachusetts.

TABLE I

Mammals Studied

Arranged in Order of Increasing Body Size

Common name	Scientific name	No. of animals	Body weight* Range	Body weight* Average
			kg	*kg*
Group A:				
Little brown myotis	*Myotis lucifugus*	2	0.005 0.009‡	—
Western Harvest mouse	*Reithrodontomys megalotis*	1	—	0.008
Little pocket mouse	*Perognathus longimembris*	2	—	0.008
Arizona pocket mouse	*Perognathus amplus*	3	0.007-0.014	0.011
Great Basin pocket mouse	*Perognathus parvus*	1	—	0.013
Big brown bat	*Eptesicus fuscus*	1	0.011-0.017‡	—
Pale kangaroo mouse	*Microdipodops pallidus*	2	0.013-0.018	0.015
Deer mouse	*Peromyscus maniculatus*	2	0.014-0.018	0.016
Desert pocket mouse	*Perognathus penicillatus*	1	—	0.017
Short-tailed shrew	*Blarina brevicauda*	5	0.012-0.023‡	—
Southern grasshopper mouse	*Onychomys torridus*	1	—	0.019
Boreal redback vole	*Clethrionomys gapperi*	1	—	0.020
Marsupial mouse	*Antechinus stuarti*	2	—	0.022
White-footed mouse	*Peromyscus leucopus*	1	0.012-0.031‡	—
Albino mouse (BUB strain)	*Mus musculus*	5	0.032-0.034	0.033
Ord kangaroo rat	*Dipodomys ordii*	2	0.040-0.046	0.043
Meadow vole	*Microtus pennsylvanicus*	1	0.020-0.070‡	—
Great Basin kangaroo rat	*Dipodomys microps*	2	0.052-0.065	0.058
Group B:				
Eastern chipmunk	*Tamias striatus*	2	—	0.07†
Golden hamster	*Mesocricetus auratus*	2	0.097-0.113‡	—
Whitetail antelope squirrel	*Citellus leucurus*	2	—	0.111
Desert woodrat	*Neotoma lepida*	1	—	0.190
Albino rat	*Rattus norvegicus*	14	0.300-0.400	0.350
Grey squirrel	*Sciurus carolinensis*	1	0.340-0.681‡	—
Guinea pig	*Cavia cobaya*	1	—	0.837
Brush-tail possum	*Trichosurus vulpecula*	2	1.97-3.24	2.60
Cat	*Felis catus*	1	2.5-4.0‡	—
Opossum	*Didelphis virginiana*	4	1.81-5.45‡	—
Dog	*Canis familiaris*	1	3.64-7.86‡	—
Albino rabbit	*Oryctolagus cuniculus*	4	3.63 9.09‡	—
Beaver	*Castor canadensis*	1	13.6-27.3‡	—
Grey kangaroo	*Macropus canguru*	2	40-46	43.0
Man	*Homo sapiens*	1	68-91‡	—
Sheep	*Ovis aries*	1	—	79.5
Group C:				
Pig	*Sus scrofa*	1	—	100
Cow, steer	*Bos taurus*	3	363-659	492

* Unless otherwise designated, body weight represents actual weight of the animals studied.

‡ Range or average of body weight expected for the species or variety (e.g. dog) obtained from various sources.

tional areas of the fibers, and fiber diameter was calculated. Data presented in Table II represent measurements from a typical member of each species. Fiber diameters for shrew and albino rat represent an average measurement from two animals of each species; for cow and steer, three animals; and for rabbit, four animals.

RESULTS

Rationale for the Use of Sudan Black to Distinguish Fiber Types

Fiber types in the albino rat diaphragm can be identified in cytological preparations which demonstrate mitochondria (Padykula and Gauthier, 1963). In the present investigation, staining with Sudan black was especially suitable for this purpose because specimens obtained in the field or from the abattoir could be preserved in formalin and, at a later time, embedded in gelatin and stained. When sections are treated with acetone before staining, triglyceride droplets are extracted, but phospholipid components of structures such as mitochondria remain (Figs. 1 to 4). The pattern of fiber distribution in a given specimen remains the same after acetone extraction, since mitochondria predominate in fibers of small diameter. The same pattern exists also when the mitochondrial enzymes, succinic dehydrogenase and adenosine triphosphatase, are localized by histochemical procedures or when the fibers are observed with the electron microscope (Fig. 5).

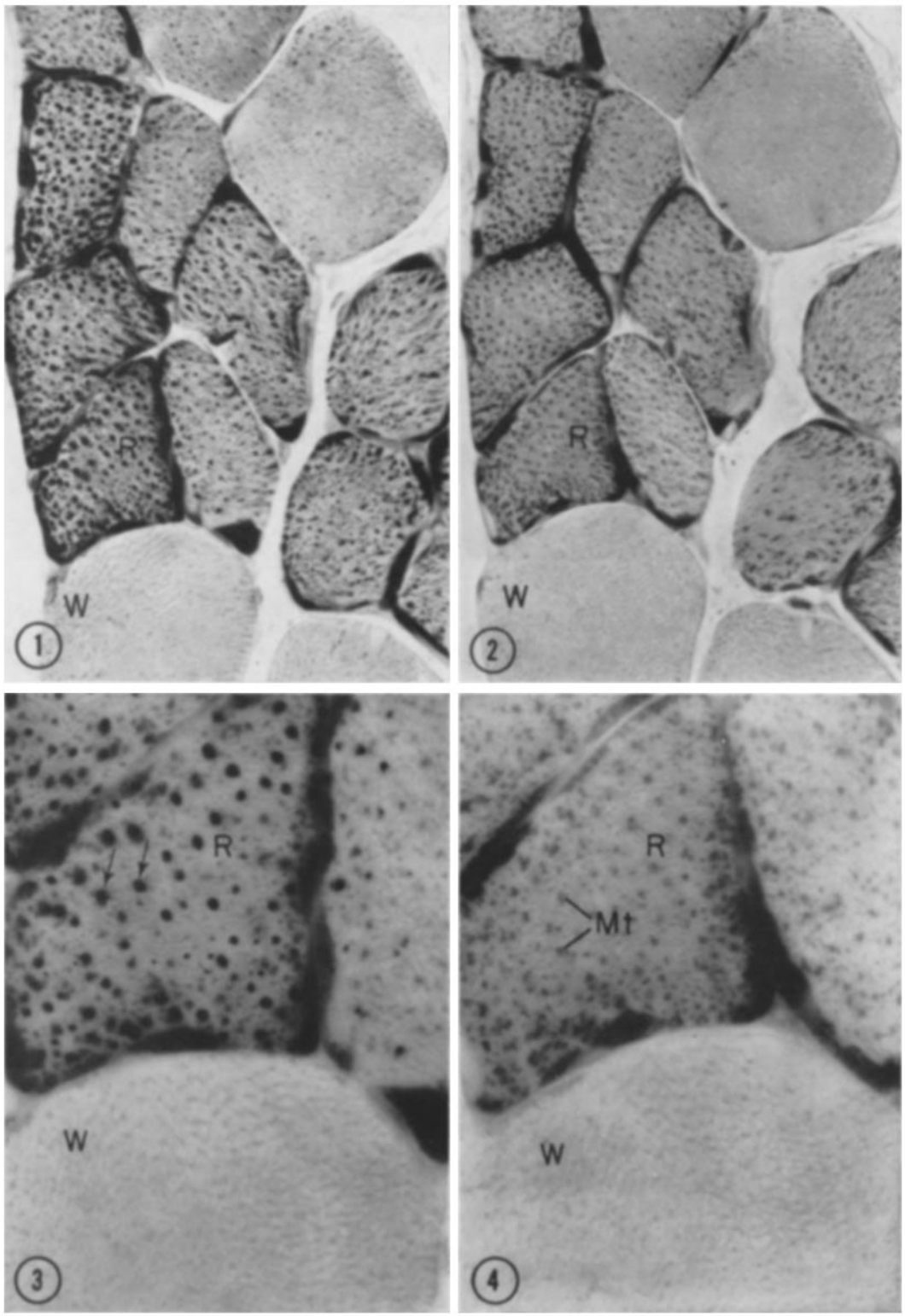
Furthermore, triglyceride droplets can be distinguished from mitochondria after staining with Sudan black without acetone extraction since they are intensely black, while mitochondria are a lighter gray color (Figs. 3 and 4). Sudan black can be used, therefore, to demonstrate the distribution of mitochondria in the diaphragms of different species (Fig. 8 *a* to *h*). The amount of triglyceride may vary among the members of a given species, according to physiological state, but the mitochondrial content appears to be constant. *Mitochondrial "content" is used throughout this study to express mitochondrial mass relative to the amount of myofibrillar material; this is a visual estimate and does not represent a mathematical analysis.*

Principal Cytological Characteristics of the Fibers of the Rat Diaphragm

The majority of the fibers in the rat diaphragm correspond to classical descriptions of red and white fibers, also referred to as dark and light fibers. Small red fibers comprise about 60% of the fibers in this muscle. 20% are classified as large

FIGURES 1 to 2 Diaphragm, albino rat. Adjacent transverse sections, Sudan black. In Fig. 1, small red fibers (*R*) are darker than large white fibers (*W*) because of a high concentration of both triglyceride droplets and mitochondria. In Fig. 2, the sections were treated with acetone before staining. Triglyceride droplets are extracted, but phospholipid components of structures, such as mitochondria, remain. The patterns of fiber distribution in Figs. 1 and 2 are the same, since mitochondria predominate in the small red fibers, and form conspicuous aggregations at the periphery. The same pattern exists when the mitochondrial enzymes, succinic dehydrogenase and adenosine triphosphatase, are demonstrated. Staining with Sudan black can, therefore, be used to demonstrate the distribution of mitochondria in muscle fibers of the diaphragm. × 625.

FIGURES 3 to 4 Same as Figs. 1 and 2, but the higher magnification permits identification of mitochondria and triglyceride droplets. In Fig. 4, intensely black triglyceride droplets (arrows in Fig. 3) have been extracted with acetone, but the lighter gray mitochondria (*Mt*) remain. Both circular and filamentous mitochondrial profiles are apparent in the red fibers (*R*) while only filamentous profiles are apparent in the white fiber (*W*). Conspicuous subsarcolemmal aggregations of mitochondria are evident in red fibers in both Figs. 3 and 4. In Fig. 3, mitochondria and triglyceride droplets (arrows) can be distinguished because of a color difference after staining. Note that no triglyceride droplets are present in the large white fiber, and thus the fiber appears the same in both photographs. Compare with Fig. 5. × 1,200.


R
W
1
R
W
2
R
W
3
R
Mt
W
4

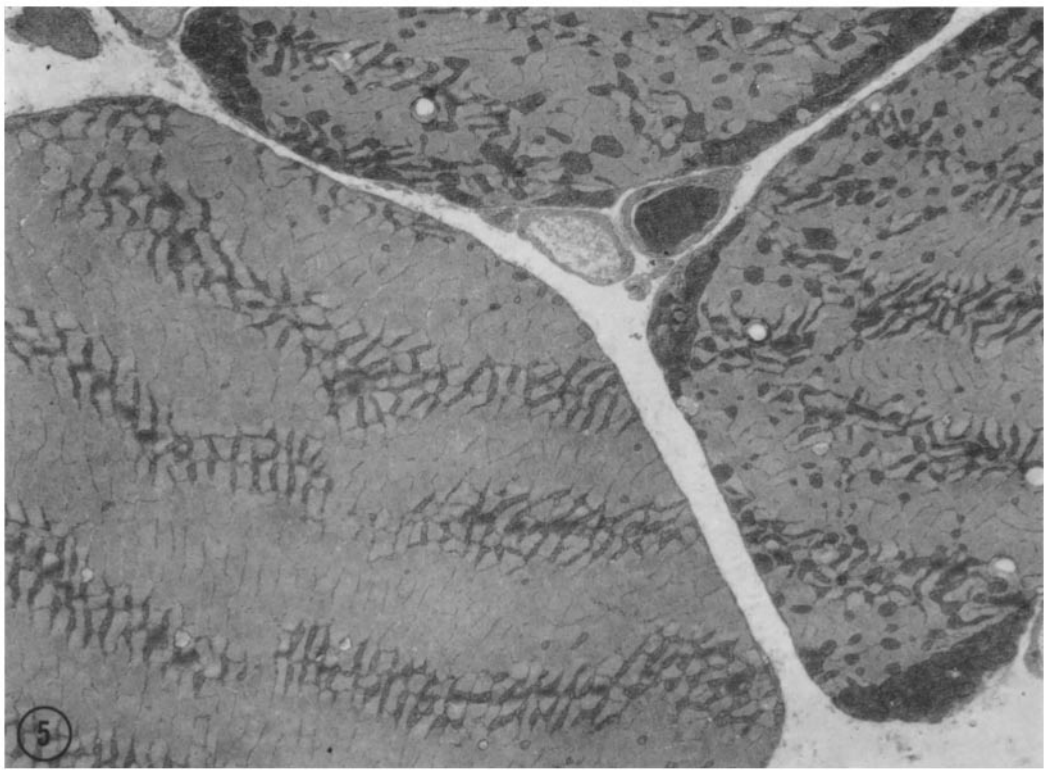
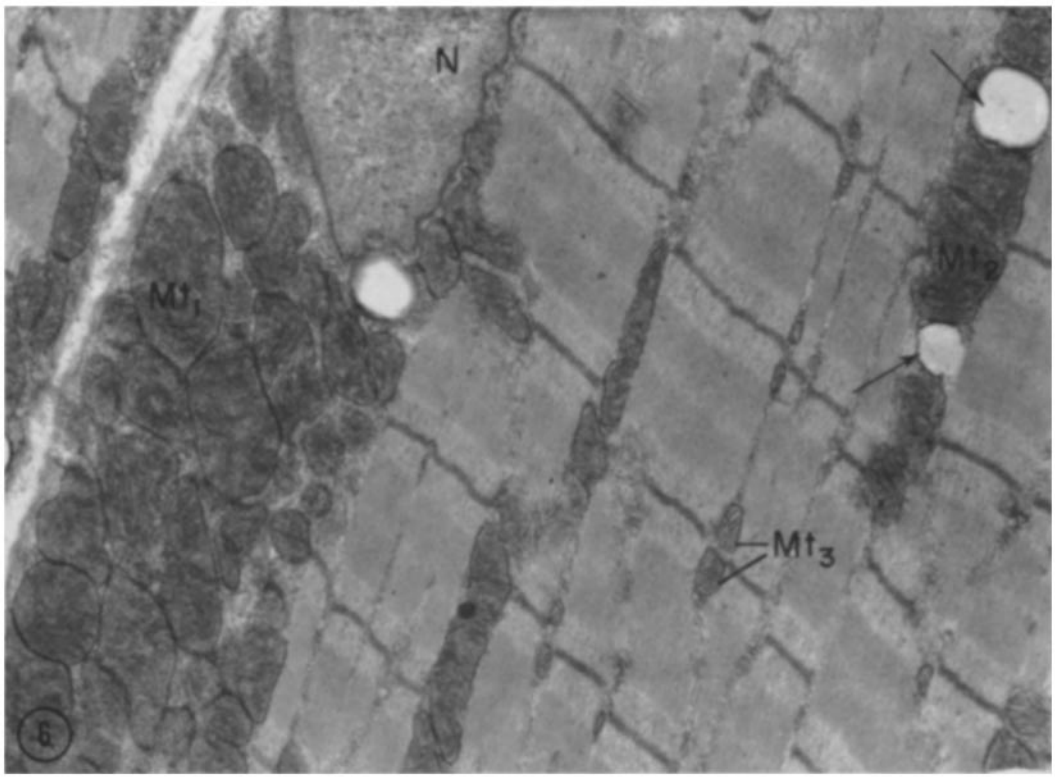
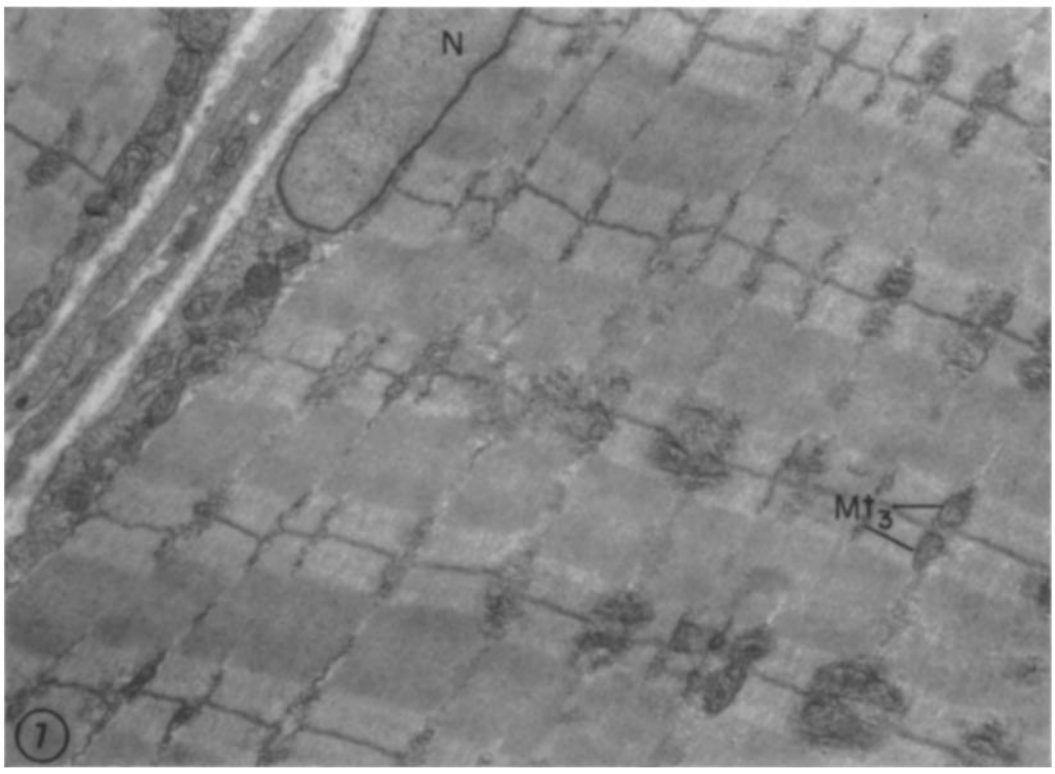


FIGURE 5 Diaphragm, albino rat. This low magnification electron micrograph, in nearly transverse section, shows portions of two red fibers at the upper right and one white fiber at the lower left. Mitochondria predominate in the two red fibers. Circular profiles of large, more or less spherical mitochondria form conspicuous subsarcolemmal aggregations in the red fibers. Both circular and filamentous profiles are apparent in the interior of these two red fibers. These mitochondrial profiles represent transverse sections through longitudinal chains and transverse branches, respectively (see Figs. 6 and 7). In the white fibe small filamentous mitochondria predominate (compare with Figs. 3 and 4). × 2,000.

FIGURES 6 to 7 Diaphragm, albino rat. These electron micrographs of longitudinal sections through a red (Fig. 6) and a white (Fig. 7) fiber are of equal magnification, and both represent the nuclear region of the fiber. In the red fiber, large mitochondria are aggregated beneath the sarcolemma (Mt_1), and form chains (Mt_2) between myofibrils. Triglyceride droplets (arrows) are often closely associated with these large mitochondria in particular. In both types of fibers, paired mitochondria are aligned with the I bands (Mt_3). These elliptical profiles represent sections through filamentous mitochondria which encircle the myofibrils transversely (see Figs. 4 and 5). In the white fiber, mitochondria are sparse, and cristae are less abundant than in the red fiber. Mitochondria are, for the most part, filamentous in form, and they encircle myofibrils transversely at the I bands (Mt_3). Triglyceride droplets and chains of large mitochondria are absent. Note that in the nuclear region, where subsarcolemmal aggregations of large mitochondria tend to occur in red fibers, there are only a few small mitochondria. × 8,800.


N
Mt1
Mt2
Mt3
6


N
Mt3
7

white fibers, and 20% as intermediate fibers. A detailed analysis of fiber types in the rat diaphragm will be presented in a separate report. Only certain of the cytological features are presented here as a standard for comparison.

When stained with Sudan black, red fibers are darker than white fibers because of a high concentration of both triglyceride droplets and mitochondria (Figs. 1 to 4). A conspicuous feature of the red fiber is the accumulation of large mitochondria beneath the sarcolemma. This peripheral aggregation imparts a rimmed appearance to transverse sections of this type of fiber. Subsarcolemmal mitochondria in the red fiber are large and more or less spherical (Fig. 22), and thus usually appear as circular profiles in electron micrographs (Figs. 5 and 6). In the interior of the fiber, large mitochondria form chains running longitudinally among myofibrils (Fig. 6). Filamentous branches extend transversely, encircling the myofibrils at the I bands. In transverse sections stained with Sudan black, these mitochondria appear as granules and delicate filaments, respectively, in the interior of the fiber (Fig. 4). The large mitochondria, in particular, are often closely associated with triglyceride droplets (Fig. 6).

In the white fiber, triglyceride droplets are rare (Figs. 1 and 3) and mitochondria are consistently thin, filamentous, and contain fewer cristae than do those of the red fiber (Figs. 3 and 7). Subsarcolemmal accumulations and longitudinal chains of mitochondria are usually absent.

A third type of fiber, which is intermediate in size, has cytological characteristics intermediate between those of red and white fibers. Subsarcolemmal aggregations of mitochondria occur, but are less conspicuous than in red fibers. In general, mitochondrial content is lower than that of the red fiber, but greater than that of the white fiber.

In all three types of fibers, transverse mitochondria at the I bands are associated intimately with terminal cisternae of the sarcoplasmic reticulum. Differences exist in the sarcoplasmic reticulum of typical red and white fibers. In both red and white fibers the sarcoplasmic reticulum has a distribution similar to that found in many other mammalian skeletal muscle fibers, but in the red fiber a dense network of tubules is present in the H-band region, while in the white fiber a broad transverse cisterna occupies the same position.

Comparative Cytological Features of the Diaphragm in Various Mammalian Species

GENERAL APPEARANCE: When the cytological characteristics of the diaphragm of the albino rat are compared with those of the diaphragms of various species ranging in body size from 5 g to 659 kg, it becomes apparent that the heterogeneous structure of this diaphragm is representative of a group of mammals of intermediate body size. The diaphragm in smaller mammals is homogeneous and consists entirely of small fibers with abundant mitochondria. As body size increases, the diaphragm becomes heterogeneous, and fibers of larger diameter become more frequent. The component fibers of the heterogeneous muscle differ in both size and mitochondrial content, as in the rat. In very large mammals, fibers of large diameter with a low mitochondrial content in relation to myofibrillar mass predominate. Fig. 8 illustrates the diaphragms of eight representative mammals arranged in order of increasing body size. These photographs are of equal magnification. The diaphragm of the harvest mouse and shrew exemplify the appearance of this muscle in the small mammals listed in Group A, Table I. They consist entirely of typical red fibers with the usual rich subsarcolemmal accumulations of mitochondria. These fibers are even smaller than red fibers of the albino rat diaphragm. Although the albino mouse diaphragm is somewhat more heterogeneous with respect to mitochondrial content, the fibers are, nevertheless, uniformly small and generally rich in mitochondria. In the albino rat and grey squirrel, fibers are larger and differences in both fiber size and mitochondrial content are obvious. These species are representative of mammals of intermediate size (Group B, Table I). In the cat and in man, fibers tend to be quite large, more uniform in size, mitochondrial content is lower than in the rat and squirrel, and subsarcolemmal accumulations of mitochondria are less prominent. Because of the heterogeneous pattern of the diaphragm of the cat and man, these species are included with the intermediate group. In the cow (Group C, Table I) all fibers are large, mitochondria are sparse, and subsarcolemmal accumulations of mitochondria are thin and occur infrequently. In specimens in which enzymic activity was demonstrated, the localization of the mitochondrial enzymes, succinic dehydrogenase

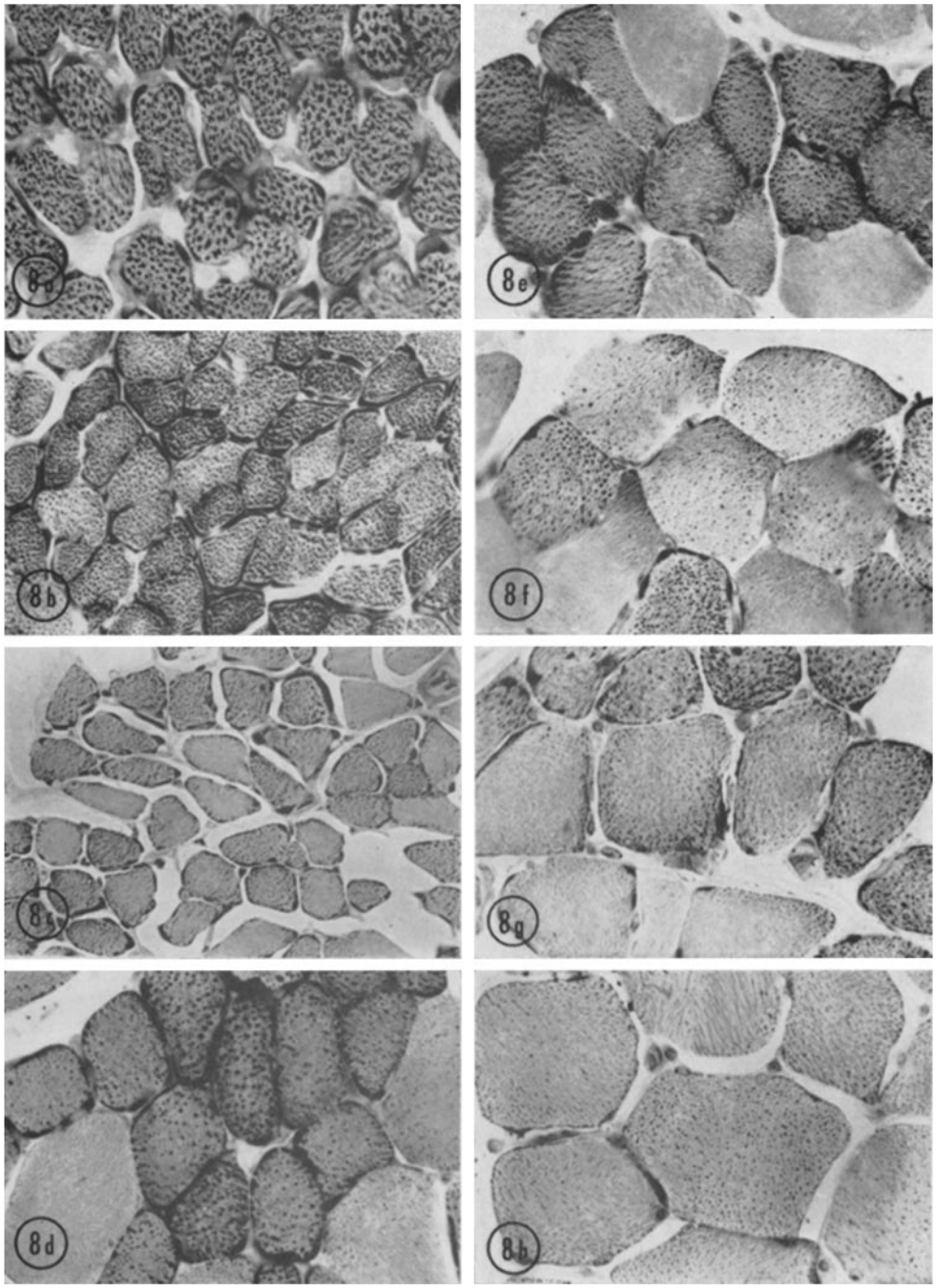


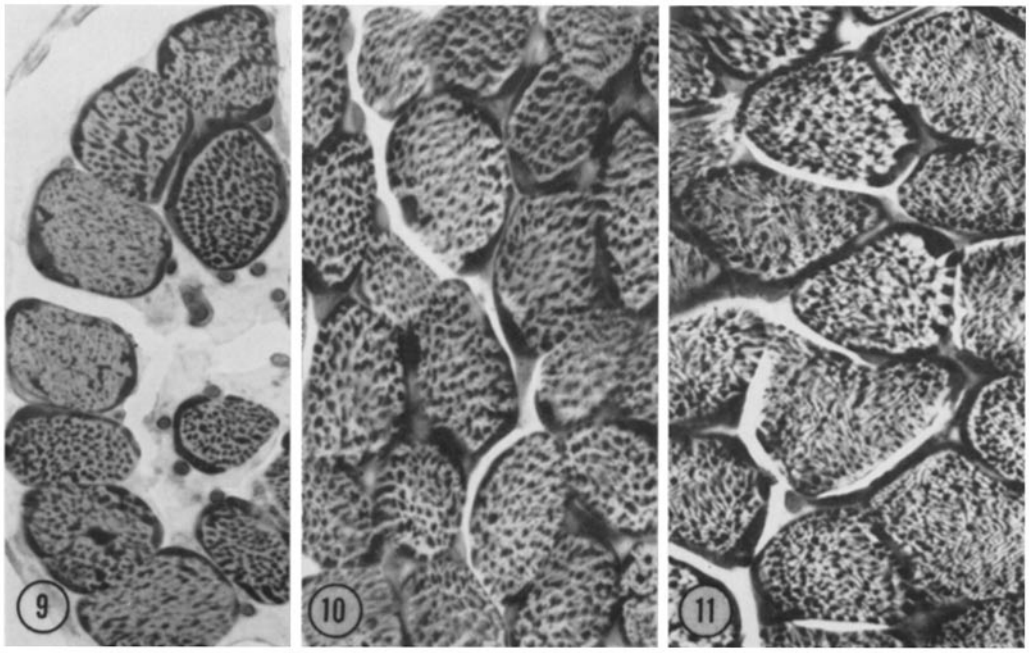
FIGURE 8 Diaphragm, Sudan black. *A*, harvest mouse, *B*, shrew, *C*, albino mouse, *D*, albino rat, *E*, squirrel, *F*, cat, *G*, man, *H*, cow. All are transverse sections photographed at the same magnification, and arranged in order of increasing body size. Fiber types are distinguished on the basis of differences in sudanophilia, as demonstrated in Figs. 1 to 5. In the smallest mammals, such as the harvest mouse and shrew (*A*, *B*), fibers are uniformly small and rich in mitochondria. As body size increases, fiber diameter increases, and the diaphragm becomes heterogeneous with respect to both fiber diameter and mitochondrial content. The albino rat diaphragm (*D*) exemplifies this type of mixed muscle. In still larger mammals, fibers of large diameter and low mitochondrial content become more frequent. In the cow (*H*), fibers are almost entirely large, and mitochondrial content relative to myofibrillar mass is low. Compare with Table II. × 350.

and adenosine triphosphatase, confirmed the pattern of mitochondrial distribution observed after staining with Sudan black.

These groups represent an arbitrary division of animals arranged in progressive order according to body size but separated at points at which heterogeneity of the diaphragm begins and ends (Table I). Very small and very large animals have uniformly small and large fibers, respectively (Table II). Animals of intermediate size, on the other hand, have a more heterogeneous population of fibers, and the general appearance of the diaphragm among members of this group is more variable. This relationship of fiber type to body size is apparent not only in the Eutherian mammals illustrated in Fig. 8, but also in a sample of Metatheria (opossum, brushtail possum, marsupial mouse, grey kangaroo).

To determine whether this trend is peculiar to diaphragm muscle, certain hind limb muscles were examined as well. A similar trend toward greater differences in fiber types with increase in body size is apparent in hind limb muscles of the seven mammals examined. However, although fibers of the five small mammals are clearly smaller than the fibers of the same muscles from the two larger mammals, a heterogeneous pattern is observed even in the smallest mammals.

SMALL MAMMALS: Muscle fibers from the diaphragm of mammals weighing between 5 and 65 g (Table I) are strikingly similar. Figs. 9 to 11 illustrate the fibers of three representative species from this group, a bat, pocket mouse, and kangaroo rat. It is readily apparent that the fibers comprising these diaphragms are uniformly small. Fibers of similar small mammals listed in Table II have an average diameter of about 22 μ. All fibers are rich in mitochondria, and triglyceride droplets are usually abundant also. Occasionally, there is an apparent variation in fiber type, but this is usually the result of a difference in triglyceride content of the fibers. For example, in the



FIGURES 9 to 11 Diaphragm, bat (Fig. 9), pocket mouse (Fig. 10), kangaroo rat (Fig. 11). All are transverse sections stained with Sudan black and photographed at the same magnification. In these three representative small mammals the diaphragm muscle is similar in structure. In all three animals the fiber population is homogeneous. All fibers are small and uniformly rich in mitochondria, and usually have a high content of triglyceride droplets. Note the conspicuous subsarcolemmal rims of mitochondria. These small fibers resemble red fibers of the albino rat diaphragm but are even smaller and the mitochondrial content is higher. Compare with Fig. 1, which is the same magnification. × 625.

photomicrograph of pocket mouse diaphragm (Fig. 19), only certain of the fibers contain triglyceride droplets, but all are rich in mitochondria. The diaphragm of the albino mouse may seem to be an exception to this grouping, because certain fibers have a lower mitochondrial content than other fibers and the subsarcolemmal accumulations of mitochondria may be less conspicuous (Fig. 8 *c*). However, fibers are uniformly small, and mitochondria are not so sparse as in the large white fibers of the albino rat. This species, therefore, resembles other small mammals in Group A, with respect to fiber size and over-all abundance of mitochondria.

An outstanding characteristic of the fibers in the diaphragm of small mammals is the abundance of large mitochondria widely distributed throughout the sarcoplasm (Figs. 9 to 11). Extensive aggregations of large spherical mitochondria are present beneath the sarcolemma of all fibers (Figs. 12, 19, 20). Oxidative machinery is thereby heavily concentrated beneath the plasma membrane of these muscle cells, and most likely imparts special properties to the cell surface. In addition, large spherical or elongated mitochondria form longitudinal chains between myofibrils (Fig. 12). These are similar to chains of mitochondria present in red fibers of the rat diaphragm but are even more abundant. They may be continuous along a considerable length of the interfibrillar space except for the presence of numerous interspersed triglyceride droplets which are crowded between these mitochondria and are closely associated with them. As in the red fibers of the albino rat diaphragm, these droplets occur close to the I-band region of the myofibril. Smaller elliptical profiles of mitochondria are aligned as pairs on either side of the Z line, and these represent sections through long braceletlike mitochondria which encircle myofibrils transversely at the I-band level (Fig. 13). The elliptical configuration of these profiles (Fig. 12) indicates that the mitochondria are flattened against the myofibrils which they encompass. These mitochondrial bracelets arise as branches from the longitudinally oriented chains of mitochondria. This is especially apparent when seen in transverse sections of the fibers. Attenuated projections of a large mitochondrion partially encircle myofibrils in the region of the I band in Fig. 15. It appears, then, that transverse bands of mitochondria are, at certain points, continuous with longitudinally oriented mitochondria, as in the rat. In all species examined with the electron microscope (bat, shrew, pocket mouse), the mitochondria contain abundant closely packed cristae (Figs. 12 to 14). These cristae appear to consist of fenestrated sheets as well as of tubules and prisms (Fig. 14). Long fingerlike profiles are interpreted as perpendicular sections through the sheets or longitudinal sections through tubules. Small circular and angular profiles most likely represent transverse sections through tubules and prisms, as described in other tissues (Revel, Fawcett, and Philpott, 1963).

The sarcoplasmic reticulum has been examined in the bat, pocket mouse, and shrew. Its form and distribution resembles closely that of the red fiber of the albino rat diaphragm. Triads are precisely aligned with large braceletlike mitochondria encircling the myofibrils, and terminal cisternae are connected through the region of the A and H bands by an elaborate system of tubules resembling that of the red fiber (Fig. 13).

The fibers comprising the diaphragm of small mammals are similar in many respects to red fibers of the rat diaphragm, but the characteristics of "redness" are exaggerated in the fibers of these small mammals. The fibers themselves are even smaller, and they tend to have a much higher content of mitochondria relative to myofibrillar mass; and the mitochondria are, for the most part, larger than in red fibers of the rat diaphragm.

AN INTERMEDIATE GROUP OF MAMMALS: The intermediate group of mammals listed in Table I (Group B) includes a broad range of body size (70 g to 80 kg), and considerable variation exists in the appearance of the diaphragm. However, in all cases, the diaphragm is heterogeneous with respect to fiber type, and thus resembles that of the albino rat (Figs. 16 to 18; Fig. 8 *d* to *g*). Diaphragms of the various species in this group differ primarily in the degree of heterogeneity. The opossum diaphragm, for example, has less prominent subsarcolemmal aggregates of mitochondria than does the rabbit diaphragm, and, in addition, differences among fiber types are somewhat less striking than in the rabbit (Figs. 17 and 18). Despite considerable variation in fiber size among members of this group, the average diameter is, in most cases, intermediate between that of the small and large mammals. The average fiber diameter for the intermediate mammals in Table II is 32 μ.

For the most part, small fibers tend to have a higher mitochondrial content than do larger fibers

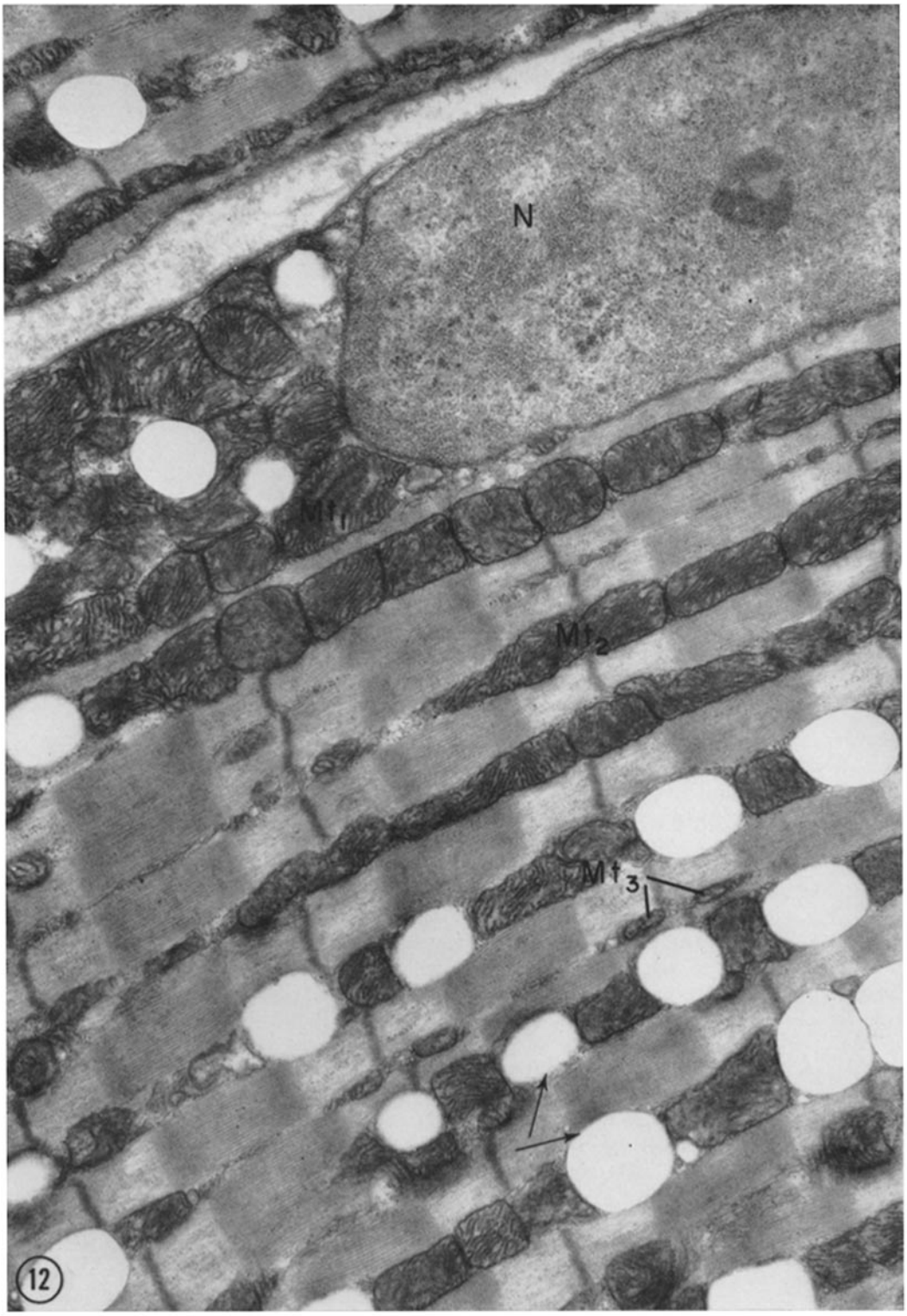


FIGURE 12 Diaphragm, shrew, longitudinal section. This electron micrograph illustrates the typical appearance of the fibers in the diaphragm of small mammals. Large mitochondria (Mt_1) are accumulated beneath the sarcolemma near the nucleus (N), and chains of large mitochondria (Mt_2) are especially prominent. These large mitochondria have abundant closely packed cristae. Numerous triglyceride droplets (arrows) are crowded among the mitochondria, and tend to be aligned close to the I bands. Small elliptical profiles of mitochondria (Mt_3) represent filamentous pairs which encircle the myofibrils at the I bands. × 13,000.

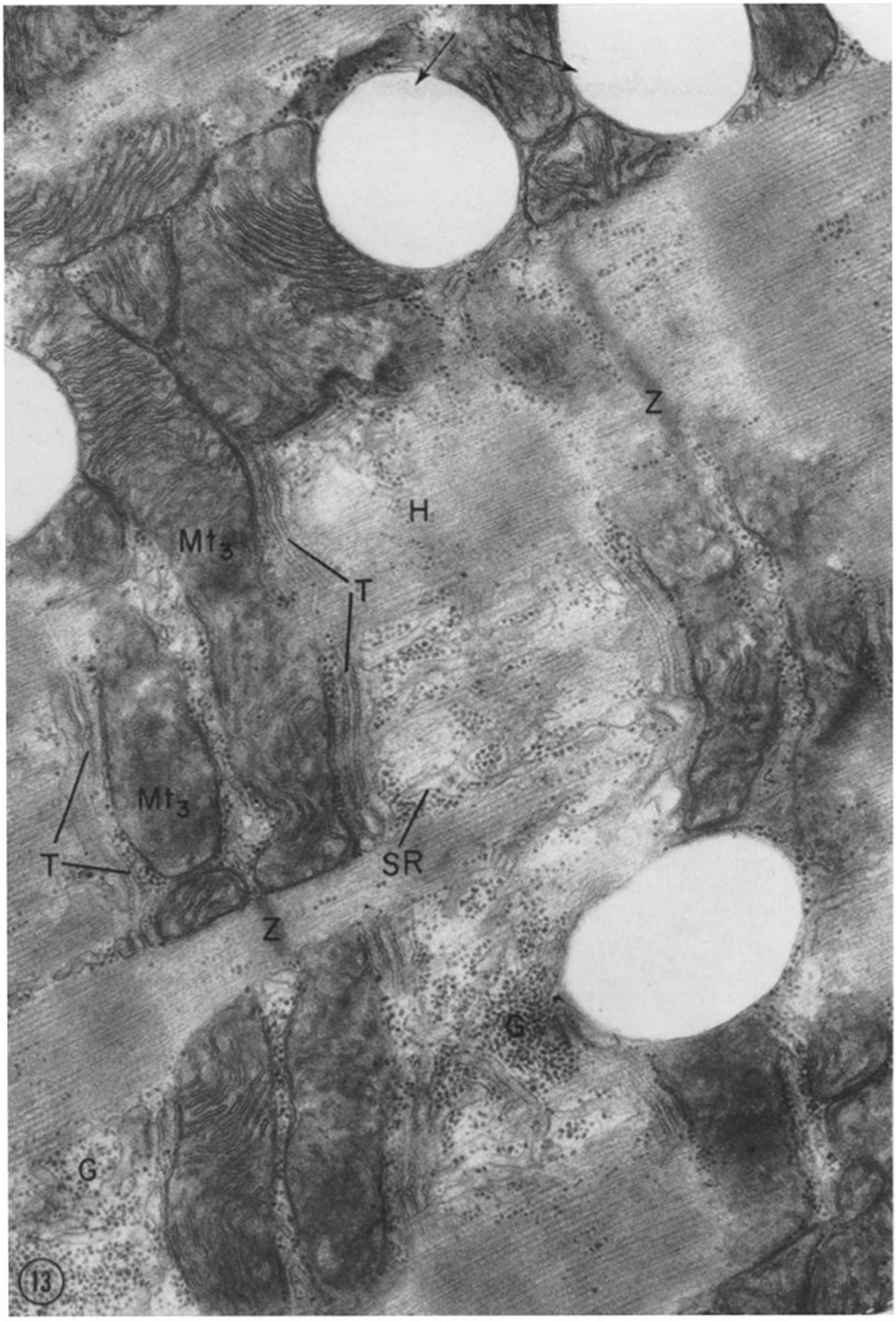


FIGURE 13 Diaphragm, shrew. This electron micrograph shows the appearance of a fiber sectioned longitudinally and, in part, tangential to the surface of the myofibrils. Paired ribbonlike mitochondria (*Mt*$_3$), seen as elliptical profiles in Fig. 12, traverse the myofibrils and are intimately associated and aligned with triads (*T*) of the sarcoplasmic reticulum. Extending from the triads, longitudinal tubular elements of the sarcoplasmic reticulum (*SR*) give rise to a complex network in the region of the H band (*H*). Glycogen particles (*G*) are especially abundant in the intervening sarcoplasmic spaces. Large triglyceride droplets (arrows) are located near the A-I junctions. × 29,500.

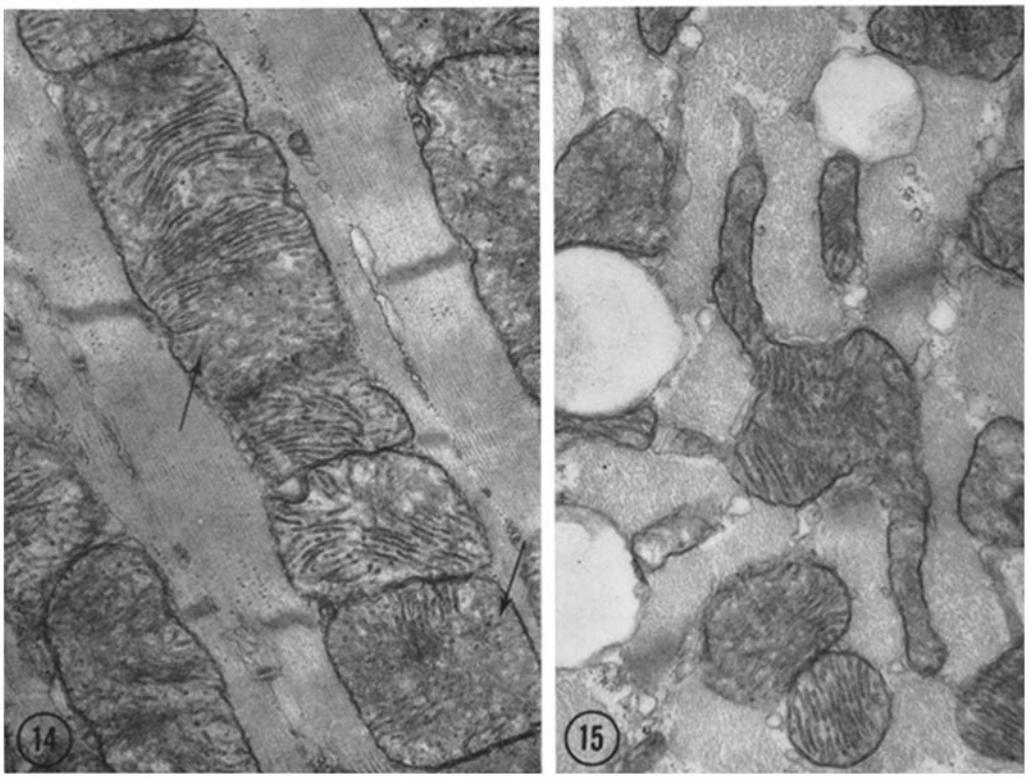
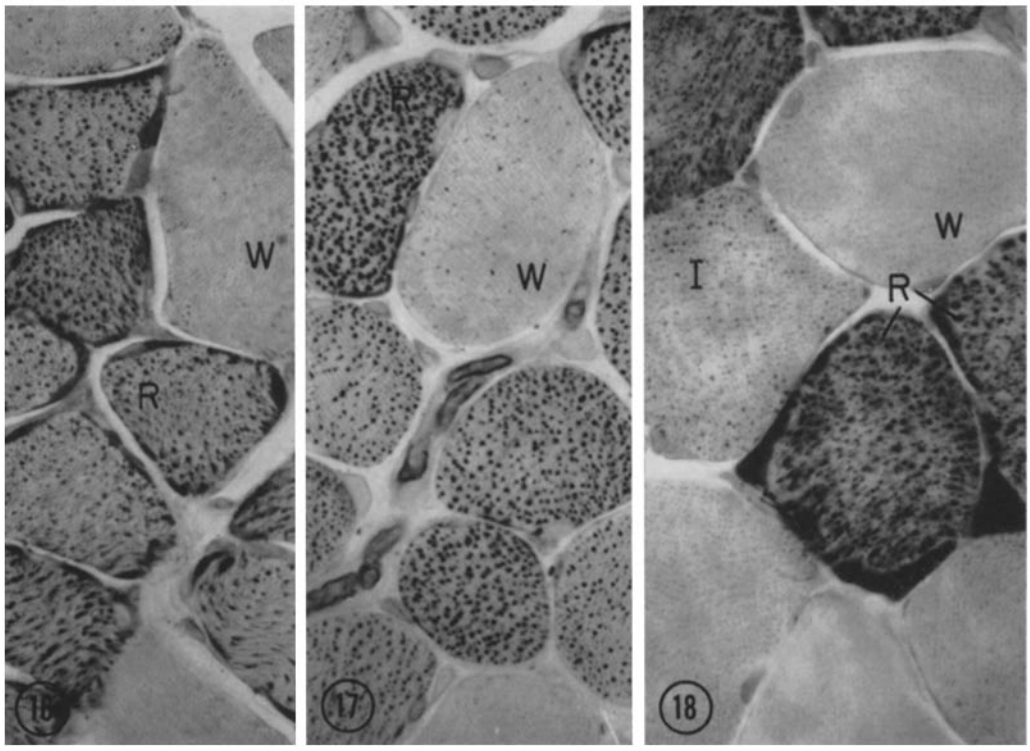


FIGURE 14 Diaphragm, bat. This electron micrograph shows the typical appearance of chains of large mitochondria which run longitudinally between myofibrils in all fibers of the small mammals studied. Note especially the abundance of closely packed cristae. In some areas the plane of section is such that the cristae (arrows) appear as fenestrated sheets, and many of the nearby paired membranes are interpreted as perpendicular sections through these sheets. Circular and angular profiles, interpreted as transverse sections through tubules and prisms, are present also, but are difficult to see at this magnification. × 22,000.

FIGURE 15 Diaphragm, shrew. This electron micrograph of a nearly transverse section illustrates the branching nature of large mitochondria at the level of the I bands. The mitochondrion at the center of the photograph has two conspicuous extensions. These form the braceletlike mitochondria which encircle the myofibrils in pairs at this level. Similar branches are seen traversing the myofibrils in Fig. 13 and are sectioned in profile in Fig. 12. × 24,000.

in the same species, and in general they are richer in triglyceride than are large fibers. Usually, large spherical mitochondria are accumulated in distinct masses beneath the sarcolemma (Fig. 21), and appreciable numbers of large mitochondria are present in the interior of the fiber, scattered among smaller, more filamentous profiles. These small fibers are equivalent, therefore, to red fibers of the rat diaphragm. Mitochondria in the large fibers are usually filamentous throughout, and form only scant subsarcolemmal aggregations (Figs. 16 to 18). These large fibers appear to be equivalent to white fibers of the rat diaphragm. Fibers with intermediate characteristics similar to those of intermediate fibers in the rat are especially apparent in the rabbit diaphragm (Fig. 18).

The fiber types differ to varying degrees among species, as, for example, in the opossum and rabbit (Figs. 17 and 18). In some species (the whitetail antelope squirrel, for example), large mitochondria are conspicuous in all fibers, but are especially abundant in the small fibers (Fig. 21). Fibers differ markedly in size, and those of large diameter do not possess subsarcolemmal accumulations of



FIGURES 16 to 18 Diaphragm, woodrat (Fig. 16), opossum (Fig. 17), rabbit (Fig. 18). All are transverse sections stained with Sudan black and photographed at the same magnification as Figs. 9 to 11. In all three animals the fiber population is heterogeneous, and in this respect these diaphragms resemble the albino rat diaphragm (Fig. 1). In general, small fibers (*R*) are richer in mitochondria and triglyceride droplets than are larger fibers in the same species. In the small fibers, large spherical mitochondria are usually accumulated in distinct masses beneath the sarcolemma, and large mitochondria are scattered among smaller more filamentous profiles in the interior of the fibers, as in the red fibers in the albino rat. In the larger fibers (*W*), mitochondria are usually filamentous throughout, and form very scant subsarcolemmal aggregations, if any. These fibers appear to be equivalent to white fibers of the albino rat diaphragm. One of the fibers (*I*) in Fig. 18 has intermediate characteristics. × 625.

mitochondria. The fiber types in this squirrel are, therefore, similar to red and white fibers of the rat diaphragm, but, as with the opossum, heterogeneity is less striking than in other members of the group. While the diaphragm of the opossum tends to be "whiter," that of the whitetail antelope squirrel tends to be "redder" than the rat diaphragm.

Ultrastructural details of the fibers in this group of mammals were studied only in the albino rat.

LARGE MAMMALS: Muscle fibers of the diaphragm of cow, steer, and pig (Table II) are almost entirely of large diameter (about 52 μ). Mitochondrial content is relatively low, and triglyceride droplets are usually sparse or absent (Figs. 23 and 25). Occasional subsarcolemmal accumulations of mitochondria are seen, but these are thin and relatively inconspicuous. Even when sectioned through the nuclear region of the fiber, which is the expected site of such accumulations, they are often absent (Fig. 23). When present, however, mitochondria comprising them are usually small and contain only few to moderate numbers of cristae. Mitochondria in the interior of the fibers are almost entirely small and filamentous (Fig. 23). When seen in longitudinal section, small elliptical profiles are aligned on either side of the Z line (Fig. 27). These represent sections through braceletlike mitochondria encircling the I bands of the myofibrils, as demonstrated in other species. Longitudinal chains of mitochondria are infrequent. In general, cristae are not abundant in the

TABLE II

Comparative Dimensions of Skeletal Muscle Fibers of the Diaphragm

The relationship of fiber diameter to body size, metabolic activity, and rate of breathing

Fiber diameter			Heat production (from Benedict, 1938, *Carnegie Inst. Publ. No. 503*)			Breathing rate (from Crosfill and Widdicombe, 1961, *J. Physiol.*, **158**, 1–14)		
Species	Body weight	Average fiber diameter	Species	Body weight	Cal/kg/24 Hr	Species	Body weight	Breaths/Min.
	kg	*μ*		*kg*			*kg*	
Harvest mouse	0.008	24	Mouse	0.021	158	Mouse	0.032	109
Pocket mouse	0.008	25	Rat	0.400	82	Rat	0.25	97
Shrew	0.012–0.023	18	Guinea pig	0.410	85	Guinea pig	0.69	42
Albino mouse	0.033	18	Rabbit	2.6	44.5	Rabbit	2.4	39
Albino rat	0.350	34	Marmot	2.65	28.0	Monkey	2.45	33
Squirrel	0.340–0.681	31	Macaque	4.2	47.9	Cat	3.7	30
Guinea pig	0.837	25	Cat	3.0	49.8	Dog	12.6	21
Cat	2.5–4.0	30	Dog	14	34.7	Man	70	16
Opossum	1.81–5.45	32	Goat (doe)	36	22.2			
Rabbit	3.63–9.09	36	Chimpanzee	38	28.5			
Man	68–91	34	Sheep	45	35.5			
Pig	100	60	Woman	56	22.6			
Cow, steer	492	44	Man	65	25.3			
			Cow, steer	500	12.5			
			Horse	500	17.0			
			Bull	600	20.0			

mitochondria of diaphragm muscle of the pig, cow, or steer.

The fibers comprising the diaphragm of these large mammals are similar in many respects to white fibers of the albino rat diaphragm, but are usually even larger in diameter. Some fibers resemble intermediate fibers of the rat more closely, but are usually larger. Mitochondrial content relative to myofibrillar mass varies somewhat, but in no instance is it as high as that of red fibers.

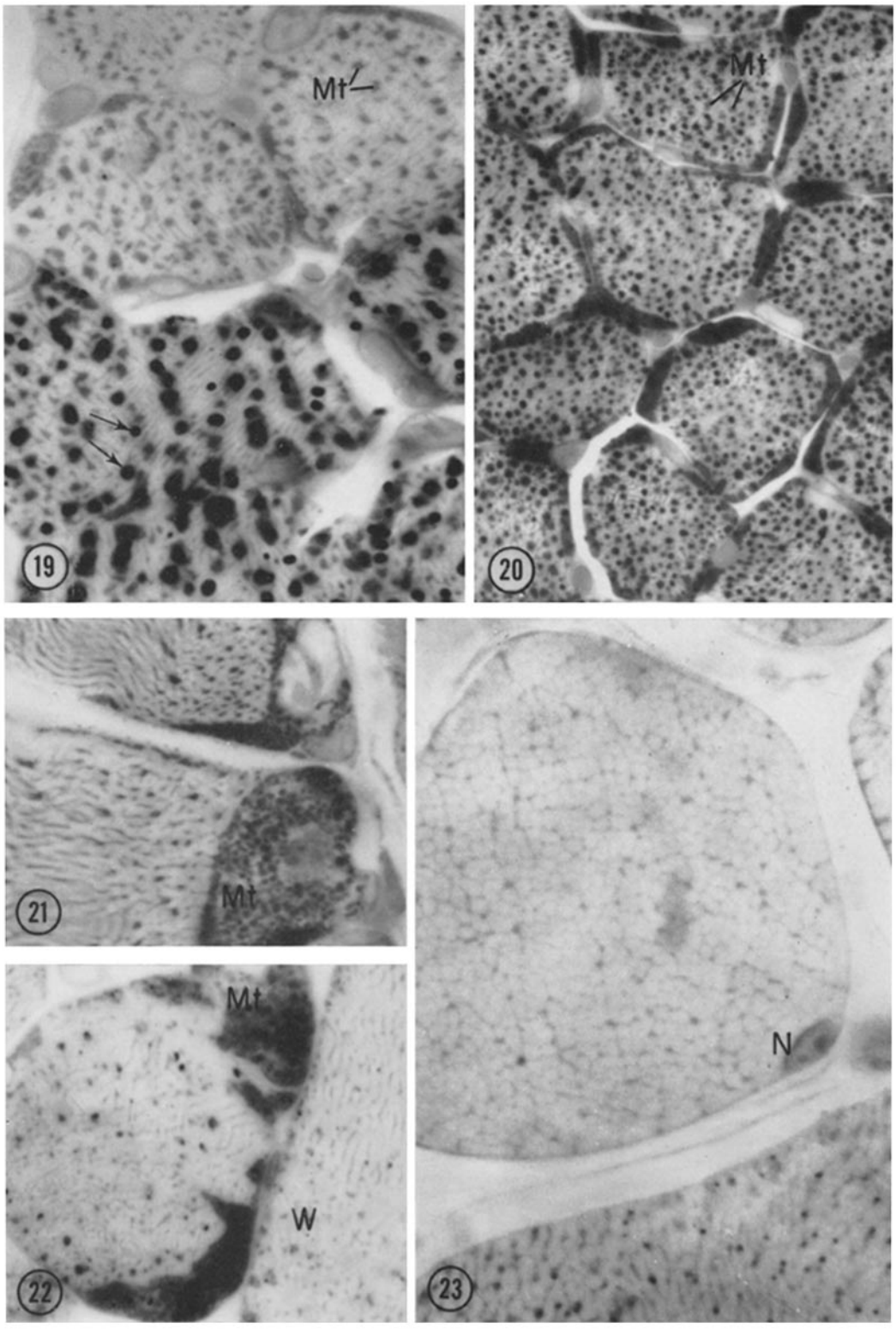
Large white fibers appear, then, to be characteristic of the diaphragm of large mammals,

FIGURES 19 to 23 Diaphragm, pocket mouse (Fig. 19), shrew (Fig. 20), whitetail antelope squirrel (Fig. 21), albino rat (Fig. 22), cow (Fig. 23). All are transverse sections stained with Sudan black and photographed at the same magnification, using an oil-immersion lens.

Figs. 19 and 20 illustrate some of the cytological features of the fibers of small mammals. Although there is an apparent variation in fiber type in the pocket mouse, this is the result of a difference in triglyceride content of the fibers only. The fibers in the lower part of the photograph contain triglyceride droplets (arrows), while those in the upper part do not. All fibers have an abundance of large mitochondria (*Mt*).

Figs. 21 and 22 are from mammals of intermediate body size. Large spherical mitochondria at the periphery of small red fibers are especially conspicuous (*Mt*). Note that in Fig. 22 the white fiber (*W*) has only a few mitochondria present at the periphery.

Fig. 23 illustrates the typical low mitochondrial content of fibers in the large mammal. Even in the nuclear region (*N*), there are no aggregations of mitochondria, and mitochondria present in the interior tend to be small and filamentous. Triglyceride droplets are usually sparse or absent. × 1400.


Mt
19
Mt
20
Mt
21
Mt
W
22
N
23

while small red fibers are characteristic of the diaphragm of small mammals. The average fiber diameter of cow and steer diaphragms is more than twice that of the shrew diaphragm (Figs. 24 and 25; Table III), and the average cross-sectional area of fibers from these two species differs by nearly 6-fold (Table III). The mitochondrial content is obviously greater in the diaphragm with the small fibers (compare Figs. 24 and 25, and Figs. 26 and 27). Mammals of intermediate size display variable mixtures of red and white fibers, as well as fibers possessing intermediate characteristics (Figs. 16 to 18). When actual measurements of fiber diameter are examined (Table II), it is apparent that fiber size is directly related to body size, and thus mitochondrial content of the fibers is inversely related to body size. Metabolic rate and breathing rate are likewise inversely related to body size (Benedict, 1938; Crosfill and Widdicombe, 1961). Small red fibers are characteristic, therefore, of mammals with a high metabolic rate and fast rate of breathing, while large white fibers predominate in mammals known to have a relatively low metabolic rate and slow rate of breathing.

DISCUSSION

Marked differences are known to exist among the component fibers of most mammalian skeletal muscles, but the functional significance of these differences is not understood. Ranvier (1874) observed that, in the rabbit, red muscles contracted in a slower and more sustained manner than did white muscles of the same animal. Biochemical measurements and histochemical localization indicate that a high content of mitochondrial enzymes is characteristic of red muscles (Green, 1951; Nachmias and Padykula, 1958). In general, fiber diameter within individual skeletal muscles of the mammal is inversely related to their mitochondrial content. This has been demonstrated by both histochemical and ultrastructural procedures (Porter and Palade, 1957; Nachmias and Padykula, 1958; Ogata, 1958; Dubowitz and Pearse, 1960; George and Susheela, 1961; Padykula and Gauthier, 1963). Small fibers with a high content of mitochondria, known as red fibers, are believed, therefore, by many investigators, to be characteristic of slow muscles. However, a fast muscle, such as the cricothyroid of the bat, has ultrastructural features (Revel, 1962) which resemble those of red fibers. Observations on frog muscle lend support to such a direct relationship between mitochondrial content and speed of contraction. Differences in the speed of individual fibers in a single muscle have been demonstrated in the frog (Kuffler, 1953). The ultrastructural appearance (Peachey and Huxley, 1962) of equivalent frog muscle fibers suggests a higher mitochondrial content in fast than in slow fibers (Page, 1965). These conflicting observations

FIGURES 24 to 25 Diaphragm, shrew (Fig. 24) and cow (Fig. 25). Transverse sections, Sudan black. These two photomicrographs, taken at the same magnification, show the contrasting appearances of the diaphragm in a small and large mammal. In the shrew all fibers are small and are rich in mitochondria, while in the cow the fibers are almost entirely large and the mitochondrial content is relatively low. Where subsarcolemmal accumulations of mitochondria exist, they are thin and relatively inconspicuous. Compare with Figs. 20 and 23. × 312.

FIGURES 26 to 27 Diaphragm, shrew (Fig. 26) and cow (Fig. 27). Longitudinal section. These two electron micrographs are magnified to the same extent. The fiber from the shrew muscle contains abundant large mitochondria and triglyceride droplets, while in the fiber from the cow muscle the mitochondria are almost entirely small and triglyceride droplets are rare. Despite the occurrence of contracture in the specimen of cow diaphragm (Fig. 27), it is apparent that the predominant type of mitochondrion in this large mammal is the braceletlike filament encircling myofibrils at the I bands. Paired elliptical profiles of mitochondria are present on either side of the Z lines (Mt_3). Note the relatively small size of these mitochondria when compared to equivalent paired mitochondria in the shrew muscle in Fig. 26 (Mt_3). Occasional chains of larger mitochondria (Mt_2) are likewise smaller in the cow than in the shrew. × 10,000.

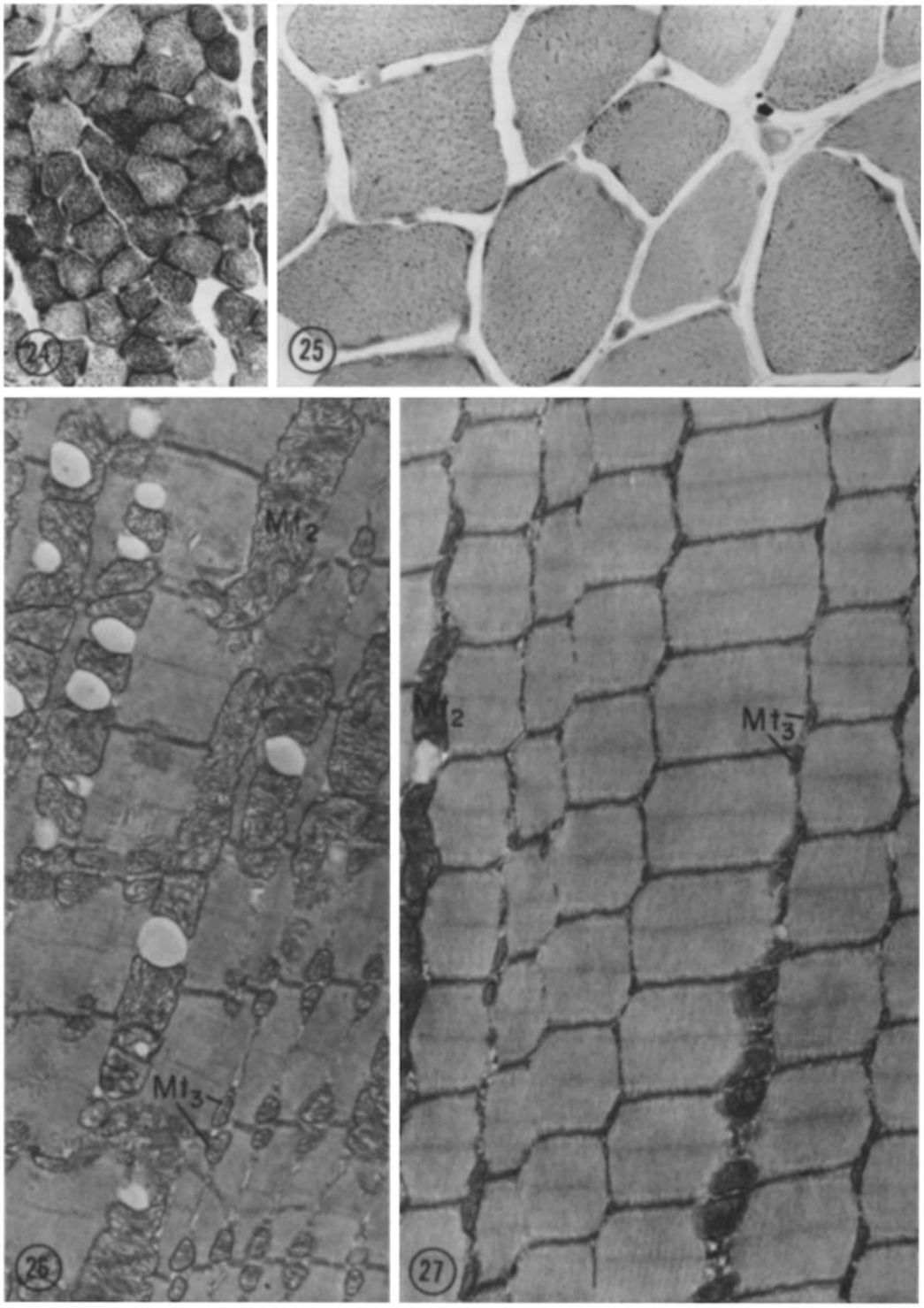

24
25
Mt2
Mt3
26
Mt2
Mt3
27

TABLE III

Comparative Cross-Sectional Dimensions of Diaphragm Fibers

Species	Diameter	Area
	μ	μ^2
Shrew	18	276
Cow, steer	44	1572

on mammalian and amphibian muscle indicate that the exact relationship of speed of contraction to fiber type remains unclear. However, in the present study of the mammalian diaphragm, a definite trend in functional activity with respect to fiber type is demonstrated.

The mammalian diaphragm has a similar function in all species, but its activity, which is continual throughout life, varies in a quantitative manner. It is apparent that, in this muscle, red fibers are characteristic of small mammals, and, therefore, of mammals with high metabolic activity and high rate of breathing. Large white fibers, on the other hand, predominate in the diaphragm of large mammals with relatively low metabolic activity and low breathing rate. These morphological findings are believed to be related more closely to body size of the animals than to such factors as diet, environment, or taxonomic groupings. Four members of the subclass Metatheria, for example, display the same trend of decreasing proportions of red fibers with increasing body size. Several desert rodents, all with similar dietary habits, exhibit this trend also, depending on body size. Mammals of approximately equivalent body size, on the other hand, have diaphragms with similar cytological characteristics. The shrew and harvest mouse, for example, have diaphragms composed entirely of small red fibers, though they belong to different orders and have different dietary habits. Factors other than body size cannot be ignored, however. Discrepancies in the trend shown in Table II might, therefore, be related, in part, to activities peculiar to a given species.

The functional significance of fiber size and mitochondrial content remains uncertain, but various important relationships are apparent. Fibers of small diameter present a greater surface area for exchange of gases, ions, and metabolites than does an equivalent total mass of large fibers. The layer of large mitochondria which intervenes between the plasma membrane and the contractile substance of red fibers may function in providing a readily available supply of energy at the cell surface. The energy requirements of the cell surface, furthermore, may be greater in red fibers than in white fibers. Additional large branching mitochondria in the interior of the fiber provide ready access to the enzymic machinery required for contractile activity. The close association of numerous triglyceride droplets with mitochondria in red fibers suggests extensive oxidative activity. Large mitochondria occupy much of the interfibrillar space longitudinally, and branches from them encompass the myofibrils transversely at each I band. These transverse mitochondrial bands are situated close to the triads of the sarcoplasmic reticulum and, furthermore, are precisely oriented with both the triads and the contractile apparatus of the A-I junction. The triads are believed to be the sites of transverse conduction of impulses which elicit contraction (Huxley and Taylor, 1958; Franzini-Armstrong and Porter, 1964; Huxley, 1964). These ultrastructural relationships are compatible with the coordinated activities necessary to effect muscular contraction.

The morphological features of small red fibers are compatible also with a high degree of metabolic activity. Krebs (1950) suggested that the striated musculature might be the system chiefly responsible for the reciprocal variation of metabolic rate with body size. The diaphragm muscle is, in fact, involved in such a relationship. Respiratory rate of diaphragm muscle from developing rats measured in vitro is inversely related to body size (Bertalanffy and Pirozynski, 1953). However, this relationship may pertain specifically to the diaphragm, since similar experiments with hind limb muscles showed a less striking relationship between metabolic rate and body size (Bertalanffy and Estwick, 1953). Our observations, likewise, indicate a less striking relationship between fiber composition of hind limb muscles and body size than was demonstrated in the diaphragm. Although fibers tend to be smaller in the small mammals, the population of fibers is, nevertheless, heterogeneous in all seven species examined. It might be concluded that the diaphragm muscle in particular reflects the degree of over-all metabolic activity of the animal. Alternatively, both the in vitro measurements and the morphological features of the diaphragm might be related more specifically to breathing rate or to the particular metabolic requirements of this muscle. Thus, the small

red fiber might be a specialization related to a relatively high frequency of contraction, since small mammals breathe more rapidly than do large mammals (Crosfill and Widdicombe, 1961). The elaborate nature of the sarcoplasmic reticulum in the diaphragm of small mammals supports this possibility. Extensive development of the sarcoplasmic reticulum is believed to be related to high rates of contraction (Peachey and Porter, 1959; Fawcett and Revel, 1961; Porter, 1961), although this has recently been questioned by Bergman (1964), who reported that the sarcoplasmic reticulum is as highly developed in a slow as in a relatively fast fish muscle. Our suggestion that red fibers are faster than white fibers in the diaphragm is compatible with the observation in frog muscle that, within a single muscle (Page, 1965) fibers known to be fast have characteristics which resemble those of red fibers of the diaphragm, while slow fibers resemble white fibers more closely.

The diaphragm is a relatively slow muscle when compared to fast mammalian muscles such as the cricothyroid of the bat. It is believed that the cricothyroid is involved in the emission of sounds, which may be as rapid as 200 times per sec in the bat. On the other hand, the diaphragm of one of the fastest breathing mammals studied here (albino mouse) is involved in a physiological event that takes place less than two times per second (*see* Table II). Yet ultrastructural features of the bat circothyroid (Revel, 1962) resemble those of red fibers, which predominate in the diaphragms of small mammals, including the bat. Thus, within a broad range of "slowness," diaphragms involved in higher breathing rates show characteristics resembling those of truly fast muscles. Since the fast cricothyroid and relatively slower diaphragm muscle of the bat resemble each other closely, this structural similarity may reflect also the overall high metabolic activity of the bat.

In evaluating the functional significance of fiber types, it is difficult to assign a specific functional role to a particular cytological variation. Metabolism of the particular muscular tissue and of the whole animal as well as the various aspects of muscle contraction must be considered. Speed of an individual twitch response must be distinguished from frequency of contraction and from duration of contraction. The particular role of a given skeletal muscle may vary among species. In the diaphragm, the function is similar in all species, but its primarily quantitative variation among species makes it possible to relate cytological characteristics to degree of activity where one kind of function is involved.

The general trend toward increase in fiber diameter with increase in body size is of interest, because magnitude of the animal body is usually viewed as a reflection of cell number rather than cell size (Thompson, 1917). Large animals, therefore, have a larger number of cells comprising a given tissue than do small animals, but the size of the cells usually is similar. It has been emphasized, however, that striated muscle fibers do not proliferate in mature animals, and that, in this respect, they resemble neurons (Goss, 1964). In both these cell types, increase in cell size is related to increase in body size, though within certain limits. The present study has established that a considerable difference in fiber diameter exists in the diaphragms of small and large mammals, the shrew vs. the cow, for example (Figs. 24 and 25). If, however, the skeletal musculature is more intimately related to metabolic activity than are other tissues, as suggested by Krebs (1950), then the small diameter of skeletal muscle fibers in small mammals would contribute to surface area, a characteristic favoring high metabolic activity. The inverse relationship between total body surface and body mass is thus manifested, at the cellular level, in a similar inverse relationship between surface area of muscle fibers and size of the animal.

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