Cellular organization of rhesus extraocular muscle

James E. Miller

Rhesus extraocular muscle appeared to be divided into three zones: an outer small-fiber layer, an intermediate area containing a mixture of small and large cells, and a large-cell area in the center of the muscle and adjacent to the globe. The outer and intermediate layer had the histochemical and electron microscopic characteristics of red muscle, while the large-cell area appeared to be white muscle. The small cells were of two types with varying mitochondrial configuration and histochemical characteristics. The sarcotubular apparatus of both cells was limited almost entirely to the I band. The M line was absent from the center of the A band. The large cells of the intermediate zone contained abundant columns of mitochondria with the sarcoplasmic reticulum outlining the entire length of the myofibrils, and the M line was absent. The large cells were found in the white portion of the muscle with the addition of two other cellular types. A Felderstruktur type fiber contained a smaller number of transverse tubules and triads. A reduced number of mitochondria were paired on either side of the Z line and partially encircled the I band. The M line was either absent or poorly formed, and all enzymatic determinations were reduced. The majority of the white portion of the muscle consisted of typical twitch fibers with a well-developed M line, sarcoplasmic reticulum and transverse tubular system, fewer mitochondria, and the histochemical characteristics of white fibers. It is proposed that the outer red portion of the muscle is utilized for slower eye movements and the inner white portion for faster movements. The variation of sarcoplasmic reticulum and enzymatic content of the cells without an M line may also represent a modification of slow fibers with different degrees of slowness.

The extrinsic eye muscles appear to be the most highly organized of all striated muscles. The innervational ratio, the number of muscle cells to one nerve, is described as the lowest in all muscular systems. Nerve supply, both afferent and efferent, is extremely diverse and ranges from the small nonmyelinated gamma system to large, heavily myelinated alpha fibers. A large variety of motor end plates[1-4] and stretch receptors[5] are present. Different types of muscle fibers were identified by light microscopy,[6, 7] histochemistry,[8-10] electron microscopy,[1, 3, 11, 12] and by their pharmacological[13, 14] and electrophysiological properties.[11]

Included in extrinsic eye muscle are slow fibers[1, 11] which may account for many of the unusual characteristics of eye muscle. Slow fibers are a unique type of striated muscle cell found in fish, amphibians, and reptiles. Each fiber is supplied by multiple motor end plates, often called "en grappe,"

From the Department of Ophthalmology, Albany Medical College, Albany, N. Y.

This investigation was supported in part by research grants NB 04936 and NB 05794 from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, United States Public Health Service, and the National Society for the Prevention of Blindness, Inc.

and is capable of a graded contracture.[15] Shortening is not accompanied by a propagated action potential and would be difficult to identify by electromyography. Slow fibers also contract when exposed to cholinergic compounds;[16] a similar response was observed in extraocular muscle.[13, 14] Present knowledge indicates that mammalian slow fibers are located only in the extraocular muscles and the tensor tympani[17] of the middle ear.

One of the microscopic characteristics of slow fibers appears to be absence of the M line. This observation was originally made in frog slow fibers classified by electrical stimulation,[18] and was later considered the major distinguishing feature of this cell.[19] Micrographs of longitudinal sections from mammalian slow fibers identified by intracellular recording[11, 17] fail to show an M line. Another characteristic of mammalian slow fibers is branching of myofibrils to form an interlacing network between large masses of mitochondria in the interfibrillar spaces.[17] Z bands were originally described as jagged or broken,[1] but this feature was not emphasized in a later report.[11] The sarcoplasmic reticulum and transverse tubular system are not as well developed as in twitch fibers, indicating poorer excitation-contraction coupling.

Slow fibers resemble Felderstruktur muscle fibers, a light-microscopic classification based on the shape of the myofibrils.[7] In cross-section, Felderstruktur fibers contain large block or ribbonlike myofibrils in contrast to small discrete myofibrils of Fibrillenstruktur fibers. Differences in the distribution of the sarcoplasmic reticulum of slow and twitch fibers may account for the shape of the myofibrils. In twitch fibers the sarcoplasmic reticulum encircles the myofibrils, whereas slow fibers have a patchy distribution of sarcoplasmic reticulum with the contractile elements incompletely divided into irregular or large myofibrils. Felderstruktur fibers correlate with slow contraction and Fibrillenstruktur with fast contraction. Unfortunately, the shape of the myofibrils cannot be used to distinguish slow fibers, since Felderstruktur muscle cells have been found in muscles that consist entirely of twitch fibers.[20]

Histochemical methods have been used as a system to classify different types of muscle fibers. This concept is based on high levels and intense histochemical staining of oxidative enzymes and reduced amounts and staining of glycolytic enzymes in red muscle and red fibers. White muscle and white fibers contain greater amounts of glycogen and glycolytic enzymes and less oxidative enzymes. Intermediate forms between red and and white fibers are also recognized. A number of cytochemical relationships have been described, but the more frequently used is the inverse staining proportion between succinic dehydrogenase and phosphorylases[21] or alpha-glycerophosphate dehydrogenase.[22] Furthermore, only amphibian and fish muscles were found to contain small cells with high levels of both succinic dehydrogenase and phosphorylase which may represent slow fibers as these were described as small fibers.

The rhesus monkey has eye movement and a muscle fiber content similar to man.[6] This animal would appear to be suited for morphological and histochemical evaluation of eye muscle and serve for further exploration. When the above histochemical methods were applied to rhesus extraocular muscle, a number of fibers were identified distributed between red, white, and the unusual fiber of amphibians and fish. The fibers were arranged in a histochemical gradient from a slow or red type exterior to a fast or white portion adjacent to the globe.

Method

Progressive degeneration has been described in extraocular muscle. The earliest changes appear around the age of puberty in man[23] and laboratory animals.[24] In an effort to eliminate aging changes from this study, eleven prepubertal monkeys were used. The muscles were obtained within five minutes after decapitation or dissected from animals anesthetized with sodium amytal.

The specimens were prepared by several methods in order to recognize morphologic changes

produced by muscle contraction. A perforated support was placed behind the muscle in situ and the origin and insertion clamped with hemostats. The supported muscle was excised and fixed at resting length with 5 per cent glutaraldehyde or 10 per cent formalin. For further relaxation, other clamped muscles were incubated for ten minutes in 0.5 per cent cold buffered lidocaine or 1 per cent tetracaine prior to fixation. Measured portions of the muscle were also excised in situ. These were anesthetized and re-extended to the original length. The best orientation and relaxation for phase microscopy was obtained by clamping the muscle in situ and incubating in local anesthetic. Satisfactory orientation and relaxation were also observed in anesthetized portions of muscle re-extended to the original length.

For frozen sections, the ends of the muscles were tied at resting length with sutures across bent copper rods. The entire specimen was placed in cold anesthetic for ten minutes and/or dipped in talcum powder and frozen in liquid nitrogen.[25] Specimens for longitudinal sectioning were cemented to a slice of frozen liver with gum tragacanth and solidified on a brass object disk by the quick-freeze block of an International cryostat. Material for transverse sectioning was prepared by dividing the frozen muscle into thirds with a jeweler's saw maintained in the cryostat. Origin, middle, and insertional portions of the muscle were mounted on liver with gum tragacanth and frozen on the object disks. Sections were cut at -28° C. at thicknesses of from 2 to 30 μ. Sections 6 μ thick were screened with Gomori's trichrome stain applied to unfixed frozen sections.[26] This stain distinguishes the orange to red sarcoplasm from the greenish-blue myofibrils. Fibrous tissue stained light green, myelin sheaths of nerves orange, and the enclosed axons light green. In thin, well-oriented transverse sections, the myofibrils were surrounded by a fine reddish network that corresponded to the sarcoplasmic reticulum. The best differentiation was obtained by staining 6 μ sections for five minutes.

Longitudinal frozen sections from muscles clamped in position or extended after excision had comparable cellular organization by bright-field microscopy. Minimal improvement in morphology was noted from bathing muscle strips in local anesthetic. In order to eliminate any effect of the anesthetic agent, the histochemical determinations were made on untreated muscle.

Serial sections were cut from at least half of each frozen block and prepared in the following repeating sequence:

(1) The first section (6 μ) was stained with Gomori's trichrome.

(2) Succinic dehydrogenase was demonstrated by incubating the second 12 to 30 μ section for 30 minutes to 1 hour in 37° C. substrate media containing 0.05 molar sodium succinate, 0.05M phosphate buffer, pH 7.6, 7.5 per cent polyvinylpyrrolidone (PVP, m.w. 10,000), and 1 mg. per milliliter of either nitro blue tetrazolium (NBT) or MTT tetrazolium. The sections were fixed in 10 per cent formalin for 10 minutes, washed for 5 minutes with 15 per cent alcohol, and mounted with a cover slip and glycerol jelly.

(3) The third section (12 to 30 μ) was treated to show alpha-glycerophosphate dehydrogenase (α-GPD) by incubating for 30 minutes in 37° C. substrate media consisting of 0.1M disodium alpha-glycerophosphate, 0.1M nicotinamide adenine dinucleotide (NAD), 0.01M sodium cyanide, 0.005M magnesium chloride, 0.05M Tris buffer, pH 7.6, 1 mg. per milliliter of MTT or NBT, and 7.5 per cent PVP. The sections were prepared and mounted as in (2) above.

(4) The fourth section (12 to 30 μ) was incubated in the above media for 30 minutes with the addition of 1 mg. per milliliter of phenazine methosulfate.[27]

(5) The fifth section (12 to 30 μ) was handled as in (3) above, with 0.1 mg. per milliliter of menadione substituted for NAD.[28]

(6) The sixth section (12 to 30 μ) was prepared to localize with glucosan transphosphorylase and transglycosylase by incubating for 2 hours at 37° C. in substrate media containing 0.13 mM. of dipotassium glucose-1-phosphate, 0.03 mM. of adenosine-5-phosphate, 0.04M acetate buffer, pH 6, and primed with 1 to 2 mg. of glycogen and 1 drop of U-40 regular insulin. Sections were hydrated by passage through 40 per cent alcohol and water, dried at 37° C., and fixed for 3 minutes in absolute alcohol. Color was developed by an overlay of 0.1 strength Gram's iodine for 5 minutes, and the section was mounted with glycerin containing 0.1 strength Gram's iodine and a cover slip. The presence of glucosan phosphorylase or straight chain enzyme was indicated by a dark blue color within the cells. Transglycosylase or branching enzyme produced a reddish hue that resisted digestion with beta amylase in the following section.

(7) The seventh section was prepared as in (6) above but incubated in beta amylase at 37° C. for 5 hours before iodination.

Histochemical reactions were absent when substrates were omitted from the incubating media or beta glycerophosphate was substituted for alpha glycerophosphate. The histochemical staining for transglycosylase was eliminated by adding 20 per cent ethyl alcohol to the reaction and all phosphorylase end product was abolished by digestion for 1 hour in alpha amylase.

Histochemical determinations for oxidative enzymes may be influenced by the dye marker utilized in the reaction.[29] The effect of NBT and MTT tetrazolium upon the intensity of the reaction was compared in alternate succinate media. The

relative intensity of cells appeared equal in serial sections.

The cytochemical staining for α-GPD may also be altered by the type of hydrogen (electron) acceptor used.[27] Three different reagents were alternately tried: NAD, NAD and phenazine methosulfate, and menadione. Mitochondrial localization was accentuated in media containing NAD and sarcoplasm when phenazine methosulfate was included. This enzyme was localized in both sarcoplasm and mitochondria with menadione. The variation in intensity between layers and cells was visible in all of the preparations but was more marked in the menadione slides.

The prepared sections were viewed and photographed with a Leitz Labolux microscope using Adox KB-14 film and a Zeiss VG-9 filter. Selected muscle fascicles were identified in each transverse preparation and photographed with the same shutter speed and intensity of illumination. The 35 mm. frames were enlarged to 5 by 7 inches. Exposure and development time were identical for all prints. Each cell was numbered and followed throughout in the photographs. The same cells were identified for distances of 500 μ or longer with similar repeating morphological and histochemical characteristics. All cells showed some activity for the various enzyme systems and were graded from 1 to 4+ by two observers.

With continuing transverse sections it was possible to follow a single cell for any change in histological or histochemical characteristics. This was not possible with longitudinal sections, since many cells measured less than 20 μ in diameter. However, the same characteristics were maintained throughout their longitudinal extent. Longitudinal sections were used primarily for identifying the relationship between the A, I, and Z bands and mitochondria.

Clamped muscles were fixed in 10 per cent buffered formalin for 24 hours, dehydrated, and embedded in a combination of butyl and methyl methacrylate. Prior to polymerization, the muscles were cut transversely into three pieces: origin, middle, and insertion. Each portion was placed in a gelatin capsule and oriented for transverse sectioning. The capsule was filled with partially polymerized methacrylate mixture and covered with the other end of the capsule. Polymerization was completed in a drying oven at 60° C. for 12 hours. Full cross-sections were cut on a Sorvall MT-1 ultramicrotome with glass knives at thicknesses of 2 μ or less. Sections were transferred to glass slides, stained with toluidine blue, and mounted in immersion oil under a cover slip. Myofibrils, nuclei, and cellular membranes stained blue; details were further accentuated by phase microscopy.

Relaxed extended muscle was also fixed in 6 per cent glutaraldehyde and 2 per cent acrolein for 1 hour. Aldehyde-fixed tissue was stored in 0.2M Sørensen's phosphate buffer, pH 7.4, and selected portions were taken at various depths within the muscle from origin, middle, and insertion. These were postfixed in Dalton's fixative for 30 minutes. The tissue was dehydrated in ascending strengths of ethyl alcohol and polymerized in Epon 812 or Maraglas. The embedded muscle strips were cut from the resin block and oriented in gelatin capsules. The capsules were filled with the appropriate resin and repolymerized.

Phase and thin sections were cut on a Sorvall MT-1 or MT-2 ultramicrotome with glass or diamond knives. Thick sections were picked up on glass slides and stained with hot 1 per cent aqueous paraphenylenediamine and viewed directly by oil-immersion bright-field or phase microscopy. Mitochondria, nuclei, and Z bands stained dark brown, myofibrils were less densely stained; the A, I, and M bands and H zone could be distinguished in longitudinal sections by phase microscopy. The sections were viewed and photographed with a Zeiss GFL phase microscope with a 100× Neofluar objective and a V-Z oil immersion condensor. Thin sections were floated onto uncoated copper grids and stained with 0.2 per cent uranyl acetate for 15 minutes and Reynolds lead citrate for 4 to 5 minutes. An RCA EMU-3F or 3-G electron microscope was used to examine and photograph the sections.

Result

The exterior of an extraocular muscle was covered with a small-cell layer that was more prominent on the orbital surface (Figs. 1 and 2), and absent or irregularly distributed adjacent to the globe. In cross section this layer was C-shaped in outline. The nerve penetrated the muscle within the incomplete portion of the "C" in the posterior third of the muscle. Penetration occurred in the middle third of the inferior oblique. The small-cell layer was 1 to 3 fascicles in depth, with each fascicle containing 8 to 25 cells. Beneath the small cells was an intermediate zone of both large and small fibers, 1 to 6 fascicles across. This portion was well developed in the obliques and medial and lateral recti. The intermediate layer combined with the central portion of the muscle consisting of larger cells which progressively increased in size toward the globe (Figs. 1 and 3). The change in cellular size was more obvious in the middle third of a rectus.

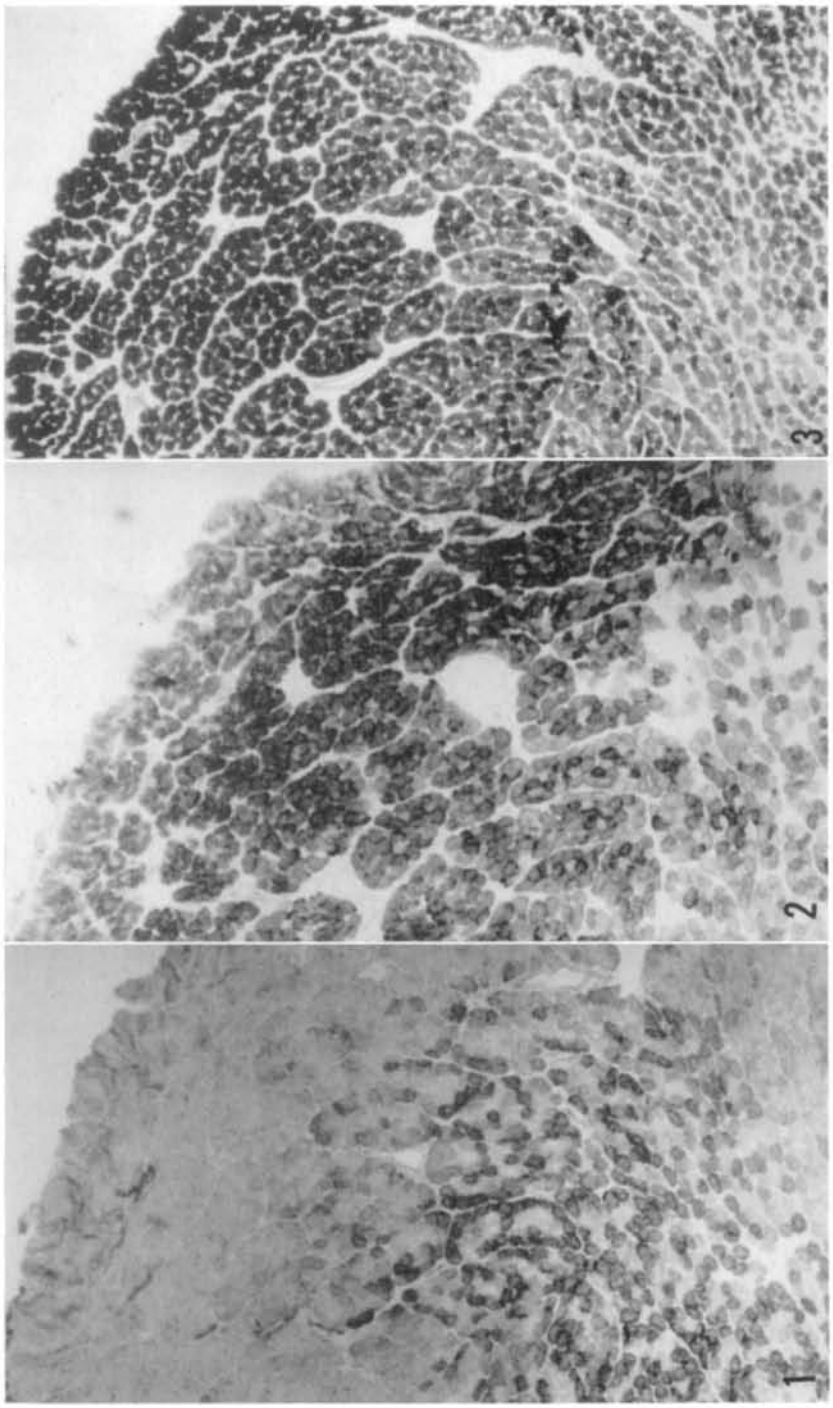

Fig. 1. Transverse sections of superior rectus temporal margin, insertional end. (×45.) *1*, α-GPD histochemical preparation. The most intense localization is in the central portion of the muscle. The periphery is uniformly low in activity. 2, Succinic dehydrogenase. The highest level is located between the small cells of the periphery and the central part. 3, Phosphorylases. The small marginal cells are the most reactive.

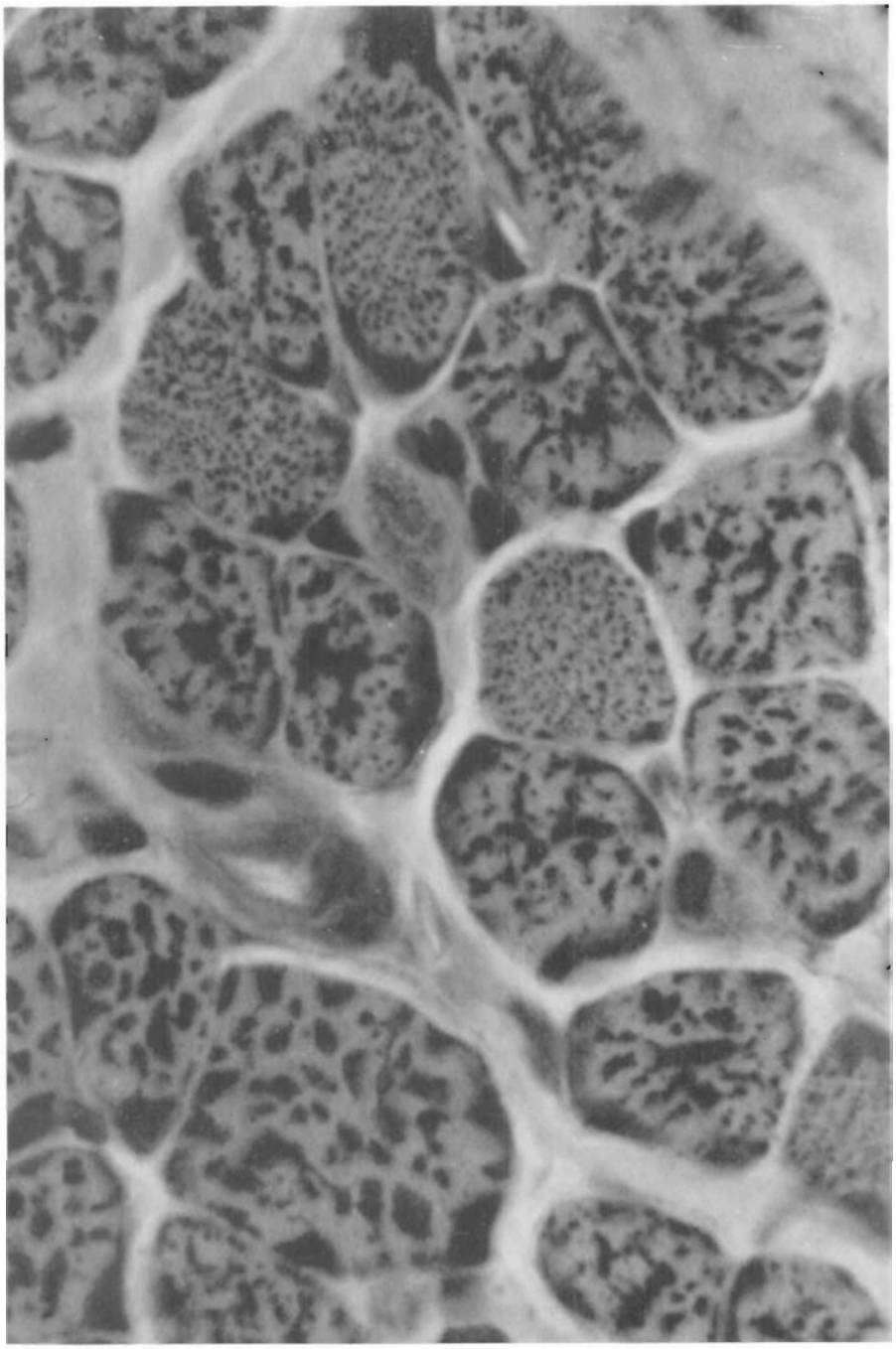

Fig. 2. Slightly oblique section of the outer fibers of the superior oblique, middle portion. Fibers located about the periphery are smaller and consist of two types. The predominating cells contain scattered irregular areas of dark-staining cytoplasm and occasional fine linear structures consistent with sarcoplasmic reticulum. The other small cells have punctate areas of sarcoplasm connecting with a few fine lines of sarcoplasmic reticulum. In the upper left hand corner is a large cell of the intermediate zone which includes large areas of sarcoplasm and a network of sarcoplasmic reticulum. (Gomori's trichrome stain. ×1,250.)

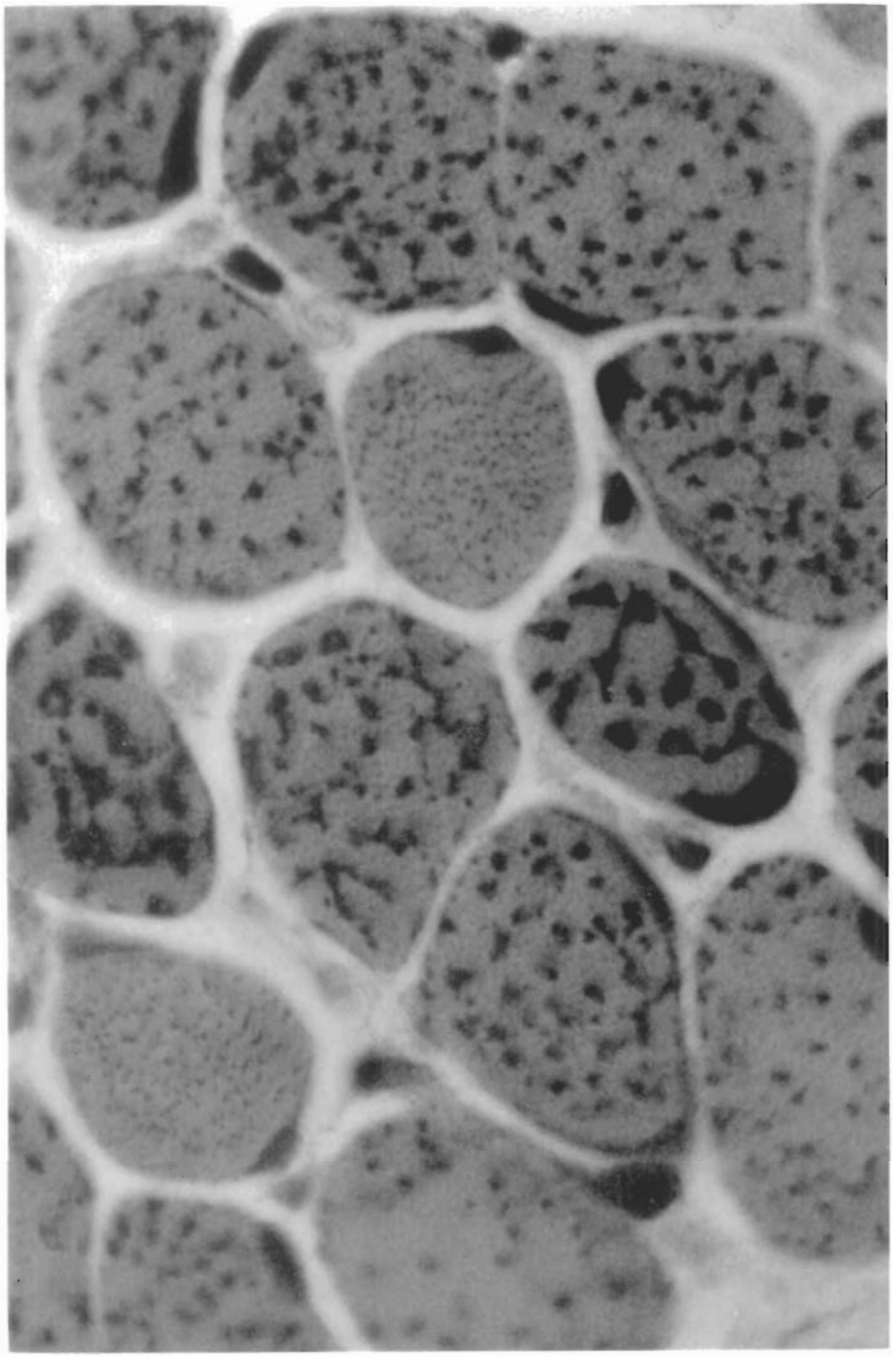

Fig. 3. More centrally located cells in the same section as Fig. 2. The central and global margin contains three cells with a variation in sarcoplasm. Two of the cells are composed of large myofibrils surrounded by a sarcoplasmic network interspersed with small oval mitochondria. Immediately adjacent to the latter fibers are cells with large sarcoplasmic areas and a fine reticulum outlining the myofibrils. The remaining cells contain less sarcoplasm and a prominent reticulum. (×1,250.)

Although the size distribution was not absolute, the smallest cells were usually located around the periphery and the largest adjacent to the globe. The levator varied from the other muscles in that the periphery had only scattered areas of small cells and the intermediate zone was not recognizable.

Histochemical determinations indicated that the variation in cell size was accompanied by enzymatic alterations as well (Figs. 1, 4, and 5). The small-cell layer and the intermediate zone were higher in succinic dehydrogenase and phosphorylase activity than the remaining portion of the muscle. However, comparison between these two layers showed more succinic dehydrogenase reaction and less phosphorylase in the intermediate area. The small-cell layer was characterized by a greater localization of phosphorylases and slightly less succinic dehydrogenase. The greatest reaction for α-GPD was in the global and central portion of the muscle with the intermediate and outer fiber layer appearing as a homogeneous, faintly staining mass (Fig. 1). Greater diversity of all histochemical reactions was also present in the central and global portion of the muscle.

The small cells located about the periphery consisted of two types (Fig. 2). The more frequent cell contained an abundance of sarcoplasm distributed in large, irregular spaces, often occupying one third of the cross sectional area. In trichrome slides the cell did not appear to have a well-developed sarcoplasmic reticulum, since the myofibrillar areas were not outlined by the fine, honeycomb pattern observed in other cells more centrally located. Phase cross-sections also indicated a loose areolar pattern of contractile material rather than separate myofibrils. In longitudinal phase sections the periphery of the cell was characterized by scattered nuclei, sarcoplasmic masses, and lipid droplets (Fig. 6, *A*). The myofibrils were also separated by lipid droplets and columns of interfibrillar sarcoplasm high in mitochondrial content. Prominent Z bands divided the longitudinal sections into regular sarcomeres. The center of the sarcomere contained a well-defined H zone without an M line.

Similar features were observed in electron micrographs (Figs. 7 and 8). The sarcoplasmic reticulum was almost entirely limited to the A bands, and consisted of a network of tubules projecting through the Z line and surrounding the thin filaments. A few scattered longitudinal tubules extended into the thick filaments and there was almost total lack of a sarcoplasmic reticulum sleeve throughout the A band. The absence of a sarcoplasmic reticulum sheath surrounding the entire fibril would also account for the lack of separate myofibrils noted in phase or trichrome cross-sections. The remainder of the sarcotubular apparatus was well developed, with numerous transverse tubules and triads adjacent to the margins of the A bands. Oval or globular mitochondria with highly developed cristae were interspersed with lipid droplets in the subsarcolemmal, interfibrillar, and perinuclear spaces. Often the mitochondria numbered 2 to 3 across the sarcomeres and in many areas the transverse tubules traversed the mitochondrial collections. The center of the A band contained an H zone but no M line. Between the mitochondrial masses the Z lines were usually in register and continuous, except for small interruptions by the sarcoplasmic reticulum. Glycogen was heavily localized in sarcoplasmic areas along the sarcotubules and between mitochondria (Fig. 8).

This cell showed intense localization for straight and branched chain phosphorylases, moderately high succinic dehydrogenase, and low alpha-glycerophosphate (Fig. 4, Table I). The minimal sarcoplasmic reaction for α-GPD was particulate in outline, suggesting mitochondrial localization. In longitudinal sections, succinic dehydrogenase activity was limited to the interfibrillar and subsarcolemmal cytoplasm.

The other cell located within the periphery was usually smaller and more ovoid in outline, with a thin broken layer of sarcoplasm and thin elongated nuclei adjacent

Text continued on p. 31

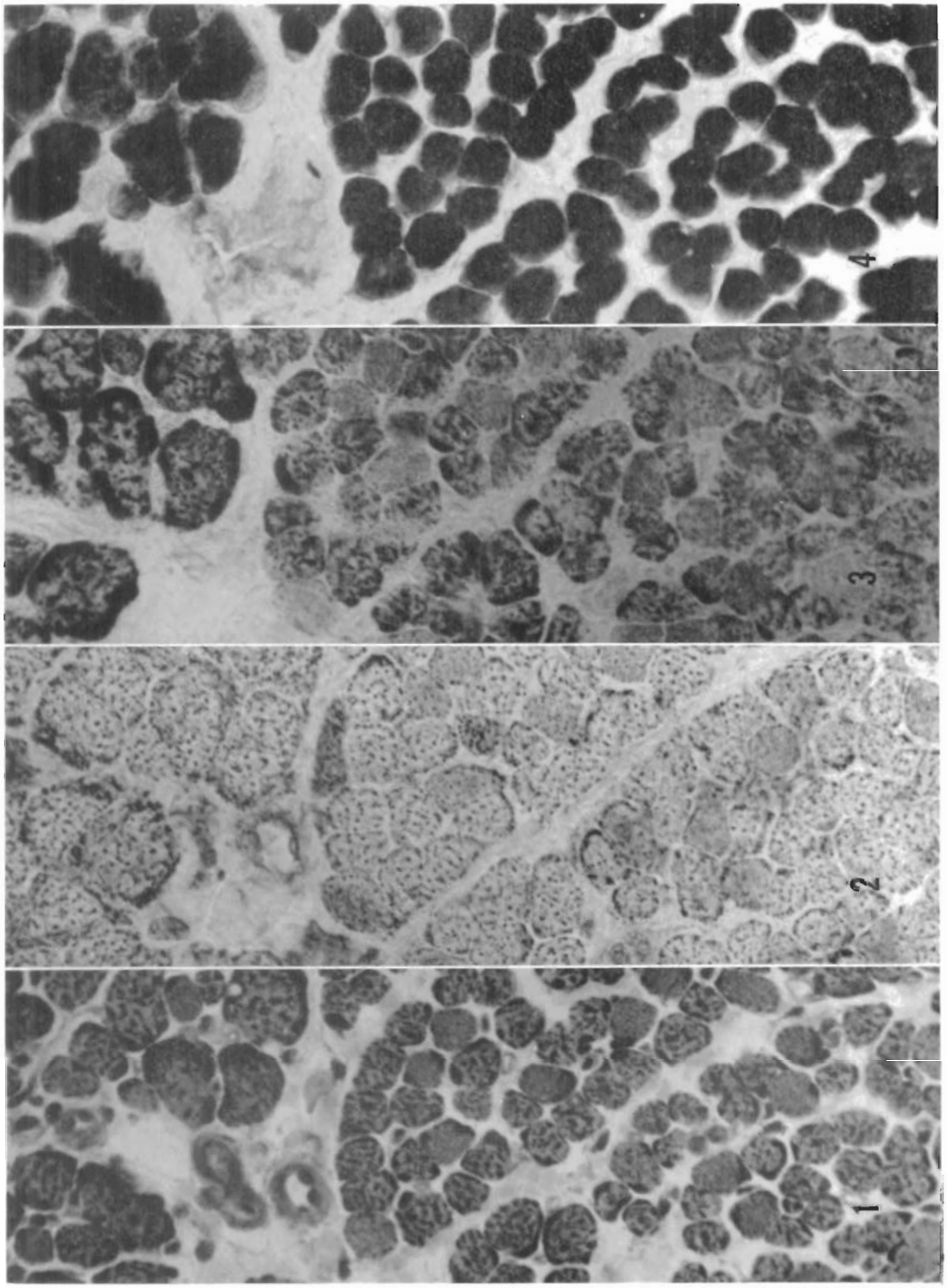

Fig. 4. Cells along the temporal edge of Fig. 1. Small cells are below; large cells of intermediate zone are above. (×300.) *1*, Gomori's trichrome. *2*, Alpha-glycerophosphate dehydrogenase. *3*, Succinic dehydrogenase. *4*, Phosphorylases.

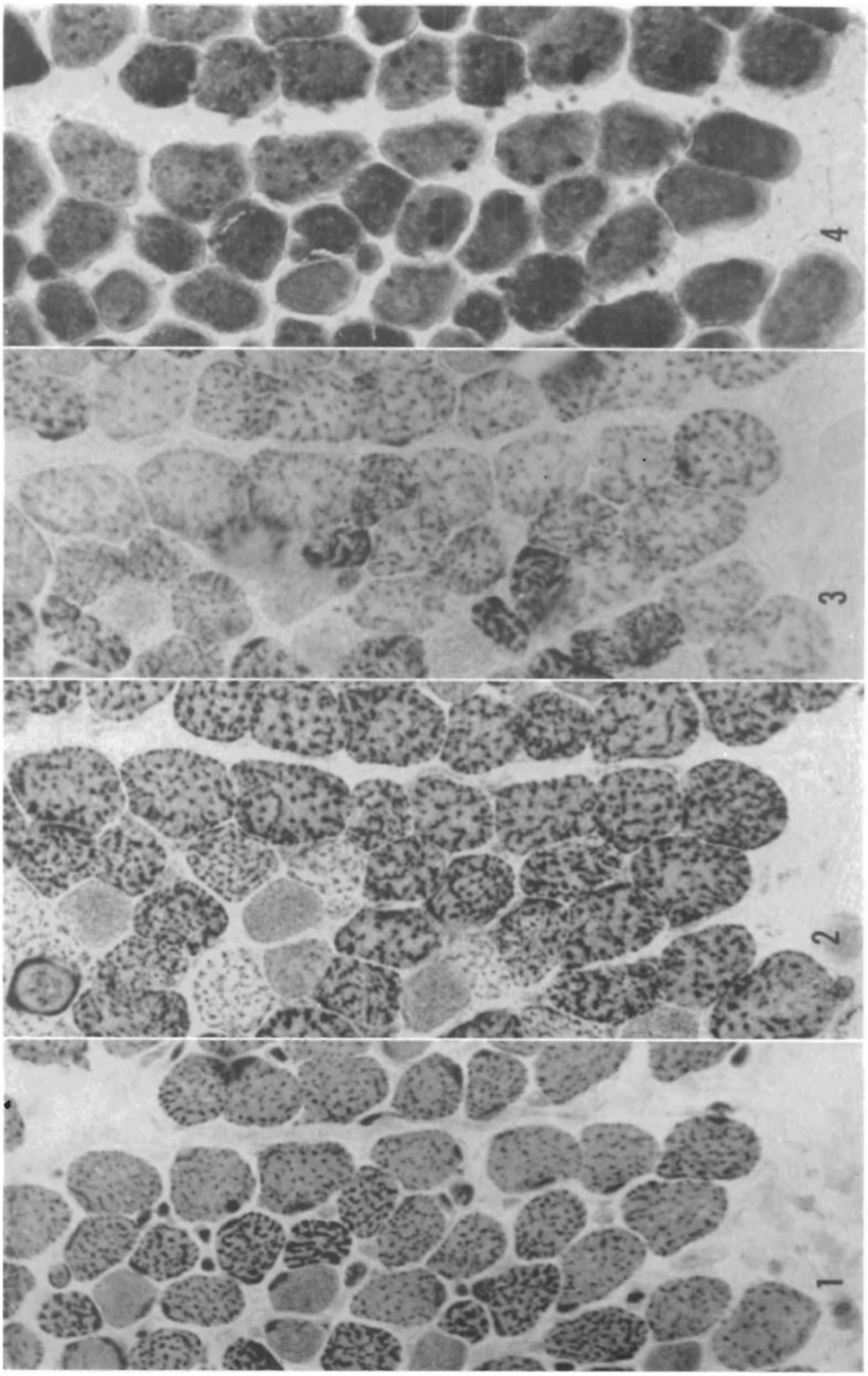

Fig. 5. Central area of the muscle just beneath the intermediate cell layer of Fig. 1. (×300.) *1*, Comori's trichrome. *2*, Alpha-glycerophosphate dehydrogenase. *3*, Succinic dehydrogenase. *4*, Phosphorylases.

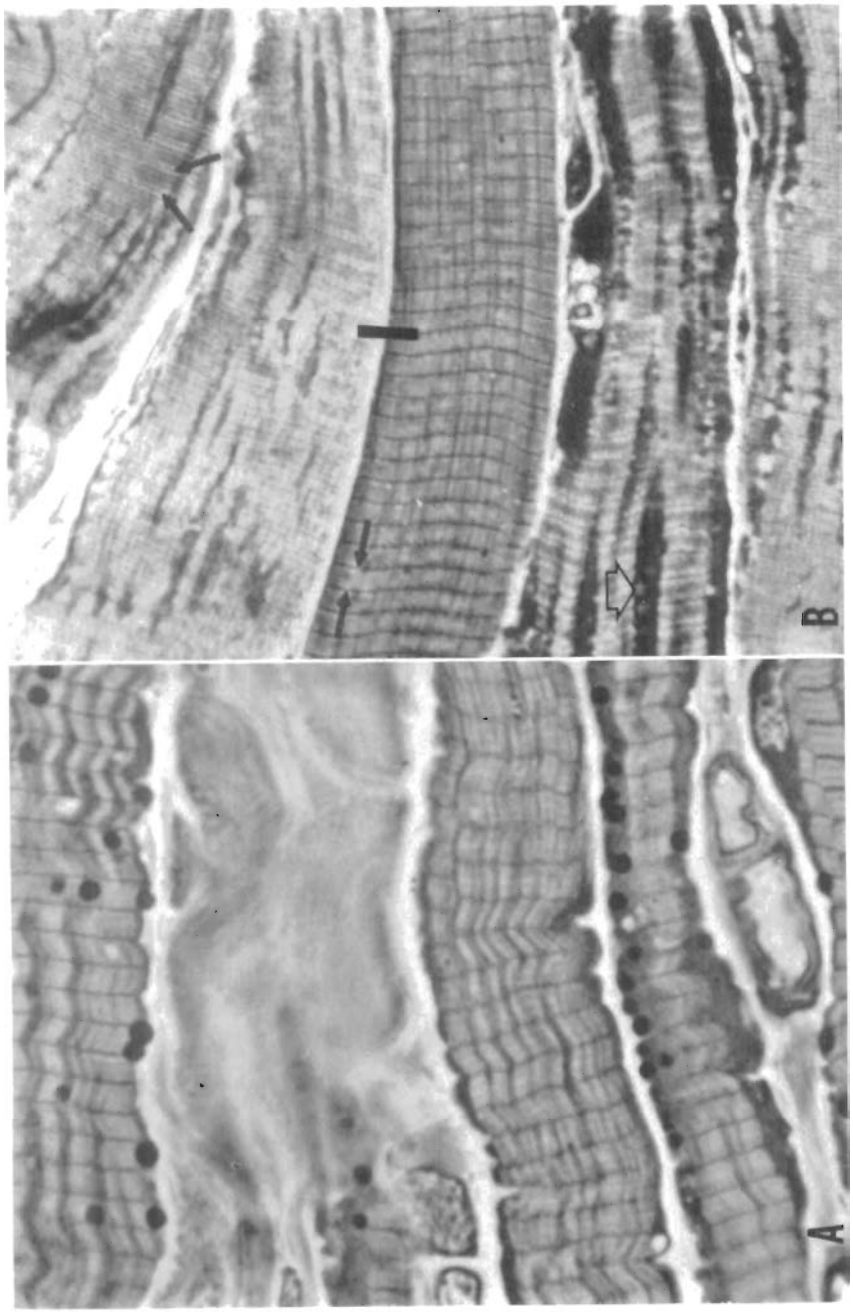

Fig. 6. Epon-embedded longitudinal section of inferior oblique stained with paraphenylenediamine. Phase microscopy. The dark staining Z lines (solid arrows) divide the fibers into regular sarcomeres. *A*, Outer fibers. The cells with larger columns of sarcoplasm contain lipid droplets. Both cell types show a light H zone in the center of the sarcomere. (×1,250.) *B*, Inner fibers. The cell with the largest amount of sarcoplasm contains lipid droplets (open arrow) and branched myofibrils. In the Felderstruktur fiber a faint M line is visible between the two horizontal arrows. Oval mitochondria are located along each side of the Z band. The remaining fibers contain M lines in the center of the dark A bands (between diagonal arrows). (×1,000.)

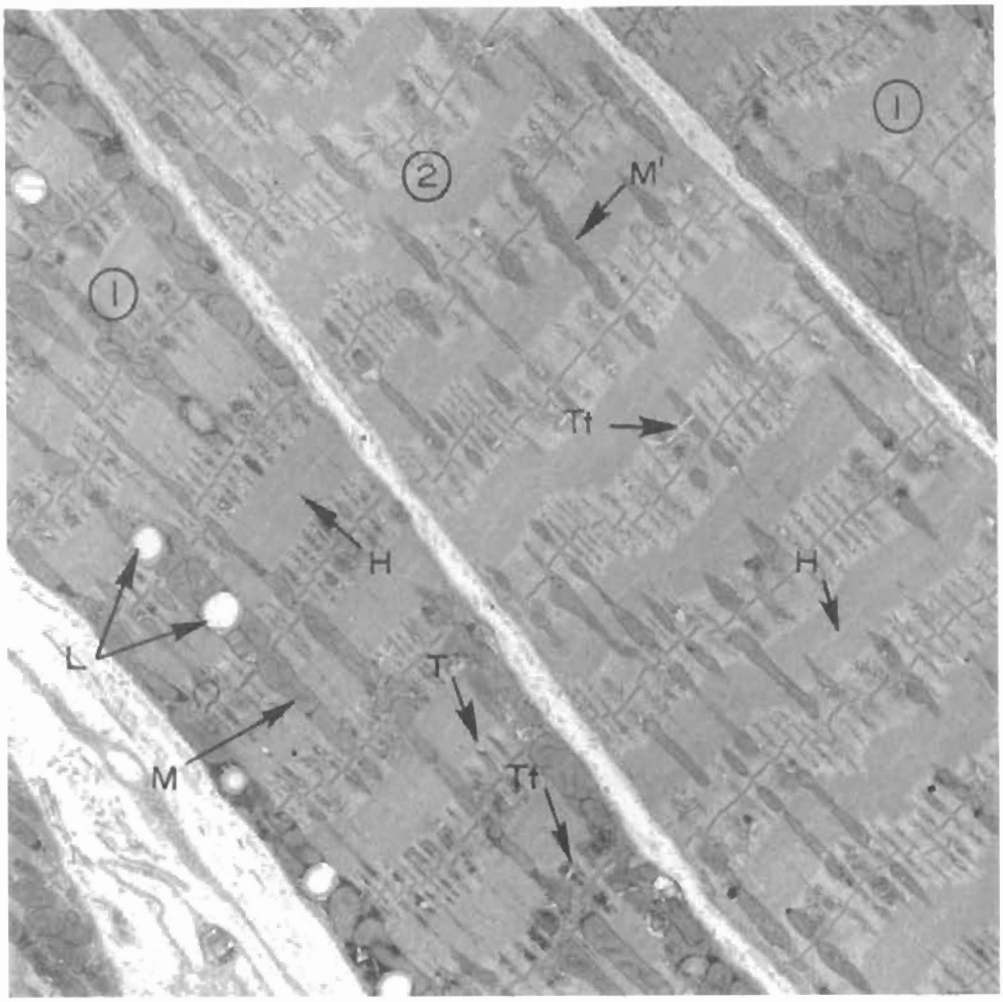

Fig. 7. Two cells in the periphery of the superior rectus. (×3,000.) Cell No. *1*, This fiber occurs more frequently in the periphery of an extraocular muscle and contains sarcoplasmic masses of ovoid mitochondria *(M)* and lipid droplets *(L)* between myofibrils and beneath the sarcolemma. The sarcoplasmic reticulum is almost entirely limited to the I band, and consists of a series of parallel tubules *(T)*, or sleeves, dividing the I band into small units. Transverse tubules *(Tt)* and triads are present in each sarcomere in front of the A band. The A band is interrupted by an occasional longitudinal tubule and contains an H zone without an M line. Cell No. 2, Rod-shaped mitochondria with enlarged ends *(M')* are interrupted at the Z line. The sarcoplasmic reticulum is similar to the first cell, with most of the network in the I band, except for isolated longitudinal tubules dividing the A band. Transverse tubules *(Tt)* and triads are located in the I band on either side of the Z line. The thick filaments comprising the A band are free of an M line.

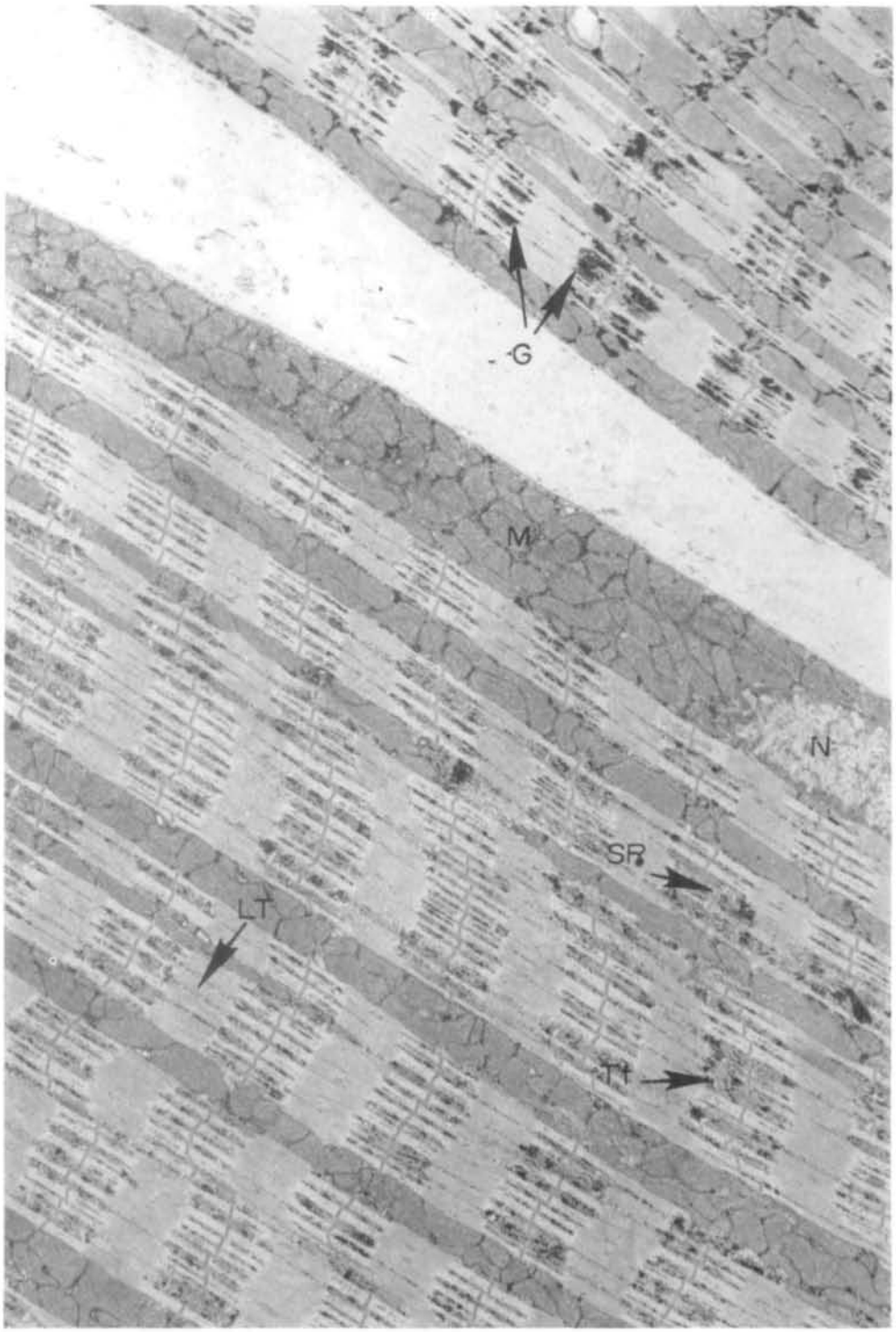

Fig. 8. Two cells located beneath the small fiber layer. The fiber in the upper part of the photograph is similar to the fiber No. 1 in Fig. 7. A heavy layer of glycogen *(G)* is located in sarcoplasmic reticulum areas and between mitochondria. The lower fiber is a larger cell containing numerous mitochondria in the subsarcolemmal, perinuclear, and intermyofibrillar areas. The sarcoplasmic reticulum *(SR)* consists of an extensive network of tubules that include longitudinal tubules *(Lt)* dividing each sarcomere into small separate myofibrils. Transverse tubules *(Tt)* and triads are adjacent to the A bands. In the center of many myofibrils is a faint H zone without an M line. Glycogen is also located in the sarcoplasmic reticulum and between mitochondria. (×4,000.)

Table I. Regional variation of fibers in extraocular muscle

Layer	*Size*	*Sarcoplasm*	*M line*	*Sarcoplasmic reticulum*	*Succinic dehydrogenase*	*Phosphorylase°*		*α-GPD*
						S	*B*	
Outer fiber	1. Small	++++	-	++	+++	++++	++++/+++	+
	2. Small	+++	-	++	++	+++	++	+
Intermediate†	Large	++++	-	+++	++++	+++	++	+
Inner fiber	1. Medium	+	-/+	+	+	+	+	++
	2. Large cell of intermediate	++++	-/+	+++	++++	+++	++	+
	3. Large	++	+	++++	++	++	+/-	++/++++

°S, straight chain or amylophosphorylase; B, branching or transglycosylase.
†Includes small cells from outer fiber layer.

to the sarcolemma. The interfibrillar sarcoplasm was located in small, evenly distributed columns that appeared punctate in cross section (Fig. 2). Z bands and H zones were well defined in longitudinal sections; however, the M line and lipid droplets were absent (Fig. 6, *A*).

The sarcoplasmic reticulum relationship was similar to the first cell in that the sarcotubules extended through the Z line but were almost entirely limited to the I band (Fig. 7). A deficiency of longitudinal tubules was again observed with broad expanses of A band thick filaments without M lines, interrupted by occasional thin tubules. Transverse tubules and triads were frequent on either side of the A bands. The mitochondria were drumstick or cylindrical in configuration, and usually appeared to terminate within the I band. The ends of the mitochondria occurred at interruptions of the Z band; an occasional mitochondrium extended two sarcomeres with a central constriction at the intervening Z band. The sarcoplasm immediately beneath the sarcolemma was very thin, with only a few scattered rod mitochondria and elongated nuclei.

By histochemical determinations, succinic dehydrogenase staining was intermediate and located in the sarcoplasmic areas (Fig. 4). Phosphorylase activity was slightly diminished in comparison to the first cell, and consisted of both glucosan phosphorylase and transglycosylase. A minimal particulate α-GPD reaction was present.

The small cells in the outer portion of the muscle combined with larger cells to form an area of varying cellular size. The larger cells were marked by broad, irregular sarcoplasmic columns located about the periphery and within the cell (Fig. 2). This cell was also found in the remaining portion of the muscle (Fig. 3). The sarcoplasm contained lipid droplets, dense accumulations of mitochondria, occasional central nuclei, and a fine reticular network about the myofibrils (Figs. 6, *B*, 8, and 9). In longitudinal section, the myofibrils formed confluent patterns with striking I and A bands lacking an M line.

In electron micrographs the mitochondrial distribution was similar to the predominating cell in the small fiber layer. However, the sarcoplasmic reticulum appeared to be better organized, since transverse and longitudinal tubules were more numerous with a regular division into small myofibrils throughout the fiber (Fig. 8). The T tubules and triads were quite prominent in front of the A bands, as were sarcoplasmic sleeves surrounding the I bands. Glycogen was heavily deposited along sarcoplasmic reticulum areas and between mitochondria, but not as extensively as in the predominating small cell. The thick filaments comprising the A band were of uniform character throughout their longitudinal extent and did not contain an M line. The subsarcolemmal, perinuclear, and intermyofibrillar sarcoplasm consisted of ovoid mitochondria packed 2 to 3 in transverse depth.

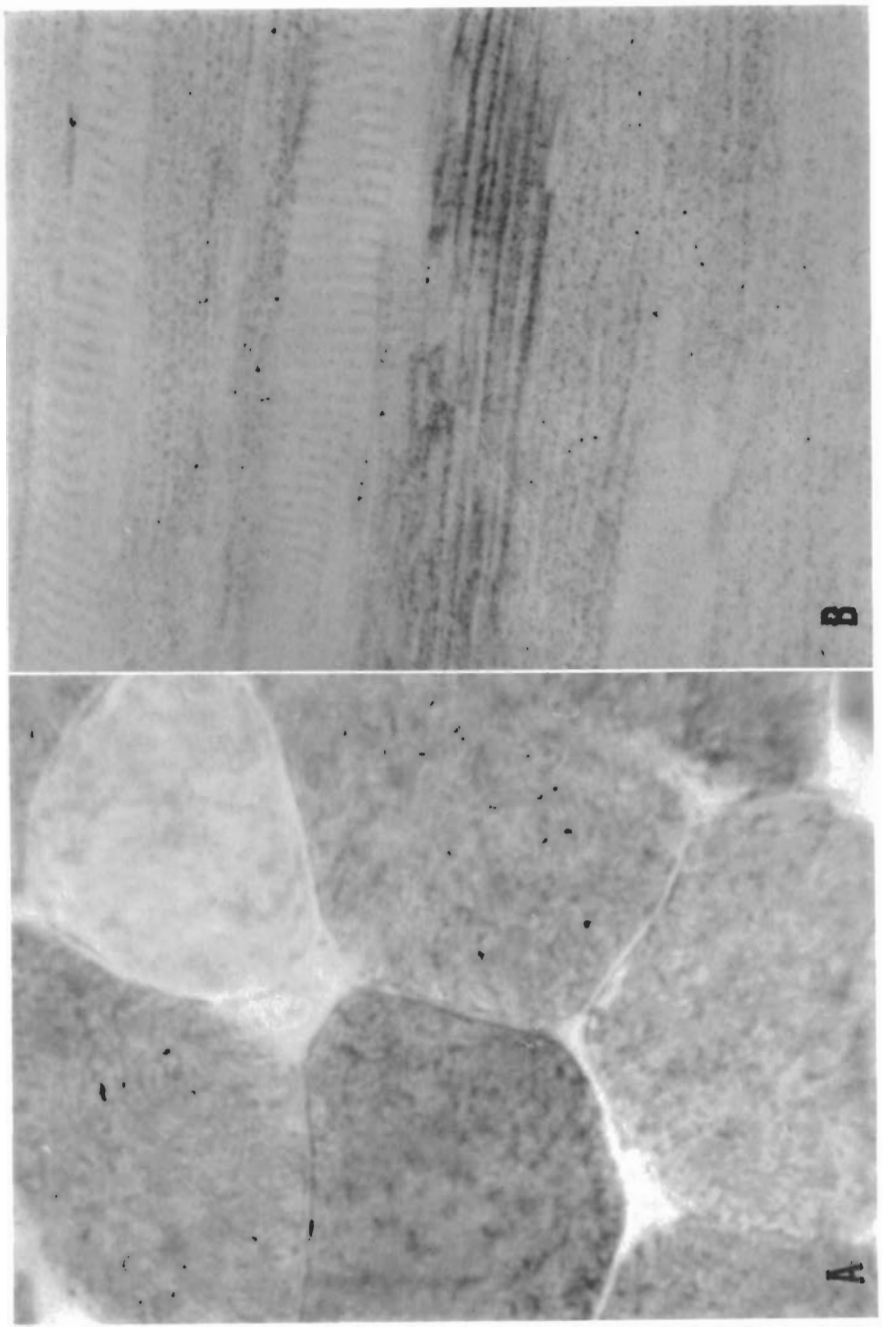

Fig. 9. *A*, Cells adjacent to the globe in the medial rectus. Transverse phosphorylase section. Three densities of reaction are present. The light cell is a Felderstruktur fiber which contains minimal linear localization. In the dark staining sarcoplasmic rich cell phosphorylase activity is greatest in the sarcoplasm and sarcoplasmic reticulum areas. The remaining cells show intermediate reaction in sarcoplasm and about the myofibrils. (×1,250.) *B*, Longitudinal section, center of the inferior oblique, insertional end. Mitochondria are indicated by succinic dehydrogenase positive granules and are distributed along sarcoplasmic columns. In the Felderstruktur fiber mitochondria are on each side of the Z band and appear as striations. (×500.)

The highest level of succinic dehydrogenase was located within this cell, and the sarcoplasm contained dense accumulations of mitochondria (Figs. 4 and 9, *B*). Phosphorylase activity was moderately high and consisted of both straight and branching enzymes. The reaction was not uniform throughout the cell, with less staining in the myofibrillar areas and an intense pattern in sarcoplasmic areas and between myofibrils (Fig. 9, *A*). The phosphorylase staining corresponded to the glycogen deposition noted in electron micrographs. Minimal α-GPD particulate deposition was present.

In one to three fascicles, another gradual transition occurred, after which three large cell types could be identified by the relative sarcoplasmic content and myofibrillar size. The cell with the least amount of sarcoplasm and the largest myofibrils resembled the Felderstruktur fiber described in extraocular muscle from human, mammalian, and other species.[7] In transverse cryostat sections, the large myofibrils were four or five sided and outlined by fine sarcoplasmic reticulum with small oval mitochondria located at the sarcoplasmic reticulum intersections (Fig. 3). Thin, elongated nuclei occurred along the margin of the cell with occasional central oval nuclei. In longitudinal sections, the sarcoplasm was arranged in slender columns with small globular mitochondria on either side of the Z band (Figs. 6, *B*, and 9, *B*). In phase sections, Z bands were regular between sarcoplasmic columns in fibers prepared in anesthetic solution, but were sinusoidal or broken and jagged in muscles extended to resting length without relaxation. A and I bands were evident; however, the M line appeared poorly developed as it was visible only in isolated areas of fortuitous sections.

The sarcoplasmic reticulum was scanty and consisted of isolated thin tubules projecting into the I bands (Fig. 10). These were continuous with thin sarcoplasmic columns, and the two in combination divided the fiber into large myofibrils. The intermyofibrillar sarcoplasm contained an occasional small longitudinal tubule, glycogen granules, and electron-lucent material. There was also a deficiency of transverse tubules and small triads in cells obtained from the global surface. Often these sarcotubules could not be identified in a single low-power micrograph, but were found in a montage. Similar cells from the center of the muscle contained a more extensive tubular network, but the transverse and triadic components were not as frequent as in other cells.

Double oval profiles of mitochondria were located on each side of interruptions of the Z line, and in some areas were vertically elongated. This diplet pattern with varying transverse length was similar to the bracelet encirclement of thin filaments described in skeletal muscle. There also were a few longitudinally placed rod mitochondria extending one or more sarcomeres and connecting with the enlarged portions of mitochondria at the Z band. The varying mitochondrial morphology and branching may represent a bracelet network with a few longitudinal interconnections. These together partially delineated the myofibrils. The closely packed thick filaments were uniform except in an occasional cell where indistinct central enlargements were found in the center of the A band.

Enzymatic staining was reduced with minimal activity of succinic dehydrogenase and phosphorylase (Figs. 5 and 9, *A*). The alpha-glycerophosphate level was slightly higher than in the peripheral red type cells. In longitudinal preparations for succinic dehydrogenase, the mitochondria were arranged in striated patterns which were located on either side of the Z band by phase microscopy (Fig. 9, *B*). This cell was the most infrequent of the interior of the muscle, and, in confirmation of a past report, was not observed in the levator.[3]

Immediately adjacent to the Felderstruktur fiber were one or more of the large cells found in the intermediate area. This association was most striking near the intermediate zone, with the Felderstruktur

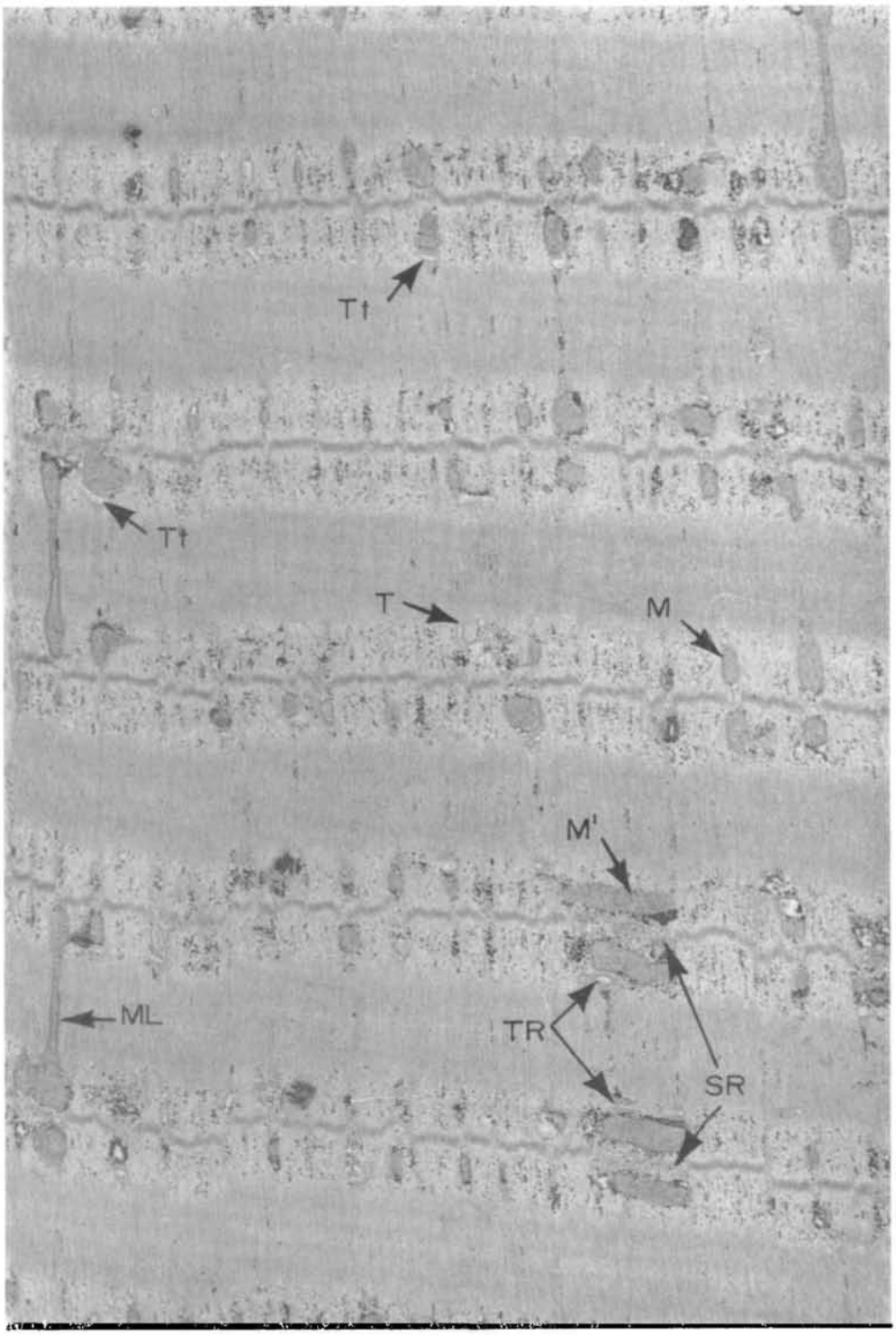

Fig. 10. Felderstruktur fiber in the center of the muscle. Myofilaments are delineated into large irregular myofibrils by thin sarcoplasmic spaces that contain a few longitudinal tubules *(T)* and glycogen granules. Transverse tubules *(Tt)* and sarcoplasmic sleeves *(SR)* are found in widely separated areas of the I bands with the triads *(TR)* lying between mitochondria and the A band. Thick filaments are closely packed without a distinct M line. Mitochondria *(M)* are paired on either side of the Z band interruptions and follow the configuration of the sarcoplasmic reticulum at *M'*. Adjacent to the triads *(TR)* they are presented in tangential view, while in the remainder of the micrograph they were cut in transverse section. The mitochondria appear to partially encircle the thin filaments similar to the sarcoplasmic reticulum. The longitudinally oriented rod-shaped mitochondria *(ML)* were continuous with oval Z band mitochondria in a few areas. (×7,500.)

fibers often surrounded by cells high in sarcoplasm. The relationship continued toward the global margin, but with fewer red type cells. At the inner edge of the muscle the association was one to one. In the center of the muscle and adjacent to the globe some of the cells with large amounts of sarcoplasm contained M lines. Usually the M lines were poorly formed and could be identified only in isolated sarcomeres.

The most frequent cell within the center and global portion of the muscle contained broad expanses of myofibrils and small areas of sarcoplasm (Figs. 3 and 6, *B*). A progressive increase of distribution was observed toward the global surface of the muscle. Striations were well developed and included an M line (Figs. 6, *B*, and 11). Nuclei were oval and located beneath the sarcolemma. At higher resolution, myofibrils were well delineated by sarcoplasmic sleeves throughout their longitudinal extent, and an extensive network of longitudinal tubules and triads was present. A well-formed M line with a pseudo H zone on either side was located in the center of the A band and recognizable even with a slightly oblique cut of the thick filaments. Interrupted masses of globular mitochondria occupied the intermyofibrillar sarcoplasm. A thin layer of glycogen granules separated mitochondria and extended along the sarcoplasmic reticulum.

Enzymatic staining consisted of moderate to high sarcoplasmic and myofibrillar alpha-glycerophosphate and low to intermediate succinic dehydrogenase and phosphorylase activity (Fig. 5). The sarcoplasmic reticulum also appeared as a honeycomb pattern in the phosphorylase preparations and Gomori's stained sections (Figs. 3 and 9, *A*).

Discussion

Eye movements are extremely diversified in both amplitude and velocity. Ocular excursions extend from the large rapid shifts of conjugate gaze to the minute movements of micronystagmus and fusional adjustments. Convergence and fusional correction vary in degree, and large movements may last two to three seconds. In contrast, the rapid shifts of saccadic and slower pursuit movements are completed in a fraction of a second. Position maintenance is accompanied by a continuous discharge of electromyographic activity similar to tonus skeletal muscle.[30-32] Electromyographic recordings indicate that different groups of muscle fibers are activated for saccadic and convergence movement and to hold each end point.[31, 32] This suggests that the gross types of eye movement are produced by motor units varying in contraction rate from slow to fast. The above evidence indicates the existence of both a tonic and phasic system in human extrinsic eye muscles. A similar hypothesis was proposed on the basis of end-plate and nerve morphology[37] and muscle electron microscopy findings.[11]

Muscle tissue may be divided into two major groups, red and white. Red muscle is darker on cut cross-section and high in mitochondria and oxidative enzymes. White muscle is pale and low in mitochondrial content but has a greater concentration of glycogen and glycolytic enzymes. Muscles, or portions of muscles, that are utilized for prolonged contraction usually have the characteristics of red muscle. Their function is that of slow movement or postural position. Muscles which perform quick or phasic movement of short duration have the enzymatic characteristics of white muscle.

There is a close correlation between the histochemistry and function of muscle. Histochemical comparisons between tonic and phasic muscle in the same animal or species have shown tonus muscle to consist of a homogeneous population of red type cells and phasic muscle of a mixed group ranging from red to white fibers. With succinic dehydrogenase staining, three types of cells were found in the fast portion of the gastrocnemius[33] and only one or two types[34] of red fibers in the slower soleus. With a battery of oxidative

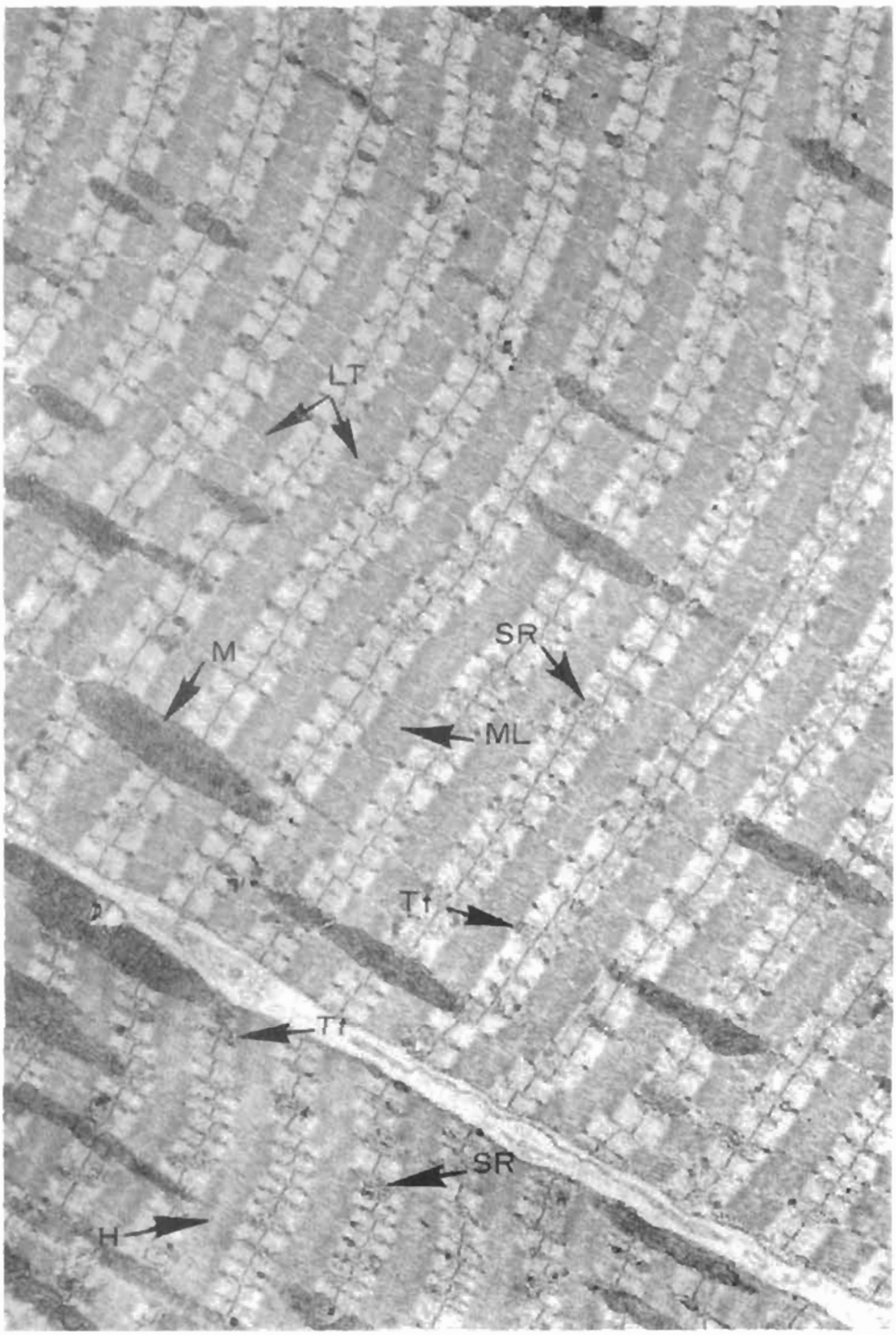

Fig. 11. The center and global portion of the muscle is mainly composed of fibers which have distinct M lines *(ML)*. Sarcomeres are divided into regular myofibrils by an extensive network of sarcoplasmic reticulum *(SR)*, including longitudinal tubules *(LT)*. The I band appears surrounded by sarcoplasmic sleeves *(SR)*. Transverse tubules *(Tt)* and triads are in front of the A-I junction. Mitochondria *(M)* are ovoid and grouped between myofibrils. (×3,000.)

and glycolytic enzyme determinations, eight types of cells were found in phasic muscles and three types in tonus muscle.[29] Electron microscopy studies of red and white muscle in the rat have indicated a variation in sarcoplasmic reticulum and mitochondrial content.[35] White type fibers had a more highly developed sarcoplasmic reticulum, which outlined the entire myofibril and contained a smaller mitochondrial mass; red fibers contained less sarcoplasmic reticulum but were higher in mitochondrial content.

The comparison between red and white muscle is on a qualitative rather than a quantitative basis, and it is impossible to classify a single cell as red, white, or intermediate without comparison with its opposite members. For example, the extremely fast cricothyroid muscle of the bat, an animal with a very high metabolic rate, was considered to be composed of red fibers.[38] However, when compared with a red fiber of the bat diaphragm[38, 39] it contains a better-developed sarcoplasmic reticulum and a smaller mitochondrial mass.

One of the determining features in the development of muscle tissue into a phasic or tonic system is the type of innervation it receives. Fast muscle is innervated by rapidly conducting nerves with heavy myelination; tonic muscle is activated by nerves with a thinner myelin sheath and slower conducting velocities. The significance of innervation may be demonstrated by transposition of nerves.[36] When a tonus nerve is transplanted to a phasic muscle, the contraction-relaxation characteristics revert to those of tonus muscle.

It would appear that, if a portion of eye muscle received the tonic discharge of convergence and position maintenance, it would be identified by the histochemical, histological, and electron microscopic characteristics of red muscle. Saccadic or phasic movements would also be produced by a white portion of the muscle. The present findings in the monkey are compatible with this interpretation. The small cell layer and the intermediate zone consist of red type fibers and the central and global portion of a diversity of fibers characterizing white muscle.

Where efforts have been made to identify slow fibers by their physiological and pharmacological properties, they appeared to be concentrated in the outer margin of extraocular muscle. They were more frequently identified in the outer fibers of the cat superior oblique by intracellular recording[11] and in the outer fibers of rabbit superior rectus in response to acetylcholine.[14] External fibers from guinea pig eye muscle also had the electron microscopy characteristics of slow fibers.[1]

The predominating cell in the outer fiber layer of monkey included many of the histological findings of slow fibers. The sarcoplasmic reticulum did not completely outline the myofibrils, thick myofilaments were distributed in broad irregular areas interspersed with large masses of mitochondria, and M lines were absent. This fiber contained high succinic dehydrogenase and phosphorylase levels similar to fibers in fish and amphibians where slow fibers are located.

If absence of an M line and elevation of succinic dehydrogenase and phosphorylase levels are indicators of slow fibers, the small-cell layer, intermediate layer, and many of the sarcoplasmic rich cells of the remaining portion of the muscle would be slow fibers. One half or more of the obliques and horizontal recti would consist of slow muscle. However, the variation in sarcoplasmic reticulum, mitochondrial content, and histochemical findings would indicate different degrees of slowness between layers and cells.

Verification of function and electrophysiological properties must await further experiments, but there appears to be morphological and histochemical evidence for a gradation of fibers in monkey eye muscle extending from slow to fast twitch. The fibers are arranged in a functional pattern with the slowest group concentrated about the periphery, the fastest adjacent to the

globe, and intermediate fibers between these two areas.

This study was made possible through the efforts of Elaine D. Miller, M.D., Mrs. Mary Etherington, and Mr. Louis Carciobolo. Appreciation for their contribution is gratefully acknowledged.

REFERENCES

1. Hess, A.: The structure of slow and fast extrafusal muscle fibers in the extraocular muscles and their nerve endings in guinea pig, J. Cell. & Comp. Physiol. **58**: 63, 1961.
2. Cheng, K.: Cholinesterase activity in human extraocular muscles, Jap. J. Ophth. **7**: 174, 1963.
3. Dietert, S.: The demonstration of different types of muscle fibers in human extraocular muscle by electron microscopy and cholinesterase staining, INVEST. OPHTH. **4**: 51, 1965.
4. Silver, A.: A histochemical investigation of cholinesterases at neuromuscular junctions in mammalian and avian muscle, J. Physiol. **169**: 386, 1963.
5. Sas, J., and Appeltauer, C.: Atypical muscle Spindles in the extrinsic eye muscles of man, Acta anat. **55**: 311, 1963.
6. Thulin, I.: Contribution à l'histologie des muscles oculaires chez l'homme et chez les singes, Soc. Biol. **76**: 490, 1914.
7. Siebeck, R., and Kruger, P.: Die histologische Struktur der auberen Augenmuskeln als Ausdruck ihrer Funktion, von Graefes Arch. Ophth. **156**: 637, 1955.
8. Nachmias, V. T., and Padykula, H. A.: A histochemical study of normal and denervated red and white muscles of the rat, J. Biophys. & Biochem. Cytol. **4**: 47, 1958.
9. Cogan, D. G., and Kuwabara, T.: Tetrazolium studies on the retina. IV. Distribution of reductase in ocular tissue, J. Histochem. & Cytochem. **8**: 380, 1960.
10. Cheng, K.: Distribution of succinic dehydrogenase activity in extraocular muscles, Jap. J. Ophth. **8**: 116, 1964.
11. Hess, A., and Pilar, G.: Slow fibers in the extraocular muscles of the cat, J. Physiol. **169**: 780, 1963.
12. Cheng, K., and Breinin, G. M.: Fine structure of nerve endings in extraocular muscle, Arch. Ophth. **74**: 822, 1965.
13. Duke-Elder, W. S., and Duke-Elder, P. M.: The contraction of the extrinsic muscles of the eye by choline and nicotine, Proc. Roy. Soc., B **107**: 332, 1930.
14. Kern, R.: A comparative pharmacologic-histologic study of slow and twitch fibers in the superior rectus muscle of the rabbit, INVEST. OPHTH. **4**: 901, 1965.
15. Kuffler, S. W., and Vaughan Williams, E. M.: Properties of "slow" skeletal muscle fibers of the frog, J. Physiol. **121**: 318, 1953.
16. Sommerkamp, H.: Das Substrat der Dauerverkurzung am Froschmuskel (Physiologische und pharmakologische Sonderstellung bestimmter Muskelfasern), Arch. f. exper. Path. u. Pharmakol. **128**: 99, 1928.
17. Erulkar, S. D., Shelanski, M. L., Whitsel, B. L., and Ogle, P.: Studies of muscle fibers of the tensor tympani of the cat, Anat. Rec. **149**: 279, 1964.
18. Peachey, L. D., and Huxley, A. F.: Structural identification of twitch and slow striated muscle fibers of the frog, J. Cell Biol. **13**: 177, 1962.
19. Page, S.: A comparison of the fine structures of frog slow and twitch muscle fibres, J. Cell Biol. **26**: 477, 1965.
20. Huxley, A. F.: *In* Rodahl, K., and Horvath, S. M., editors: Muscle as a tissue, New York, 1962, McGraw-Hill Book Company, Inc., p. 11.
21. Dubowitz, V., and Pearse, A. G. E.: A comparative histochemical study of oxidative enzyme and phosphorylase activity in skeletal muscle, Histochemie **2**: 105, 1960.
22. Pearse, A. G. E.: Direct relationship of phosphorylase and mitochondrial alpha-glycerophosphate dehydrogenase activity in skeletal muscle, Nature **191**: 504, 1961.
23. Bucciante, L., and Luria, S.: Trasformazioni nella struttura dei muscoli volontari dell' uomo nella senescenza, Arch. Ital. Anat. Embriol. **33**: 110, 1934.
24. Wohlfart, G.: Zur Kenntnis der Altersveranderungen der Augenmuskeln, Z. Mikr. Anat. Forsch. **44**: 33, 1938.
25. Moline, S. W., and Glenner, G. G.: Ultrarapid tissue freezing in liquid nitrogen, J. Histochem. & Cytochem. **12**: 777, 1964.
26. Engel, W. K., and Cunningham, G. G.: Rapid examination of muscle tissue, Neurology **13**: 919, 1963.
27. Van Wijhe, M., Blanchaer, M. C., and St. George-Stubbs, S.: The distribution of lactate dehydrogenase isozymes in human skeletal muscle fibers, J. Histochem. & Cytochem. **12**: 608, 1964.
28. Wattenberg, L. W., and Leong, J. L.: Effects of co-enzyme Q10 and menadione on succinic dehydrogenase activity as measured by tetrazolium salt reduction, J. Histochem. & Cytochem. **8**: 296, 1960.
29. Romanul, F. C. A.: Enzymes in muscle. I. Histochemical studies of enzymes in individual muscle fibers, Arch. Neurol. **11**: 355, 1964.
30. Breinin, G. M., and Moldaver, J.: Electromyography of the human extraocular muscles:

I. Normal kinesiology: divergence mechanism, Arch. Ophth. **54**: 200, 1955.
31. Miller, J. E.: The electromyography of vergence movement, Arch. Ophth. **62**: 790, 1959.
32. Miller, J. E.: Electromyographic pattern of saccadic eye movements, Am. J. Ophth. **46**: 183, 1958.
33. Stein,, J., and Padykula, H.: Histochemical classification of individual skeletal muscle fibers of the rat, Am. J. Anat. **110**: 103, 1962.
34. Ogata, T.: A histochemical study of the red and white muscle fibers. I. Activity of the succinoxydase system in muscle fibers, Acta med. okayama **12**: 216, 1958.
35. Pellegrino, C., and Franzini, C.: An electron microscope study of denervation atrophy in red and white skeletal muscle fibers, J. Cell Biol. **17**: 327, 1963.
36. Buller, A. J., Eccles, J. C., and Eccles, R. M.: Interactions between motoneurones and muscle in respect to the characteristic speeds of their responses, J. Physiol. **150**: 417, 1960.
37. Alpern, M., and Wolter, J. R.: The relation of horizontal saccadic and vergence movements, Arch. Ophth. **56**: 685, 1956.
38. Gauthier, G. F., and Padykula, H. A.: Cytological studies of fiber types in skeletal muscle. A comparative study of the mammalian diaphragm, J. Cell Biol. **28**: 333, 1966.
39. Revel, J. P.: The sarcoplasmic reticulum of the bat cricothyroid muscle, J. Cell Biol. **12**: 571, 1962.