

# CYTOCHEMICAL STUDIES OF ADENOSINE TRIPHOSPHATASES IN SKELETAL MUSCLE FIBERS

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## ABSTRACT

Mitochondrial ATPase and myosin ATPase have been localized in the muscle fibers of the rat diaphragm. The principal fiber type possesses a structure favorable for making this cytochemical separation with the light microscope. This small red fiber has numerous large, nearly spherical, mitochondria (*ca.* 1.5  $\mu$ ) which are aggregated beneath the sarcolemma. In the interior of the fiber, smaller paired filamentous mitochondria (*ca.* 0.2  $\mu$  diameter) are aligned with the I band. Distribution of mitochondria was determined by sudanophilia, succinic dehydrogenase activity, and by direct examination with the electron microscope. ATPase activity at pH 7.2 is located in the large peripheral mitochondria and in the smaller mitochondria associated with the I band. The alignment of the small mitochondria results in a discrete cross-striated appearance in fibers stained for this enzymic activity. This mitochondrial ATPase does not cleave adenosine diphosphate or adenosine monophosphate; it is not sulfhydryl dependent and, in fact, is enhanced by the mercurial, *p*-hydroxymercuribenzoate. It requires magnesium ion and is stimulated by dinitrophenol. It is inhibited after formol-calcium fixation, but the residual activity is demonstrable by lengthening the incubation time. At pH 9.4 the ATPase is myofibrillar in origin and is located in the A bands. This myosin ATPase activity is sulfhydryl-dependent. Mercurial at this high pH has an interesting dual effect: it suppresses myosin ATPase but evokes mitochondrial ATPase activity. A third type of ATPase activity can be demonstrated, especially in the large white fibers. This activity occurs at pH 7.2 in the presence of cysteine. Its position is manifested cytochemically as a fine reticular pattern which surrounds individual myofibrils. The distribution suggests that it may originate in the sarcoplasmic reticulum.

## INTRODUCTION

Adenosine triphosphate (ATP) is the end product of the major energy-generating systems of the cell (29). Demonstration of the sites at which this important carrier is split to release its energy aids significantly in interpretations of cellular function. In recent years the reliability of the histochemical techniques for demonstrating ATPase<sup>1</sup> activity has been illustrated repeatedly in

a variety of tissues (24, 35, 1, 6, 10). With Gomori's metal salt procedure for localizing phosphatase, ATPases active in the neutral pH range can be demonstrated by using lead ion to trap the released orthophosphate (35). In the alkaline range calcium ion is used (24). These procedures are precise enough to permit cytochemical localiza-

this paper: ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; A-5-P, adenosine-5-phosphate.

<sup>1</sup> The following abbreviations will be used throughout

tions with the light microscope over a wide range of pH (24, 36, 9, 16). Furthermore, some of the components of the enzymic reaction survive the rigorous procedures involved in specimen preparation for electron microscopy, and this ATPase activity can be localized in relation to ultrastructure (22, 7). Thus, accurate localization can be obtained and, in addition, by combining these versatile procedures many properties of the various enzymes which act on ATP can be analyzed in frozen sections.

Within skeletal muscle fibers, at least three different ATPases have been described biochemically: myosin ATPase (13), mitochondrial ATPase (3), and most recently sarcotubular ATPase (19). Of these enzymes, only myosin ATPase has been localized in sections of muscle, and this was accomplished with the light microscope by using the calcium method (24, 9). An ultrastructural localization of myofibrillar ATPase has recently been obtained by Tice and Barnett (33) using the lead method. Mitochondrial and sarcotubular ATPases of skeletal muscle are active near pH 7.0 and thus should be demonstrable by the lead method. However, the cytochemical separation of ATPases in mammalian muscle has been complicated by the heterogeneity of the fibers of most skeletal muscles (21, 32). Histologists are aware of the fact that it is possible to distinguish at least two types of fibers in most mammalian skeletal muscles. Differences in mitochondrial number and distribution constitute one of the major cytological differences between fiber types. In this study, we were able to identify mitochondrial ATPase in the rat diaphragm because in one fiber type the cellular organization permits easy distinction of mitochondrial and myofibrillar sites with the light microscope. This fiber corresponds closely to the classical red fiber in that it is small in diameter, has an abundance of mitochondria, especially in the subsarcolemmal position, and usually contains large quantities of triglyceride.

Through the use of this favorable material it has been possible to recognize mitochondrial ATPase activity and to determine some of its properties *in situ* by using frozen sections of the rat diaphragm. Criteria for distinguishing between myosin and mitochondrial ATPases are presented. In addition, some of our evidence indicates that the sarcotubular ATPase may be

demonstrated under certain conditions in frozen sections.

## MATERIALS AND METHODS

### *Preparation of Tissue*

Adult albino rats were killed with chloroform, and the diaphragm was immediately exposed by a midline incision through the abdominal wall. The animal was extended dorsally, and a small strip was cut from the abdominal surface of the diaphragm. For uniformity, a portion of the right costal region of the diaphragm was used throughout this study (Fig. 1). Two incisions were made approximately 1 to 5 mm apart and parallel to the direction of the muscle fibers, leaving the muscle attached at its origin and insertion. A flat wooden splint was applied to the surface of the strip and tied to it at points close to the origin and insertion. The strip was cut beyond each tie and placed quickly into the appropriate fixative or freezing mixture.

### *Histochemical Procedures*

**LIPIDS:** Strips of muscle fixed in 10 per cent neutral buffered formalin were washed, cut into smaller segments, and embedded in gelatin. Sections approximately 5  $\mu$  thick were cut on a freezing microtome, mounted in 0.1 per cent gelatin directly on glass slides, blotted, and allowed to dry. They were stained with Sudan black B for 7 minutes and then mounted in glycerogel. Control sections were extracted with acetone for 30 minutes prior to staining in order to remove triglycerides.

**ENZYMIC ACTIVITY:** The isolated muscle was frozen by plunging it directly into a mixture of dry ice and 95 per cent ethanol at  $-70^{\circ}\text{C}$ . After 10 minutes, the tissue was transferred to a cryostat at  $-20^{\circ}\text{C}$ , blotted free of excess fluid, and the wooden splint was removed. Sections 5 to 10  $\mu$  thick were cut and then placed on glass slides. A technical problem frequently encountered at this point in the procedure was the lack of uniform flattening of fresh, frozen sections of tissue. Since chemically fixed sections were generally flatter than unfixed ones, there is reason to believe that some of the loosening or crumpling occurs during the incubation. This uneven adhesion of muscle fibers, especially of transverse sections, obscures much of the cytological detail. Although this problem was not completely solved, it was observed that sometimes there was better flattening when a frozen section was mounted on a slide which was maintained at room temperature. This quick thaw resulted in better flattening than was obtained by mounting the frozen section on chilled slides in the cryostat ( $-20^{\circ}\text{C}$ ). In addition, flat sections were

sometimes obtained by using a fresh albumin film to affix the sections to the slides.

**ATPASES:** The sections were dried for 30 minutes and incubated directly, usually without chemical fixation. One of two procedures was followed, according to the pH at which ATPase activity was demonstrated. For localization at pH 9.4, the calcium method (24) was used; this medium contains ATP, calcium chloride, and sodium barbital as a buffer. Sections were incubated at 37°C for 10 minutes, treated with cobalt nitrate, and then with ammonium sulfide. At pH 7.2, the lead method (35) was used; this medium contains ATP, lead nitrate, magnesium sulfate, and Tris maleate buffer. Sections were incubated at 37°C for 15 or 30 minutes and then treated directly with ammonium sulfide. ATPase activity at pH 7.2 was demonstrated also after fixation of dried sections in formol-calcium (23) for 10 minutes; the resulting enzymic inhibition necessitated lengthening the time of incubation to 2 hours.

After both procedures, the washed sections were usually mounted in glycerogel without dehydration. In a few instances, the reacted sections were dehydrated and mounted in Permount. Although this yields preparations with good photographic qualities, the lead sulfide gradually dissolves in the mounting

medium and thus the preparation is impermanent. Longitudinal sections of diaphragm were studied with a polarizing microscope to localize the reaction product in relation to the sarcomere.

To determine the response to sulfhydryl compounds and sulfhydryl inhibitors, cysteine or *p*-hydroxymercuribenzoate<sup>2</sup> was added to the two media, according to the procedures described by Padykula and Herman (24). In the pH 7.2 medium, cysteine forms a precipitate in reaction with lead ion. Despite this interaction, histochemical staining resulting from enzymic activity remains strong, and there is evidence of stimulatory effects on sulfhydryl-dependent enzymes (Padykula, unpublished). The specificity of the enzymic action was further explored by replacing the ATP in the media with ADP or A-5-P. In these replacements, the time of incubation was increased to 30 or 45 minutes. Control media with no substrate were also used.

Dinitrophenol is a well known activator of mitochondrial ATPase of liver (15), heart (28), and

<sup>2</sup>This compound was originally believed to be *p*-chloromercuribenzoate (Sigma Chemical Co., St. Louis).

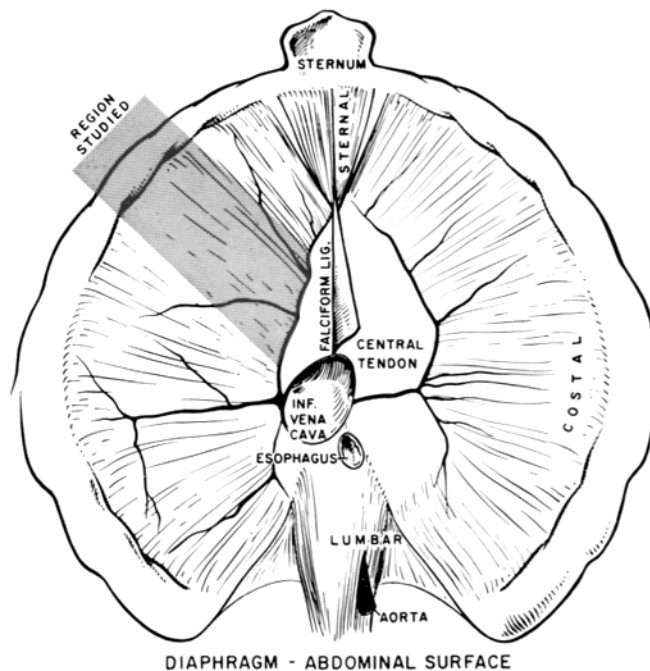


FIGURE 1

Diagram of the abdominal surface of the diaphragm of the adult albino rat. The shaded area of the right costal diaphragm represents the approximate region from which strips of muscle were isolated for study. This drawing was made by G. H. Turner.

skeletal muscle (2). At  $5 \times 10^{-4}$  M final concentration, it was demonstrated in the present study to be a very effective activator of this enzyme in frozen sections of diaphragm at pH 7.2.

Because of the problems associated with the use of fresh, unfixed sections of skeletal muscle, it is recommended that formol-calcium fixed sections be used in routine investigations of this mitochondrial ATPase. Although this prefixation will inhibit mitochondrial ATPase to a considerable degree, the activating effect of dinitrophenol or mercurial in the lead medium will result in well stained preparations after a reasonable period of incubation.

**SUCCINIC DEHYDROGENASE:** Frozen sections were mounted directly on uncoated slides, dried for 2 minutes, and incubated for 30 minutes in a medium containing nitro-blue tetrazolium, according to the method described by Nachlas *et al.* (20).

### *Electron Microscopy*

Strips of muscle approximately 1 mm wide were isolated from the animal as described above and then placed immediately into 1 per cent osmium tetroxide buffered to pH 7.5 with veronal acetate. After 1 hour, the tissue was transferred to 50 per cent ethanol, rapidly dehydrated, and embedded in methacrylate (85 per cent butyl, 15 per cent methyl) or Epon. Sections were cut on a Porter-Blum ultramicrotome and examined with an RCA model EMU-3E microscope or a Siemens Elmiskop I. Methacrylate sections were stained according to Karnovsky's method (12); Epon sections were stained according to Reynolds' method (37). Although an attempt was made to prepare sections which were truly transverse or longitudinal, in most cases the plane of section was somewhat oblique.

### RESULTS

There is reason to believe that the histochemical characteristics of the muscle fibers vary in the lumbar, costal, and sternal regions of the diaphragm of one species of rat (11), and this may be related to its complex embryological origin. Specimens of muscle used in this study were, therefore, taken from a limited, but easily accessible, region of the diaphragm (Fig. 1). After staining of the right costal diaphragm with Sudan black, at least two distinct fiber types can be recognized: small, dark, lipid-rich fibers and large, faintly sudanophilic fibers (Fig. 2). To determine the relative proportions of these two fiber types, 850 fibers were counted in a Sudan black stained section. The small fibers are the predominant type, constituting about 60 per cent of this total. The large fibers form 30 per cent of

the total, and the remaining 10 per cent have intermediate characteristics or could not be classified. It will be demonstrated that the differences in sudanophilia of the two principal fiber types are mainly the result of differences in their mitochondrial content. The degree of sudanophilia reflects triglyceride content also, but this criterion is less constant because of variation in the physiological state of the animal. The small, heavily sudanophilic fiber has a characteristic arrangement of mitochondria which facilitates the identification of enzymic activity originating in these organelles, and it was deliberately selected for the enzymic localizations described here.

### *Mitochondrial Size and Position in Small and Large Fibers*

The small fibers are characterized by the presence of large sudanophilic masses beneath the sarcolemma which impart a rimmed appearance to these fibers when viewed in transverse section (Fig. 2). The inner surface of these peripheral masses is irregular, forming extensions toward the interior of the fiber (Fig. 4). Observation at high magnification reveals that the peripheral masses consist of closely packed granules, many of which are as large as  $1.5 \mu$  in diameter (Fig. 6). The interior of the small fiber contains numerous spherical black droplets and small grey granules and filaments (Fig. 4). If a section is extracted with acetone before staining, the spherical black droplets, which most likely contain triglyceride, are removed, but the peripheral grey masses and central grey granules and filaments retain their sudanophilia (Fig. 5). This persistent sudanophilia is related to the high phospholipid content of the mitochondria which occur in these positions. In longitudinal section, the peripheral accumulations of mitochondria form a long sudanophilic band which usually encompasses the nuclei of the small fiber (Fig. 20). In the interior of the fiber, sudanophilia in the region of the I bands imparts a transverse striation. It seems likely that mitochondria aligned with the I band contribute to this sudanophilia, although it has been reported that the I band of the myofibril itself is sudanophilic (8). Triglyceride droplets in the sarcoplasm are also frequently aligned with the I band.

In Sudan black preparations, the large fibers are relatively unreactive (Fig. 2). There is a general absence of accumulations of large mito-

chondria in the periphery of the fiber. Although triglyceride droplets are rare, small filamentous mitochondria are moderately abundant, and in longitudinal section the regions of the I bands are clearly sudanophilic (Figs. 2 and 20).

To verify the differences in mitochondrial size and distribution of the two major fiber types, mitochondrial position was determined also through the localization of succinic dehydrogenase and by direct examination with the electron microscope. Succinic dehydrogenase, a mitochondrial enzyme, can be demonstrated with nitro-blue tetrazolium at sites which correspond to the distribution of mitochondria stained with Sudan black (Figs. 2 and 3). The small fibers are high in succinic dehydrogenase activity, especially in the superficial cytoplasm apart from the myofibrils (Fig. 3). In both small and large fibers, numerous blue-black granules and threads occur among the myofibrils, and undoubtedly mark the location of the internal mitochondria.

Examination of small fibers with the electron microscope further confirms the mitochondrial nature of the peripheral sudanophilic band. Beneath the sarcolemma, there are distinctly outlined dense accumulations of mitochondria (Fig. 7). The size and shape of these mitochondria are somewhat variable, and lipid droplets are occasionally interspersed among them. Many of these mitochondria are large spheres approximately 1 to 1.5  $\mu$  in diameter (Figs. 6 and 7). The cristae are numerous, lamellar, and often sinuous in form, and are usually parallel to each other. The matrix is moderately dense. These peripheral mitochondria may also assume an elongate form, especially near the surface of the myofibrils. Slender projections from the peripheral mitochondria are often seen extending toward the interior of the fiber and penetrating between myofibrils (Fig. 9). In the interior of the fiber, small filamentous mitochondria (*ca.* 0.2  $\mu$  in diameter) occur principally in pairs in the sarcoplasm between myofibrils. Each member of a pair is situated on opposite sides of the Z line (Fig. 21). The mitochondria associated with the myofibrils of the rat diaphragm have been described by Palade (25) as branching and encircling the I bands transversely. In oblique thin sections, mitochondrial profiles of various shapes are obtained (Figs. 7 and 8), and this image suggests that the filaments and granules observed in frozen sections represent different planes of section through these internal bracelet-like mito-

chondria. Thus, although there are important differences in size and shape between the peripheral and internal mitochondria of the small fiber, it is likely that there is only one type of mitochondrion which may have different forms in different locales in the muscle fiber. Electron micrographs, such as Fig. 9, which show projections from a peripheral mitochondrion extending into the myofibrillar mass, support this suggestion. The myofibrils are outlined also by various profiles of tubules and cisternae which constitute the sarcoplasmic reticulum (Figs. 10, 21), and by particles of glycogen present in the sarcoplasm.

Electron microscopic examination of the large fibers reveals a higher myofibrillar to sarcoplasmic ratio than is evident in the small fiber. The subsarcolemmal sarcoplasm contains a few small slender mitochondria and numerous glycogen particles (Fig. 8). There is no striking difference in the size or shape of the peripheral and internal mitochondria, as has been described in the small fiber. The internal mitochondria are associated with the I band as in the small fiber, but they are smaller in diameter and fewer in number. There is a conspicuous difference in the matrix of the mitochondria of small and large fibers. In the mitochondria of the large fibers the matrix is less dense than that of both the peripheral and the internal mitochondria of the small fiber and the cristae are less numerous (Figs. 10, 11). Some of the differences in the mitochondria of the two main fiber types of the rat diaphragm correspond closely to those which have been described in detail in the rat sartorius and obliqui muscles by Porter and Palade (27). In addition, glycogen particles are usually more abundant in the sarcoplasm of the large fibers.

#### *ATPase Activity at pH 7.2*

A cytochemical separation of ATPases can be achieved with respect to the above morphological characteristics. The arrangement of mitochondria in the small fiber facilitates the cytochemical separation of mitochondrial ATPase from myosin ATPase with the light microscope. At pH 7.2, ATPase activity in the small fiber is localized at the sites of peripheral accumulation of mitochondria as well as in small and large granules in the interior of the fiber (Fig. 12). Sites corresponding to the myofibrils are unreactive at this pH. When viewed in longitudinal section, ATPase activity occurs beneath the sarcolemma and also in paired, small granules which are aligned transversely in the fiber to form delicate, paired stria-

### Abbreviations Used in the Figures

<i>A</i> , A band	<i>Mt</i> <sub>2</sub> , small central Mitochondrion of small fiber
<i>I</i> , I band	
<i>L</i> , Lipid droplet	<i>Mt</i> <sub>3</sub> , Mitochondrion of large fiber
<i>M</i> <sub>1</sub> , Muscle fiber (large)	
<i>M</i> <sub>2</sub> , Muscle fiber (small)	<i>N</i> , Nucleus
<i>Mf</i> , Myofibril	<i>S</i> , Sarcolemma
<i>Mt</i> <sub>1</sub> , large peripheral Mitochondrion of small fiber	<i>Z</i> , Z line

#### FIGURE 2

Lipids. Sudan black. Cross-section.

Two types of muscle fibers are illustrated. There are large fibers (*M*<sub>1</sub>) which are faintly sudanophilic, and smaller fibers (*M*<sub>2</sub>) which are intensely reactive. The small fibers contain a greater number of distinct, black, spherical droplets which are soluble in acetone (see Fig. 5). In addition, in the small fibers there are grey sudanophilic masses (arrows) at the periphery which represent accumulations of mitochondria. Fine grey granules in the interior of the fibers also represent mitochondrial staining, and these are better seen at higher magnification in Fig. 4.  $\times 625$ .

#### FIGURE 3

Succinic dehydrogenase. Nitro-blue tetrazolium. Cross-section.

Two types of fibers can be discerned on the basis of the activity of this mitochondrial enzyme. Small fibers (*M*<sub>2</sub>) are more reactive than large fibers (*M*<sub>1</sub>). The reaction product is deposited at sites corresponding to the distribution of central and peripheral mitochondria, as observed in Sudan black preparations and with the electron microscope. Note especially the heavy peripheral enzymic activity in the small fibers (*M*<sub>2</sub>). Compare with Fig. 2.  $\times 625$ .

#### FIGURE 4

Lipids. Sudan black. Cross-section.

This oil immersion photograph shows 3 small fibers which are rich in triglyceride (*L*) and mitochondria (*Mt*). The peripheral mitochondria are large and form conspicuous granular aggregates that often have irregular contours (arrows). In the interior of the fiber, there are a few large mitochondria and numerous smaller ones. The delicate sudanophilic strands may represent longitudinal views of mitochondria or possibly the sarcoplasmic reticulum. The triglyceride droplets are black and uniformly spherical. Refer to Fig. 7 for an ultrastructural view of the interior of the fiber.  $\times 1200$ .

#### FIGURE 5

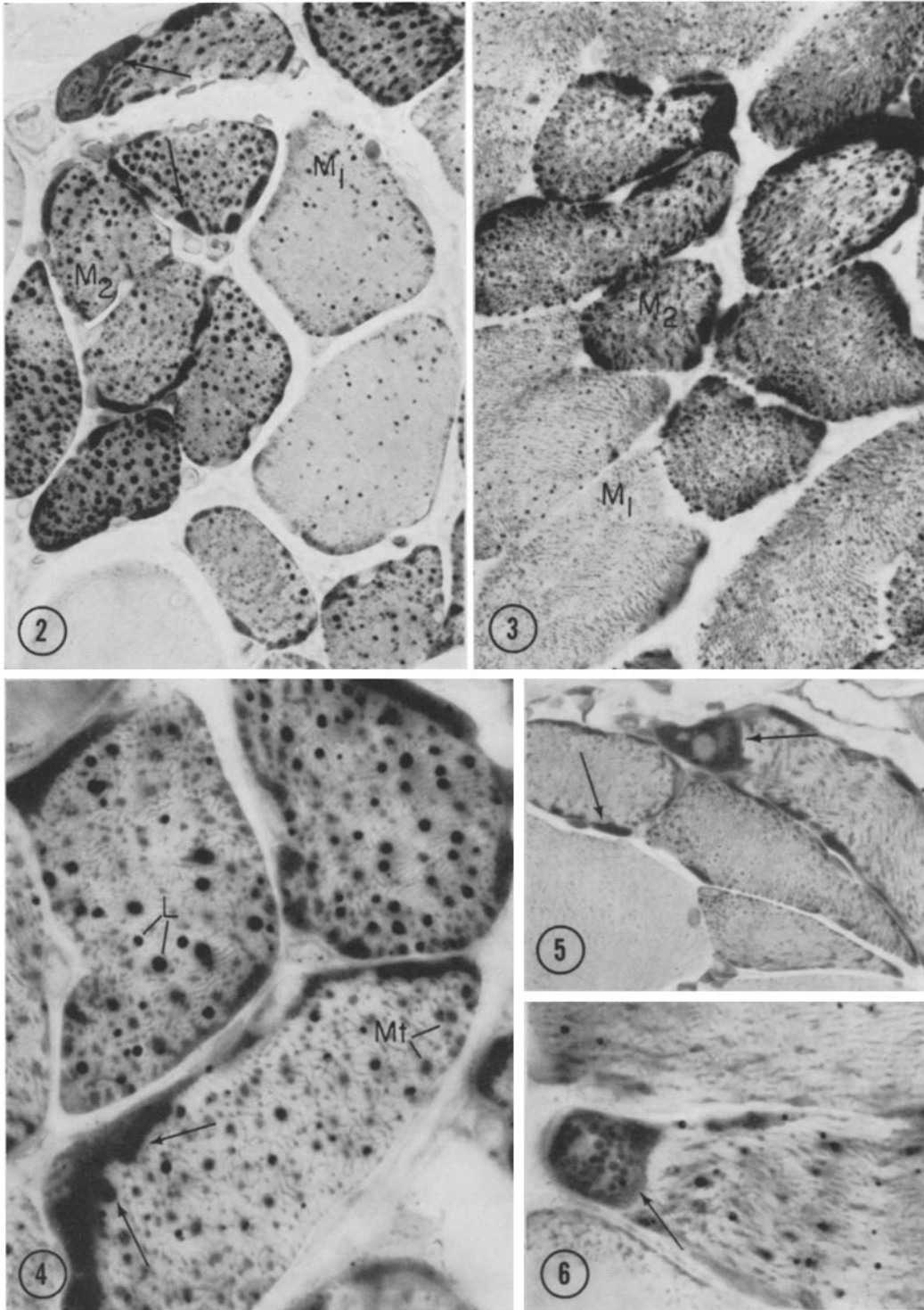
Lipids. Sudan black staining after acetone extraction of the section.

Compare with Fig. 2 to establish that acetone extraction has removed the spherical droplets of triglyceride. The remaining sudanophilia is attributed to the mitochondria which are dispersed throughout the interior of the fiber and aggregated into peripheral masses (arrows).  $\times 625$ .

#### FIGURE 6

Lipids. Sudan black.

The spherical shape and relatively large size of some of the peripheral mitochondria are demonstrated in this oil immersion photograph of a small fiber. There are also some smaller mitochondria which partially surround the larger ones (arrow).  $\times 1200$ .



tions (Fig. 22). To localize this activity with respect to the sarcomere, a section which demonstrated ATPase activity at pH 7.2 was examined both in ordinary and polarized light (Fig. 24). The anisotropic or A band is unreactive after incubation with ATP at pH 7.2. The dark or isotropic (I) band corresponds with the enzymically active region of the fiber. The reaction product is, in fact, deposited as a fine line on either side of the birefringent Z line. Comparison with the ultrastructural arrangement of the sarcomere of the small fiber (Fig. 21) indicates that part or all of this staining reaction originates in the mitochondria. In the large fiber, the distribution of ATPase activity at pH 7.2 again suggests localization in the mitochondria (Fig. 12).

Mitochondrial ATPase activity of both small and large fibers requires the presence of magnesium ion. It is activated strongly by dinitrophenol. Also, this enzymic activity could not be demonstrated with either ADP or A-5-P, and thus is a true ATPase activity in the sense that only the triphosphate is split (Figs. 18 and 19). When ATP and ADP are present in equal concentration at the start of the incubation, this mitochondrial activity is partially inhibited. Thus, ADP, one of the reaction products, is itself an inhibitor. Although this enzyme is partially inhibited by formal-calcium, its residual activity can be demonstrated by prolonging the incubation time to 1 or 2 hours. When a mercurial, such as *p*-hydroxymercuribenzoate, is added to the incubating medium at pH 7.2, there is no diminution of the staining reaction, which indicates that the mitochondrial ATPase of the fibers of the diaphragm is not sulfhydryl-dependent. This is in marked contrast to myosin ATPase of skeletal and cardiac muscle fibers which has been shown by both biochemical (30) and histochemical (24) methods to be dependent on the sulfhydryl group. Mitochondrial ATPase of the diaphragm fibers is, in fact, enhanced by mercurial. When cysteine is added to the incubating medium, the mitochondrial activity persists and there is some stimulation of activity at sites which correspond to the myofibrils. This increased staining is especially evident in the large fibers but also occurs in some of the small fibers (Fig. 13). The significance of this increased staining is not understood at the present time, although it may reflect a stimulation of sulfhydryl-dependent myosin ATPase at this lower pH. In addition to this effect, another site of enzymatic activity becomes apparent in the presence of cysteine at pH 7.2. The reaction product is deposited as a fine reticular pattern which appears to surround individual myofibrils, and this reticulum is especially evident in the large fibers (Fig. 15). This network has nodal points and is similar to that seen in metal-impregnated specimens of skeletal muscle fibers prepared according to Veratti's method (34). However, the reticular pattern obtained in the frozen sections used in the present study is believed to be enzymic in origin since it is dependent on the presence of ATP and cysteine in the medium. The distribution suggests that this ATPase activity may originate in the sarcoplasmic reticulum. In certain small fibers treated with cysteine, a similar reticular pattern of ATPase activity is evoked in addition to the usual mitochondrial activity (Fig. 14). This reticulum is less continuous than that of large fibers and consists of delicate strands which may represent components of the sarcoplasmic reticulum. However, the possibility of mitochondrial origin of this activity cannot be excluded.

*ATPase Activity at pH 9.4*

ATPase activity in the alkaline range is localized entirely within the interior of the fiber in

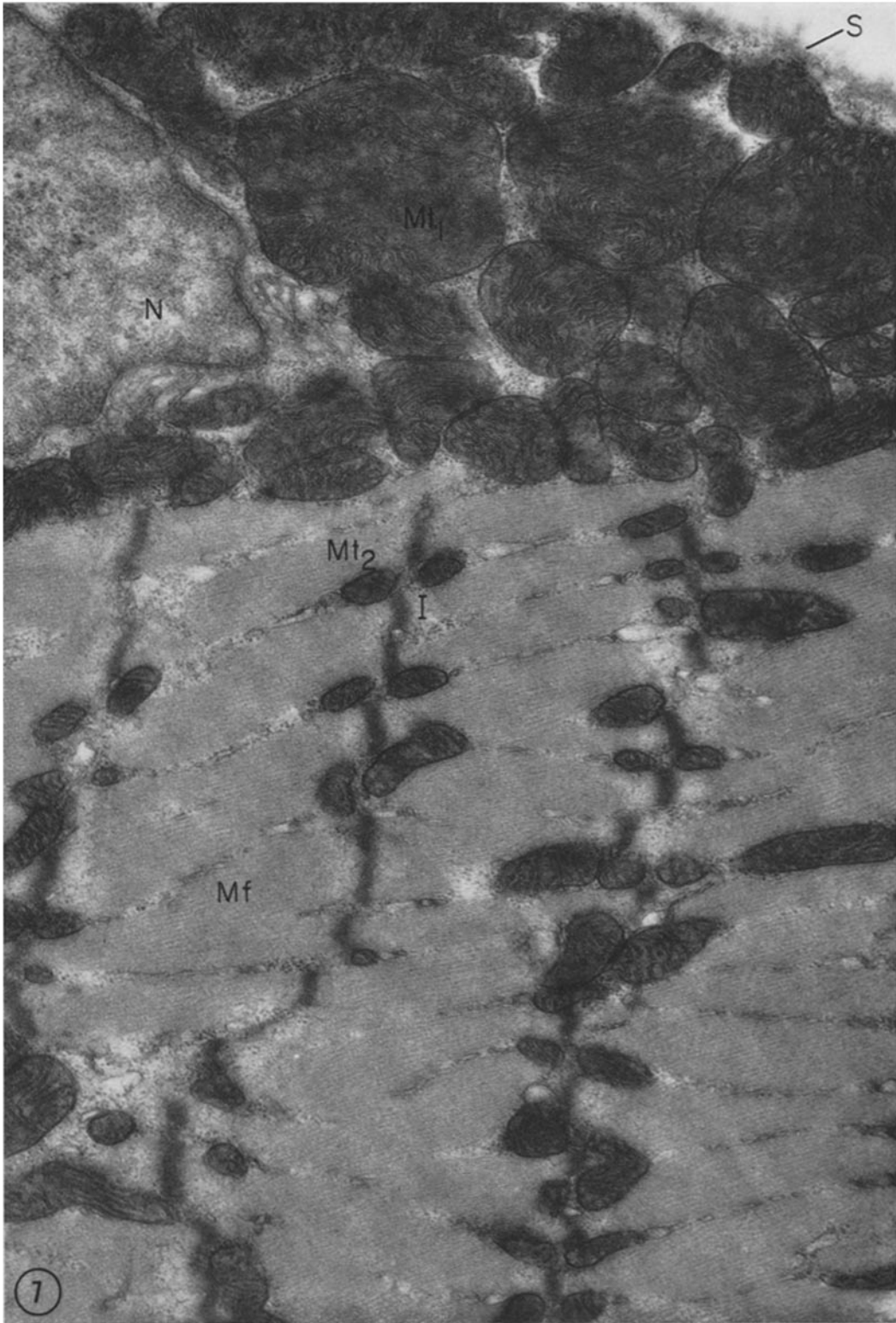
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FIGURE 7

Electron micrograph of a portion of a small fiber showing a peripheral accumulation of mitochondria. Methacrylate section.

Large mitochondria ( $Mt_1$ ) are closely packed in the perinuclear region of the subsarcolemmal sarcoplasm (upper part of the photograph). The numerous cristae of these large mitochondria are lamellar and are usually parallel to each other. They often pursue an undulating or zigzag course. The mitochondrial matrix is moderately dense. Deeper in the fiber, smaller mitochondria ( $Mt_2$ ) are located among the myofibrils ( $Mf$ ), usually as pairs in association with the I band ( $I$ ). Cisternae and tubules of the sarcoplasmic reticulum can be recognized between myofibrils.  $\times 50,000$ .





areas corresponding to the myofibrils (Figs. 16 and 23). In transverse sections, the reaction product is distributed quite uniformly throughout a given fiber in locations corresponding to the myofibrils (Fig. 16). However, fibers vary in the over-all intensity of staining, an observation which is difficult to interpret. It is important to note that there is an absence of sarcoplasmic staining, especially in the subsarcolemmal position, a locus rich in large mitochondria. This fact excludes mitochondrial ATPase activity under these conditions at high pH. In longitudinal sections the reaction product can be localized within the A bands (Fig. 23), and this imparts a distinct cross-striated pattern to the fibers. Unfixed longitudinal sections contract in this incubating medium, and this complicates the localization. This enzymic reaction is strongly inhibited by formalin, and hence fixed sections should not be used.

These observations are consistent with the belief that the enzyme demonstrated at this pH is myosin ATPase. In addition, no reaction product was deposited when ADP or A-5-P replaced ATP in the medium. Thus, only the triphosphate is split, indicating that this also is a true ATPase. Sulfhydryl dependence is manifested by the maintenance of its activity in the presence of cysteine and conversely by the strong inhibition caused by the mercurial, p-hydroxymercuribenzoate (Fig. 17). However, the addition of mercurial to the incubating medium at pH 9.4 yields a surprising result; an enzymic activity is evoked which corresponds in its position to mitochondrial

ATPase (Figs. 17 & 7). Both peripheral and internal mitochondria of small and large fibers become reactive. Thus, mercurial at high pH has a dual effect; it inhibits myosin ATPase but activates a mitochondrial ATPase. No magnesium is added to the alkaline-incubating medium, but, nevertheless, mitochondrial ATPase is active. It is possible that calcium ion substitutes for magnesium here.

Myosin ATPase of the rat diaphragm, therefore, is a sulfhydryl-dependent enzyme and exhibits optimal activity at a high pH. Mitochondrial ATPase, on the other hand, is not sulfhydryl-dependent and exhibits optimal activity at lower pH. It seems likely that the mercurial-evoked mitochondrial ATPase activity at high pH is the same one which operates in the lower range. Both myosin and mitochondrial ATPases are similar in that they act on ATP, but not on ADP or A-5-P, and in this sense are true ATPases. Sarcotubular ATPase may be demonstrable at lower pH under appropriate conditions.

## DISCUSSION

### *Mitochondrial ATPase*

Mitochondrial ATPase activity is believed to be related functionally to oxidative phosphorylation (5, 29, 17). In beef heart mitochondria, the same protein may have both coupling activity and ATPase activity (26). Furthermore, changes in mitochondrial contractility and permeability

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FIGURE 8

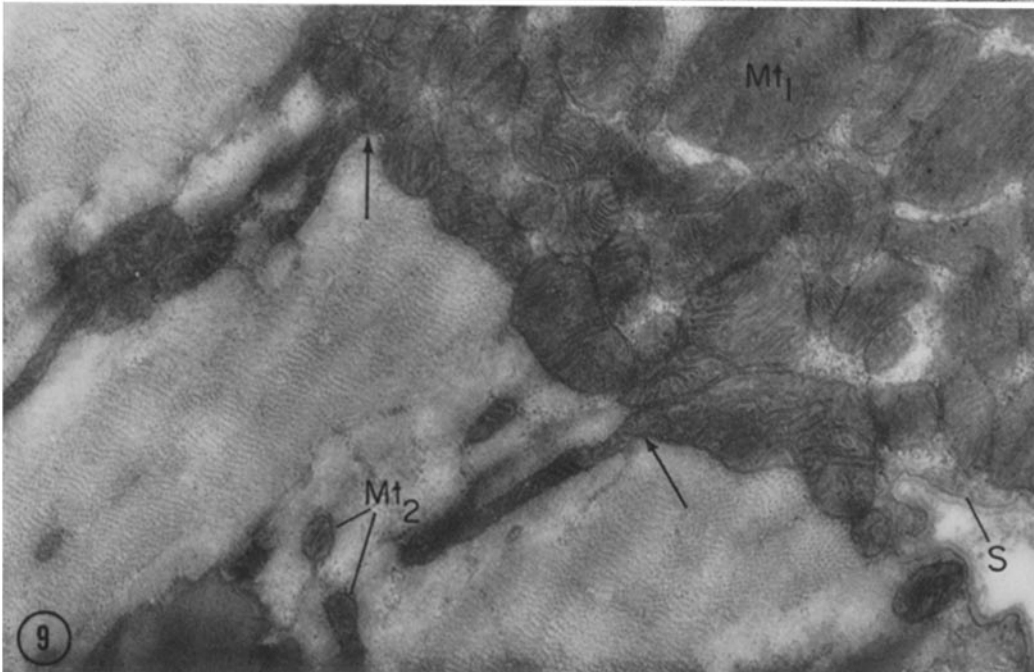
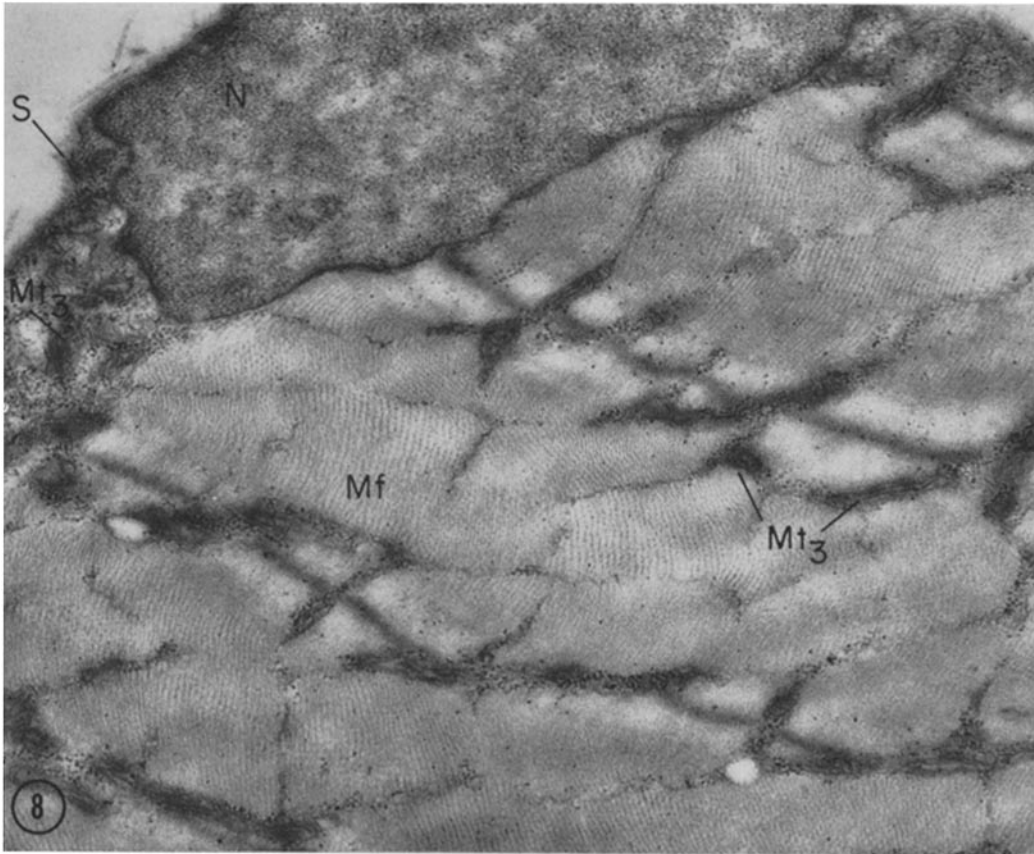
Electron micrograph of a nuclear region of a large fiber. Methacrylate section.

The perinuclear sarcoplasm contains profiles of small, slender mitochondria ( $Mt_3$ ) and glycogen particles. In the interior of the fiber, similar mitochondria are associated with the I band of the myofibrils ( $Mf$ ). These flattened slender mitochondria are surrounded by a sarcoplasm rich in glycogen particles. Thus mitochondria and components of the sarcoplasmic reticulum are somewhat obscured. The internal structure of these mitochondria may be seen more clearly in Fig. 11.  $\times 24,000$ .

FIGURE 9

Electron micrograph showing a portion of the subsarcolemmal sarcoplasm of a small fiber. Methacrylate section.

Note the large, closely packed mitochondria ( $Mt_1$ ) in the subsarcolemmal sarcoplasm. The sarcolemma ( $S$ ) is reflected over the peripheral aggregation of mitochondria, and a portion of it is shown at the lower right of the photograph. Slender projections (arrows) from two of these large mitochondria extend toward the interior of the fiber. It is possible that some of the small mitochondria ( $Mt_2$ ) represent transverse sections through such projections.  $\times 24,000$ .



may be dependent on energy derived from ATP (17), and it is possible that an ATPase may be present in the mitochondrial membrane. Although these functions are difficult to analyze both in isolated systems and *in situ*, their fundamental importance in cellular activity necessitates continued description and analysis of the enzymic mechanism.

From the present cytochemical results it is evident that mitochondrial ATPase activity of the muscle fibers of the rat diaphragm is not sulfhydryl-dependent and may occur at both neutral and alkaline pH. It is stimulated by dinitrophenol. This activity is that of a true ATPase at both pH levels, in that it attacks only ATP but not ADP or A-5-P. ADP is, in fact, inhibitory to this enzyme (28). At pH 7.2, mitochondrial ATPase in frozen sections is strongly  $Mg^{++}$ -dependent; it is possible that, at pH 9.4, calcium ion, which is the trapping agent, may be an adequate substitute for magnesium. Although most of this evidence suggests that a single enzyme is functioning at two pH levels, the possibility remains that two different enzymes may be involved. The difficult problem of the possible multiplicity of mitochondrial ATPase activity in a single cell type has been recently reviewed by Racker (29). Despite considerable biochemical evidence showing differences in ATPase activity in relation to hydrogen ion concentration or inhibitors, Racker believes there is no conclusive evidence for more than one mitochondrial protein with  $Mg^{++}$ -dependent ATPase activity in rat liver and beef heart. Both biochemical and histochemical evidence indicate, however, that mitochondrial ATPase of various tissues may be active over a wide range of pH. In histochemical demon-

strations, the mitochondria of liver, kidney, intestinal mucosa, and skeletal muscle have ATPase activity at both pH 7 and 9.

From previous histochemical experience, it was surprising to find that mitochondrial ATPase of rat muscle fibers is resistant to mercurial and may, in fact, be enhanced by it. In the kidney tubules of this species, mitochondrial ATPase activity in the alkaline range is sulfhydryl-dependent and this is inhibited by mercurial. At both neutral and alkaline pH, ATPase activity is manifested cytochemically as staining in the form of rod-like basal striations visible with the light microscope. At pH 9.4 this enzymic activity in the rat and dog kidney is known to be at least partially that of a true ATPase which is sulfhydryl-dependent (24, 10). These cytochemical results with the kidney and diaphragm of the rat suggest that tissue differences in the properties of mitochondrial ATPases exist within a single species. It has been shown by Spater, Novikoff, and Masek (31) that part of this rod-like staining at the lower pH is contributed by an ATPase activity in the basal infoldings of the plasma membrane. This enzyme may be a non-specific phosphatase, since activity of the plasma membrane of kidney tubules has also been demonstrated with phenyl phosphate (18). Although there has been no detailed comparative analysis of the rod-like staining reaction at the two levels of pH, it seems likely that it has a dual origin. Both a sulfhydryl-dependent mitochondrial ATPase and a harder phosphatase in the invaginated plasma membrane may participate in this staining reaction.

There is support in the biochemical literature for a stimulating effect on mitochondrial ATPase by mercurial (4, 28). The action of mercurial on a

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FIGURE 10

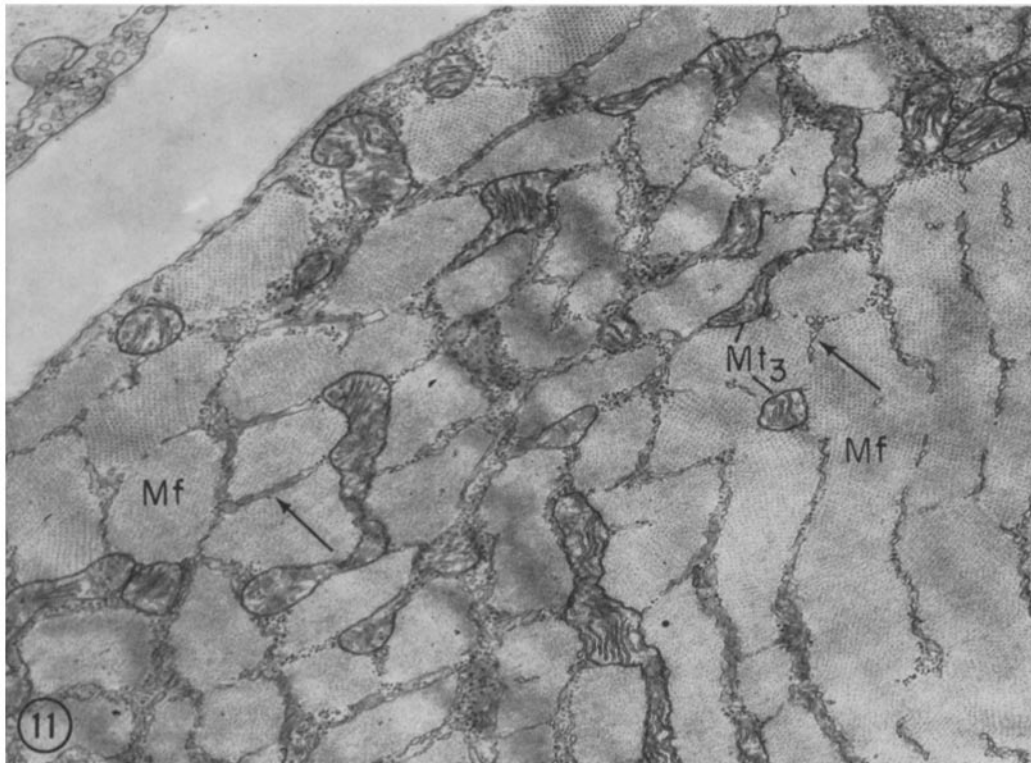
Electron micrograph of a portion of a small fiber. Epon section.

The myofibrils (*Mf*) in this longitudinal section are outlined by mitochondria (*Mt<sub>2</sub>*) and tubules of the sarcoplasmic reticulum (arrows). The small fiber is characterized by mitochondria which are larger and denser than those of the large fiber shown in Fig. 11. Compare with the light micrograph in Fig. 4 to gain an impression of the overall distribution of mitochondria in a cross-section through a whole fiber.  $\times 22,000$ .

FIGURE 11

Electron micrograph of a portion of a large fiber. Epon section.

In this oblique section, the myofibrils are outlined by mitochondria (*Mt<sub>3</sub>*) and tubules of the sarcoplasmic reticulum (arrows). The mitochondria have a less dense matrix and fewer cristae than those of the small fiber (compare with Fig. 10).  $\times 22,000$ .



soluble  $Mg^{++}$ -dependent, true ATPase from beef heart mitochondria was reported by Pullman *et al.* (28). Some of their isolated enzyme preparations were stimulated by *p*-chloro-mercuribenzoate, an observation which agrees with our findings in frozen sections of rat diaphragm. Furthermore, preliminary observations on the properties of mitochondrial ATPase in frozen sections of rat heart indicate that this activity is sustained and even enhanced by this mercurial. The biochemical and cytochemical observations on mitochondrial ATPases of rat liver (4), beef heart (28), and rat diaphragm suggest collectively that there is an inhibitory grouping close to the active site of the enzyme. The inhibitory effect can be diminished by dinitrophenol, mercurial, or  $Ag^+$  ion. The complexities of the possible interrelated effects of divalent cations, such as  $Mg^{++}$  or  $Ca^{++}$ , mercurial, and dinitrophenol,

on ATPase activity in digitonin extracts of liver mitochondria are explored by Cooper (4).

Myosin ATPase appears to have a dual response to mercurial compounds. Although *p*-chloro-mercuribenzoate is generally viewed as an inhibitor of myosin ATPase, it can also, at low concentrations, cause an activation of this enzyme (13). Kielley and Bradley (14) demonstrated a biphasic response during the titration of *p*-chloromercuribenzoate with myosin. When about half of the sulfhydryl groups have been titrated with this mercurial, there is a marked activation. Further combination with the mercurial leads to inhibition that becomes complete when all the sulfhydryl groups have reacted. In the present cytochemical study, it is clear from the sites of activity that the level of *p*-hydroxymercuribenzoate which was used inhibited myosin ATPase completely but fostered mitochondrial ATPase activity (Fig. 17).

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FIGURE 12

ATPase, pH 7.2, 30 minutes, transverse section.

In the small fibers ( $M_2$ ), enzymic activity is concentrated in peripheral masses (arrow) as well as in central granules. These sites correspond to the distribution of mitochondria, as illustrated in Fig. 4. In the large fibers ( $M_1$ ), ATPase activity is localized in central granules but there is no intense peripheral or subsarcolemmal activity.  $\times 1400$ .

FIGURE 13

ATPase, pH 7.2, 15 minutes, cysteine, transverse section.

Cysteine has two effects on the reaction: (1) there is an over-all darkening of all of the large fibers ( $M_1$ ) and some of the smaller fibers ( $M_2$ ), and (2) a prominent reticulum appears and is especially evident in the large fibers. These effects may be studied at higher magnification in Figs. 14 and 15.  $\times 625$ .

FIGURE 14

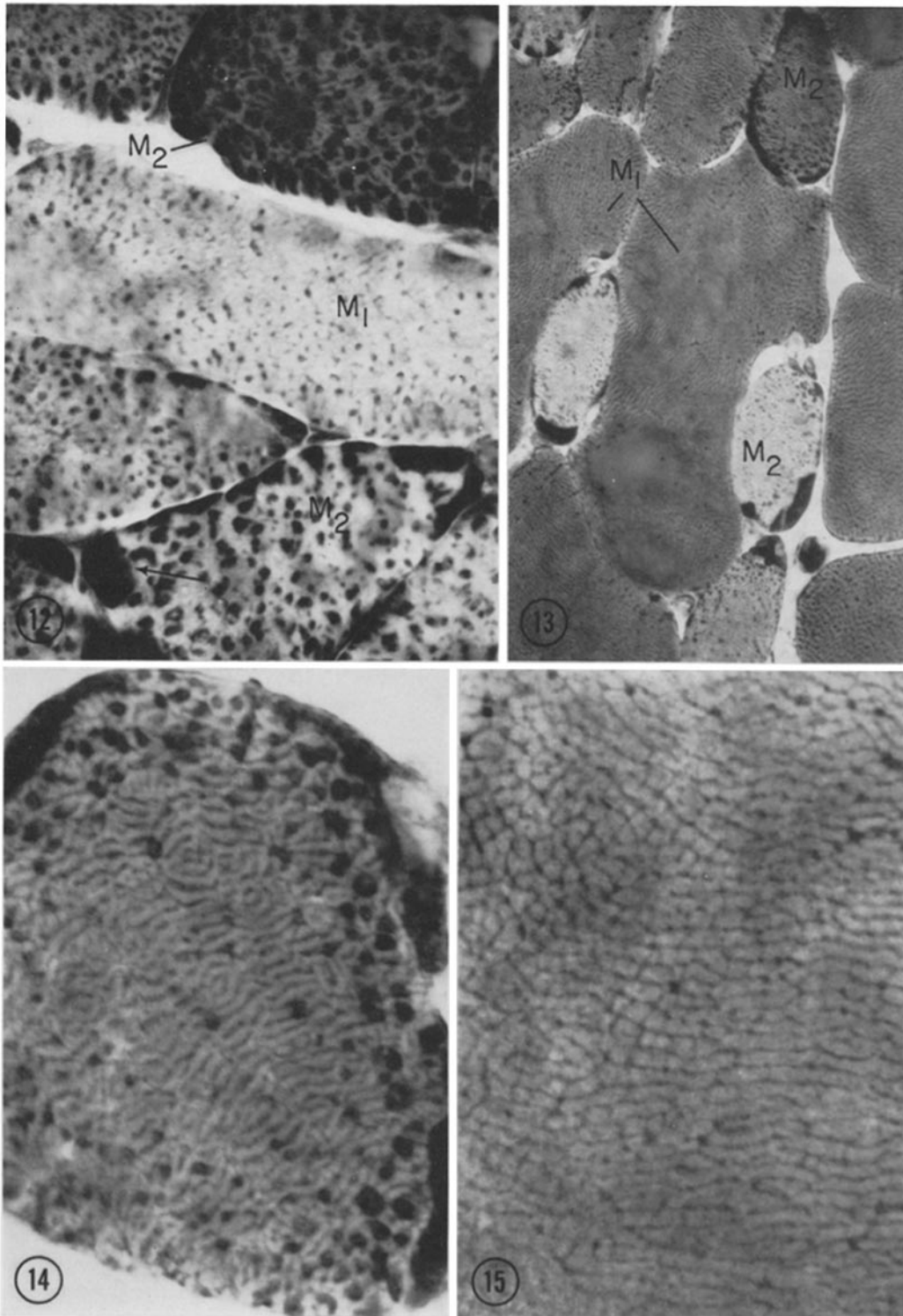
ATPase, pH 7.2, 15 minutes, cysteine, transverse section.

A section through a small fiber is shown at high magnification. In addition to subsarcolemmal ATPase activity, there are large granular sites of activity in the periphery of the fiber which correspond to mitochondria. Throughout the interior of the fiber, ATPase activity is localized in small strands which may represent small mitochondria or components of the sarcoplasmic reticulum.  $\times 2000$ .

FIGURE 15

ATPase, pH 7.2, 15 minutes, cysteine, transverse section.

A portion of a large fiber is illustrated here. The product of enzymic activity is deposited in the form of a continuous network or lattice outlining the myofibrils. Occasionally at intersections there is a heavier granular deposit or nodal point. This pattern resembles that obtained after metal impregnation, according to the procedure of Veratti (34). The similarity in the distribution of the reaction product here suggests the presence of ATPase activity in the sarcoplasmic reticulum.  $\times 2000$ .



The insensitivity of mitochondrial ATPase of rat diaphragm to a mercurial compound and formalin may explain certain previous histochemical results obtained in this laboratory. The fibers of the biceps femoris of the rat stained quite uniformly in a demonstration of ATPase activity at pH 9.4; however, in the presence of mercurial, the small fibers stained more intensely than the large fibers (Nachmias and Padykula (21), Figs. 13 and 14). These earlier preparations did not permit cytological localization of the reaction product. In the light of the present results with the rat diaphragm, however, it may be possible in retrospect to interpret our earlier results with sections of the biceps femoris as the result of simultaneous activation of mitochondrial ATPase and inhibition of myosin ATPase by the mercurial. Thus, intense staining of the small fibers reflected their higher content of mitochondrial ATPase, while diminished staining of the large fibers is related to the proportionately higher myosin ATPase activity which is inhibited by the mercurial compound. Similarly, the differential

effect of formal-calcium fixation on fibers of the gastrocnemius and soleus muscles of the rat (32) may reflect an inhibition specifically of myosin ATPase. At pH 9.4, large fibers became inactive after fixation, but small fibers retained their activity because of a high content of mitochondrial ATPase which has been shown in the present study to be a robust enzyme which survives formalin fixation (Fig. 22).

#### *Sarcotubular ATPase*

In the fibers of the rat diaphragm there is an ATPase activity at pH 7.2 which is sarcoplasmic in position and envelops the myofibrils. In demonstrations with the lead method using cross-sections of muscle fibers, the reaction product forms a reticular pattern with nodal points (Fig. 15), a configuration which closely resembles the silver-stained reticulum of Veratti (34). The comparison suggests that this ATPase may be associated with the sarcoplasmic reticulum. Since the reticulum is demonstrable through impregnation with heavy metals, it is important to consider whether the

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FIGURE 16

ATPase, pH 9.4, 10 minutes, transverse section.

ATPase activity occurs quite uniformly throughout the fibers in locations which correspond to the myofibrils. Some of the large spherical unreactive sites undoubtedly are vacuoles which are formed by removal of lipid droplets during the dehydration and clearing of the section. In addition, some of the unreactive sites represent mitochondria which do not show ATPase activity under these conditions. Compare with Figs. 12 and 17.  $\times 1600$ .

FIGURE 17

ATPase, pH 9.4, 10 minutes, *p*-hydroxymercuribenzoate, transverse section.

Compare with Fig. 16. In the presence of this mercurial, the myofibrillar ATPase is completely inhibited, and an ATPase activity is evoked, the location of which suggests that it originates in the mitochondria. The mitochondria in the peripheral position (arrow) and in the interior of the fibers have become reactive.  $\times 1600$ .

FIGURE 18

ADP, pH 7.2, 30 minutes, transverse section.

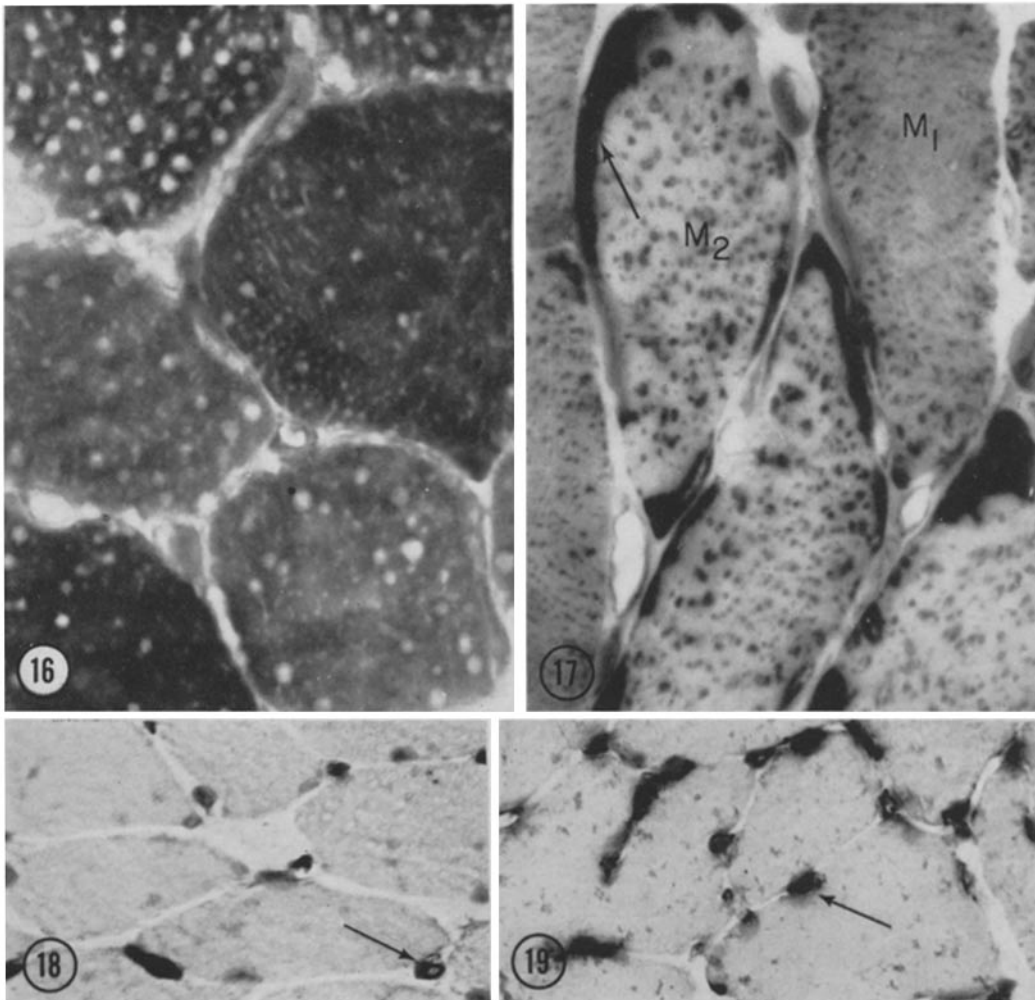
The muscle fibers are completely unreactive toward ADP. Components of the blood vessels (arrow), however, can split this substrate. The same localization is obtained at pH 9.4. Compare with Figs. 12 and 19.  $\times 625$ .

FIGURE 19

A-5-P, pH 7.2, 45 minutes, transverse section.

As with ADP, the muscle fibers are unreactive, but there is enzymic activity in the walls of the intervening blood vessels (arrow). The same localization is obtained at pH 9.4. Compare with Figs. 12 and 18.  $\times 625$ .





lead ion which is the trapping agent in this cytochemical procedure may have been bound in a non-specific manner. Because frozen sections incubated in a medium lacking ATP fail to show this reticulum, it may be concluded that its presence is substrate-dependent and thus enzymic in origin. The reticulum becomes apparent when the demonstration is performed in the presence of cysteine, which suggests the participation of a sulfhydryl-dependent enzyme. Also the reticular pattern is more prominent in the large white fibers than in the small fibers. It is not clear whether the sarcoplasmic reticulum in the large fiber is more elaborate or whether its morphological disposition is such that the pattern is more evident than in the small fiber. It seems likely that the large numbers of mitochondria present

in the small fiber could account at least in part for the more interrupted pattern shown in Fig. 14.

It is difficult to separate mitochondrial ATPase from sarcotubular ATPase in these frozen sections, since both occur in the sarcoplasm and both are active under similar conditions. It seems unlikely, however, that the extensive, continuous reticular pattern of very slender strands observed in the large fibers could be the product of mitochondrial activity. The mitochondria in the same type of fiber are arranged more intermittently, are of larger diameter, and are relatively sparse. Furthermore, reaction products at sites of mitochondria would be expected to be localized as distinct granules and threads, as seen in the large fibers in Fig. 12. The less continuous pattern observed in small fibers under the same conditions (Fig. 14)

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FIGURE 20

Lipids. Sudan black. Longitudinal section.

Both small ( $M_2$ ) and large ( $M_1$ ) fibers are illustrated, but since the plane of section is usually not radial through all the fibers, the size difference is not always apparent. In both types, the I bands ( $I$ ) are moderately sudanophilic and have a granular appearance, most likely a result of the alignment of mitochondria at this level. It has been reported, however, that the I band of the myofibril itself is also sudanophilic (8). Note the numerous lipid droplets in one of the fibers ( $M_2$ ). Along the periphery of one fiber (arrow) there is a heavy sudanophilic band which is attributed to the subsarcolemmal accumulations of large mitochondria. Note also the variation in the number of triglyceride droplets of these fibers.  $\times 625$ .

FIGURE 21

Electron micrograph of a small, lipid-rich fiber. Epon section.

In this longitudinal section, the cross-banding of the myofibrils is evident; mitochondria ( $Mt_2$ ) are usually aligned with the I band ( $I$ ), occurring often as paired elliptical profiles on opposite sides of the Z line. In addition, in the sarcoplasm there are delicate profiles of tubular and cisternal components of the sarcoplasmic reticulum.  $\times 6500$ .

FIGURE 22

ATPase, pH 7.2, 2 hours' incubation, formalin fixation, longitudinal section.

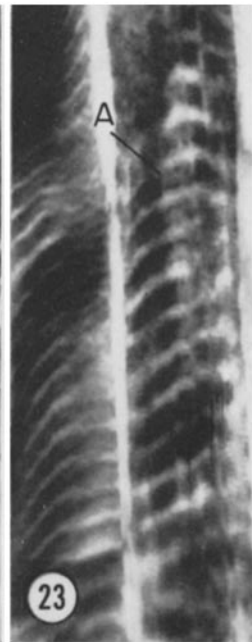
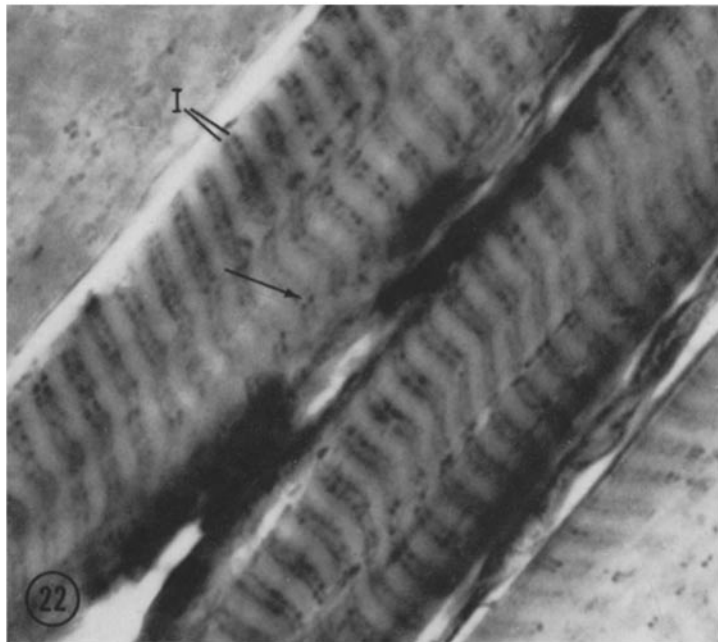
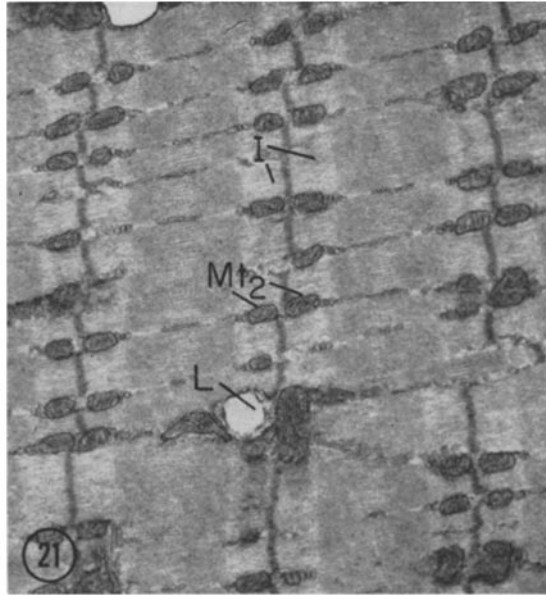
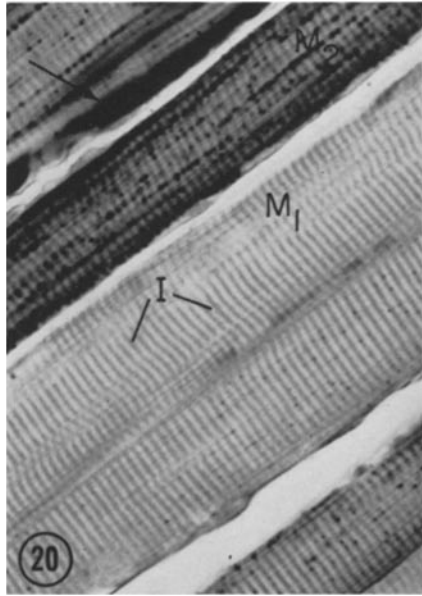
Two small fibers occupy the center of the photograph. ATPase activity is localized within paired small granules (arrow) which align transversely to form two discrete lines. These sites of activity correspond to the I band ( $I$ ), when this cytochemical preparation is examined with polarized light (See Fig. 24). Compare the distribution of these granules with the location of mitochondria shown in Fig. 21.

This cryostat section was fixed in formalin before incubation; the resulting partial inhibition required a longer incubation to demonstrate an adequately visible reaction product.  $\times 2000$ .

FIGURE 23

ATPase, pH 9.4, 10 minutes, longitudinal section.

Enzymic activity is localized within broad cross-striations which correspond to the A band ( $A$ ). The I band is unreactive. The longitudinal sections of unfixed fibers contract in this incubating medium, making precise localization difficult.  $\times 1600$ .



probably reflects an interruption of sarcothubular components by larger numbers of mitochondria present in the small fiber (Fig. 7).

The sarcoplasmic reticulum of the semitendinosus and sartorius muscles of the frog has been isolated and identified in particulate form by Muscatello, Andersson-Cedergren, Azzone, and von der Decken (19). In addition to having a relaxing effect, these isolated vesicles have a  $Mg^{++}$ -stimulated ATPase activity which is inhibited by  $Ca^{++}$ . Since mitochondrial ATPase of these frog muscles is not depressed by  $Ca^{++}$ , these investigators cite this as a major difference between the mitochondrial and sarcothubular ATPases, both of which are  $Mg^{++}$ -stimulated.

In frozen sections of rat diaphragm,  $Ca^{++}$  was without effect on the reticular pattern of ATPase activity. However, further exploration of differences between the ATPases of these two organelles might lead to their separation *in situ*.

Miss Ann G. Campbell and Miss Eileen Hall were technical assistants in the cytochemical studies. Mr. Leo Talbert prepared the light micrographs. Their generous cooperation is most gratefully acknowledged.

This study was supported by the United States Public Health Service Grant RG-3298 (C 9) and a Research Career Development Award to the senior author.

Received for publication, December 14, 1962.

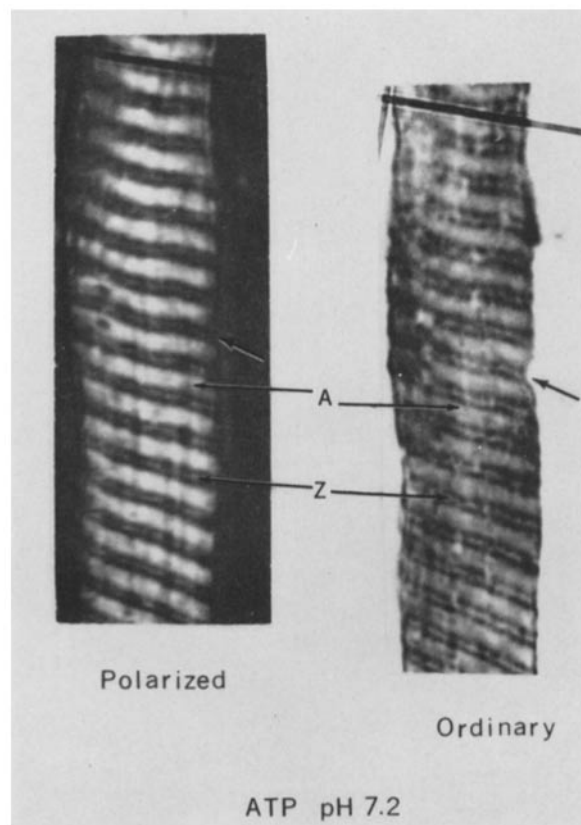


FIGURE 24

A longitudinal section of a single muscle fiber which demonstrates ATPase activity at pH 7.2.

This fiber was photographed with both polarized light and ordinary light. Arrows mark a notch present at the edge of the fiber, which may be used as a reference point. The A band (*A*) is anisotropic (left) and shows no enzymic activity (right). The I band, which is bisected by a birefringent Z line (*Z*), exhibits no birefringence itself (left), but shows intense enzymic activity (right). The reaction product is, therefore, aligned with the I bands.  $\times 2000$ .

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