THE ULTRASTRUCTURE OF THE NEUROMUSCULAR JUNCTIONS OF MAMMALIAN RED, WHITE, AND INTERMEDIATE SKELETAL MUSCLE FIBERS

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ABSTRACT

Distinct ultrastructural differences exist at the neuromuscular junctions of red, white, and intermediate fibers of a mammalian twitch skeletal muscle (albino rat diaphragm). The primary criteria for recognizing the three fiber types are differences in fiber diameter, mitochondrial content, and width of the Z line. In the *red fiber* the neuromuscular relationship presents the least sarcoplasmic and axoplasmic surface at each contact. Points of contact are relatively discrete and separate, and axonal terminals are small and elliptical. The junctional folds are relatively shallow, sparse, and irregular in arrangement. Axoplasmic vesicles are moderate in number, and sarcoplasmic vesicles are sparse. In the *white fiber* long, flat axonal terminals present considerable axoplasmic surface. Vast sarcoplasmic surface area is created by long, branching, closely spaced junctional folds that may merge with folds at adjacent contacts to occupy a more continuous and widespread area. Axoplasmic and sarcoplasmic vesicles are numerous. Both axoplasmic and sarcoplasmic mitochondria of the white fiber usually contain intramitochondrial granules. The *intermediate fiber* has large axonal terminals that are associated with the most widely spaced and deepest junctional folds. In all three fiber types, the junctional sarcoplasm is rich in free ribosomes, cisternae of granular endoplasmic reticulum, and randomly distributed microtubules.

INTRODUCTION

Cytochemical and ultrastructural investigations from this laboratory have established that three distinct types of skeletal muscle fibers are present in the diaphragm (Gauthier and Padykula, 1966; Padykula and Gauthier, 1967 *b*) and semitendinosus (Gauthier, 1969) muscles of the albino rat. These three types of fibers have been designated red, white, and intermediate, and they can be distinguished by differences in fiber diameter, mitochondrial content, width of the Z line, and form of the sarcoplasmic reticulum. Certain of these recent criteria for red and white fibers are consistent with classical descriptions that distinguish dark (red) fibers and light (white) fibers (Grützner, 1884; Knoll, 1891; Bell, 1911; Bullard, 1912). A third type, the intermediate fiber, has been described in a few studies of mammalian muscle, especially in histochemical investigations (Bullard, 1919; Ogata, 1958; Stein and Padykula, 1962), but its existence as an individual fiber type has received relatively little acceptance. The intermediate fiber has morphological and cyto-

chemical properties that are distinctive although they are, to a large extent, qualitatively and quantitatively intermediate between those of red and white fibers (Gauthier, 1969). From current evidence, it seems likely that most mammalian muscles are composed of more than one of the three fiber types, are present in varying proportions, and are arranged in characteristic geographical distribution. Furthermore, the intermediate fiber, as well as the red fiber, appears to reflect "redness" in skeletal muscles. For example, in the red region of the rat semitendinosus, it is equal to the red fiber in its contribution to the fiber population (Gauthier, 1969).

Muscles of the mammalian hind limb are characterized as fast or slow twitch muscles, and this description may be extended to include the diaphragm (Gasser and Grundfest, 1939; Ritchie, 1954). Slow tonic fibers, such as those identified in amphibian muscles, are absent in most mammalian muscles (Kuffler, 1953). However, special muscles, such as the extraocular (Zenker and Anzenbacher, 1964; Dietert, 1965; Mayr, Stockinger, and Zenker, 1966; Düring, 1967; Teräväinen, 1968) and tympanic muscles (Fernand and Hess, 1969), contain both slow tonic and fast twitch fibers, and these have, moreover, different neuromuscular junctions (*terminaison en grappe* and *terminaison en plaque*, respectively). Although a number of studies have been concerned with differences between the neuromuscular junctions of slow tonic and fast twitch fibers in amphibia and reptiles (Peachey and Huxley, 1962; Page, 1965; Page, 1968), ultrastructural identification of differences in mammalian neuromuscular junctions has been restricted to certain specialized muscles, such as the extraocular (Dietert, 1965; Mayr, Stockinger, and Zenker, 1966; Düring, 1967; Teräväinen, 1968 *a*) and tympanic muscles (Fernand and Hess, 1969).

In twitch muscles of the rat, three types of motor units have been identified and characterized as fast, intermediate, and slow in response (Close, 1967). It seems logical to expect that such significant physiological differences would be reflected in structural differences at the neuromuscular junction. Previous reports have assumed a uniform ultrastructure at the neuromuscular junction in mammalian twitch muscles (Nickel, 1966; Miledi and Slater, 1968; Nickel and Waser, 1968; Teräväinen, 1969). Thus, it seemed important to examine this relationship in the neuromuscular junctions of the three muscle fibers that are known to comprise the rat diaphragm.

In the present report, the individuality of the three fiber types in the rat diaphragm is further established by the demonstration of consistent differences in the ultrastructure of their neuromuscular junctions. In addition, the presence of cisternal forms of granular endoplasmic reticulum and of microtubules in the junctional sarcoplasm is described. The probable functional significance of the ultrastructural differences of the neuromuscular junctions is discussed.

MATERIALS AND METHODS

Preliminary Localization of Neuromuscular Junctions

The gross distribution of neuromuscular junctions within the rat diaphragm was analyzed in order to identify the region to be sampled for electron microscopy. Nonspecific esterase activity was used as a marker to demonstrate sites of neuromuscular junctions. Entire diaphragms of adult (approximately 100 days) male albino rats were excised and fixed in 10% neutral buffered formalin. Enzymic activity was demonstrated using α-naphthyl acetate as a substrate (Gomori, 1952). After incubation, the diaphragms were rinsed and examined directly with a hand lens or dissecting microscope. Reaction product reflecting the esterase activity of neuromuscular junctions was distributed in a somewhat irregular strip, approximately 0.5 mm wide, forming an arc perpendicular to the muscle fibers. In each costal region, junctions were most conspicuous in the ventral one-third, particularly at the abdominal surface, and in the dorsal one-third at the thoracic surface. In the ventral third of the right costal diaphragm, which is the region selected routinely in this laboratory, the strip of neuromuscular junctions is located slightly less than one-half the distance from the origin of the muscle fibers on the ribs to their insertion into the central tendon.

The animals used in this study (male rats, 100 days old) were killed with chloroform, and the diaphragms were exposed at the abdominal surface. In the ventral one-third of the right costal diaphragms, strips of muscle, approximately 1 mm wide, were separated by cutting parallel to the fibers, while leaving them attached at origin and insertion. A segment of each muscle strip, less than 1 cm long, midway between origin and insertion was tied to a small wooden splint and then excised beyond the ties and placed into cold fixative. This mode of isolation increases the probability of encountering the neuromuscular junctions in small samples, and minimizes contraction of the muscle fibers.

Electron Microscopy

Most of the observations were made on diaphragms fixed in 1% osmium tetroxide in Veronal-acetate buffer (pH 7.5) (Palade, 1952). Some of the specimens were fixed in 6.25% glutaraldehyde in cacodylate buffer (pH 7.4), postfixed in 1% osmium tetroxide in Veronal-acetate buffer (Sabatini et al., 1963), and stained with 1% uranyl nitrate before dehydration. While the strips of muscle were in osmium tetroxide, they were cut away from the splints, and the extreme ends of the strips, including the ties, were discarded. The remaining middle portions were cut transversely into segments less than 1 mm long. After fixation, the tissue was dehydrated and embedded in Epon. Thick sections (1.5 μ) stained with toluidine blue were used to select blocks in which neuromuscular junctions were present. Thin sections were cut on a Porter-Blum MT-1 or MT-2 ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.), stained with uranyl acetate and lead (Karnovsky, 1961), and then examined with a Siemens Elmiskop IA (Siemens America, Inc., New York).

RESULTS

Principal Cytological Characteristics of Red, White, and Intermediate Muscle Fibers

As a background for the description of the neuromuscular junctions of the three fiber types in the rat diaphragm, a brief summary of the cytological characteristics of the muscle fibers is provided here. Documentation for classification of fiber types in the rat diaphragm and semitendinosus muscles is available in earlier studies (Gauthier and Padykula, 1966; Padykula and Gauthier, 1967 *b*; Gauthier, 1969). In the rat diaphragm, red fibers constitute 60% of the fiber population, whereas white and intermediate fibers each represent 20%.

The red fiber is of small diameter (27 μ) and thus possesses a surface-to-volume ratio which is highly favorable for metabolic exchange. In all three fiber types, paired mitochondria encircle the myofibrils at the I bands on either side of the Z line. The red fiber, in particular, is rich in large mitochondria with closely packed cristae. They are aggregated in masses beneath the sarcolemma and in longitudinal rows among myofibrils. Numerous triglyceride droplets occur among the mitochondria, which suggests that metabolic energy in this fiber may be derived chiefly from the utilization of fat. The Z line of the red fiber is wider (634 A $\pm$ 31 [SD]) than that of either the white or intermediate fibers (Padykula and Gauthier, 1967 *a*; Gauthier, 1970).

In sharp contrast, the white fiber has a large diameter (44 μ), relatively few mitochondria per unit area, and it tends to store glycogen rather than lipid. Mitochondria consist almost entirely of the paired form at the I bands, and are not usually present as longitudinal rows among myofibrils. Red and white fibers can be distinguished also by the configuration of the sarcoplasmic reticulum in the region of the H band (Gauthier, 1969 and 1970). The Z line of the white fiber is narrower (339 A $\pm$ 30 [SD]) than that of the red or intermediate fibers. From these contrasting features it is to be expected that red and white fibers would differ in both their metabolic and contractile properties.

The third fiber type has been designated the intermediate fiber because its cytochemical properties, especially enzymic activities, are intermediate between those of red and white fibers. The diameter of the fiber is intermediate (34 μ) as is the width of the Z line (433 A $\pm$ 39 [SD]). The existence of this third fiber type was, however, difficult to establish by cytochemical criteria alone. Mitochondria are numerous and their distribution resembles closely that of the red fiber; however, they tend to be somewhat smaller and their cristae are less closely packed than in the red fiber. Positive identification of the intermediate fiber was achieved when differences in width of the Z line were established (Padykula and Gauthier, 1967 *a*); its width is intermediate between that of the red and the white fibers. Thus, while the intermediate fiber has many features which resemble those of the red fiber, the conspicuously narrower Z line serves as a critical distinguishing feature.

Neuromuscular Junctions

In all three fiber types in the rat diaphragm, branches of the motor nerve fibers terminate in an ultrastructural arrangement which is, in general, typical of neuromuscular junctions described elsewhere (Robertson, 1956; Reger, 1957; Andersson-Cedergren, 1959; Couteaux, 1960). The axonal ending lies in a depression of the muscle fiber surface ("primary synaptic cleft" or "synaptic gutter"). The sarcolemma of this region extends inward to form an elaborate system of infoldings ("secondary synaptic clefts" or "junctional folds"). The plasma membranes bounding the axon and

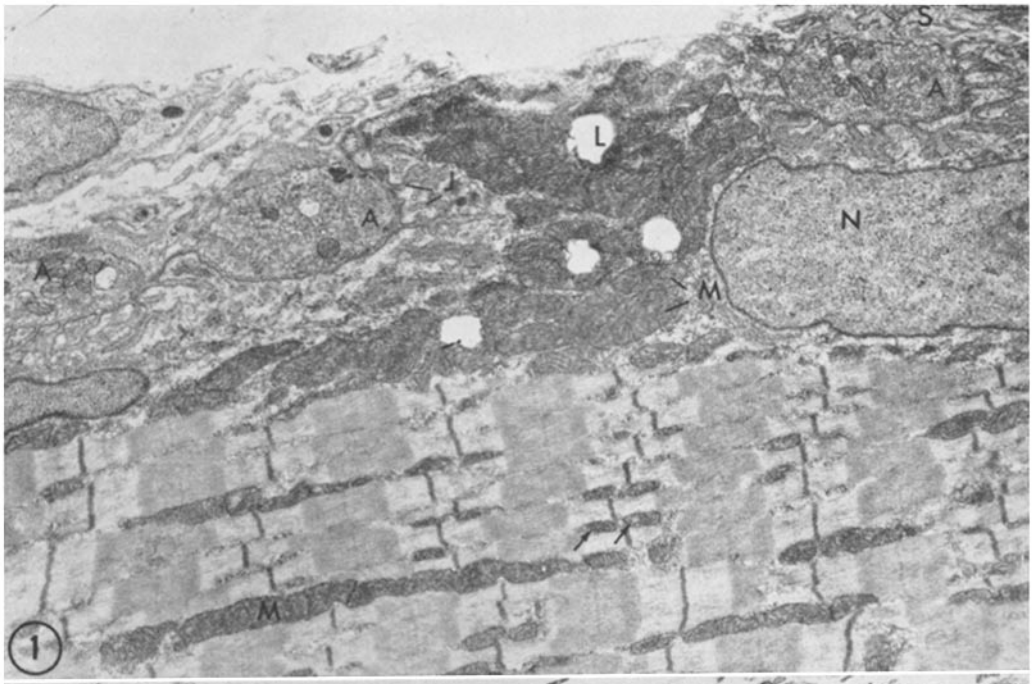
muscle fiber are separated at all points along the primary synaptic cleft by a single basal lamina which presumably represents a fusion of basal laminae from the two cells. An extension of this structure enters each secondary synaptic cleft as a single layer and continues along each wall of the cleft.

In the following descriptions, it will be demonstrated that the neuromuscular junctions of red, white, and intermediate fibers can be distinguished by differences in the shape and size of axonal endings and numbers of axoplasmic vesicles, by the distribution and spacing of the junctional folds, and by the appearance of both axoplasmic and sarcoplasmic mitochondria. The three-dimensional arrangement of the components of the neuromuscular junction is complex and difficult to analyze. Andersson-Cedergren's description (1959) of the neuromuscular junctions of mouse intercostal and abdominal muscles established that the junctional folds or invaginations are actually sections through radially arranged flat pouches. Because of the branching pattern, varying depth, and the relatively irregular radial arrangement of these pouches, only approximate measurements are presented in this study. Furthermore, it must be indicated that the search for neuromuscular junctions at the ultrastructural level is complicated by the sampling problem related to the relative proportions of the three fiber types in the rat diaphragm (60% red; 20% white; 20% intermediate). The myoneural regions of red fibers were quite readily located and numerous examples were studied; however, fewer examples were encountered in white and intermediate fibers.

RED FIBER: Profiles of axonal endings are relatively small and elliptical in outline (*circa* 2.8–4.0 μ by 1.1–1.7 μ). They occur intermittently in depressions of the muscle fiber surface (Fig. 1). At the site of nerve-muscle contact, the muscle cell surface is increased by junctional folds, whereas the surface intervening between such contacts is smooth. The sarcoplasm between areas of contact is rich in mitochondria with closely packed cristae, as is characteristic of most of the subsarcolemmal region of the red fiber (Fig. 1). The junctional folds of the red fiber are shorter, more curved, and sparser (Fig. 3) than those of the white fiber (Fig. 4). The most superficial folds are oriented more or less parallel to the muscle cell surface, and here their radial extension is greater (*circa* 1.5 μ) than in deeper locations. Along most of the deeper regions of the primary cleft, the radial extension of the folds is short (*circa* 0.5 μ). The spacing of the junctional folds is irregular and the folds themselves are branched and thus difficult to measure. The average distance between adjacent folds is, however, approximately 0.33 μ.

FIGURE 1 Red fiber. The intermittent distribution of axonal endings in the red fiber is illustrated in this low power electronmicrograph. Three elliptical endings (*A*) are located in surface depressions (primary synaptic clefts) of the junctional sarcoplasm. Junctional folds (*J*) extending from the surface depressions are sparse, shallow, and irregular when compared with those of the white fiber (Fig. 2). Two points of contact are isolated from each other by a mass of noninvaginated sarcoplasm packed with large mitochondria (*M*). Mitochondrial aggregates (*M*) at the neuromuscular junction may not be a specialization of this particular region, since they are numerous throughout most of the subsarcolemmal sarcoplasm of the red fiber. Paired mitochondria at the level of the I bands (arrows) are typical of all three fiber types. *N*, nucleus. *S*, Schwann cell. L, lipid droplet. × 8000.

FIGURE 2 White fiber. The extensive highly invaginated sarcoplasmic surface at the neuromuscular junction of the white fiber is illustrated in this low-power electron micrograph. Two long flat axonal endings (*A*) are associated with numerous closely packed, deep junctional folds (*J*). Slender mitochondria (*M*) in the superficial sarcoplasm of the junctional region form less conspicuous aggregations than in the red fiber (Fig. 1). The over-all mitochondrial content of the white fiber is low, even in the subsarcolemmal region; thus, aggregations at the neuromuscular junction are a specialization of this region in this fiber type. Interfibrillar mitochondria are primarily the paired forms at the I bands (arrows). × 4750.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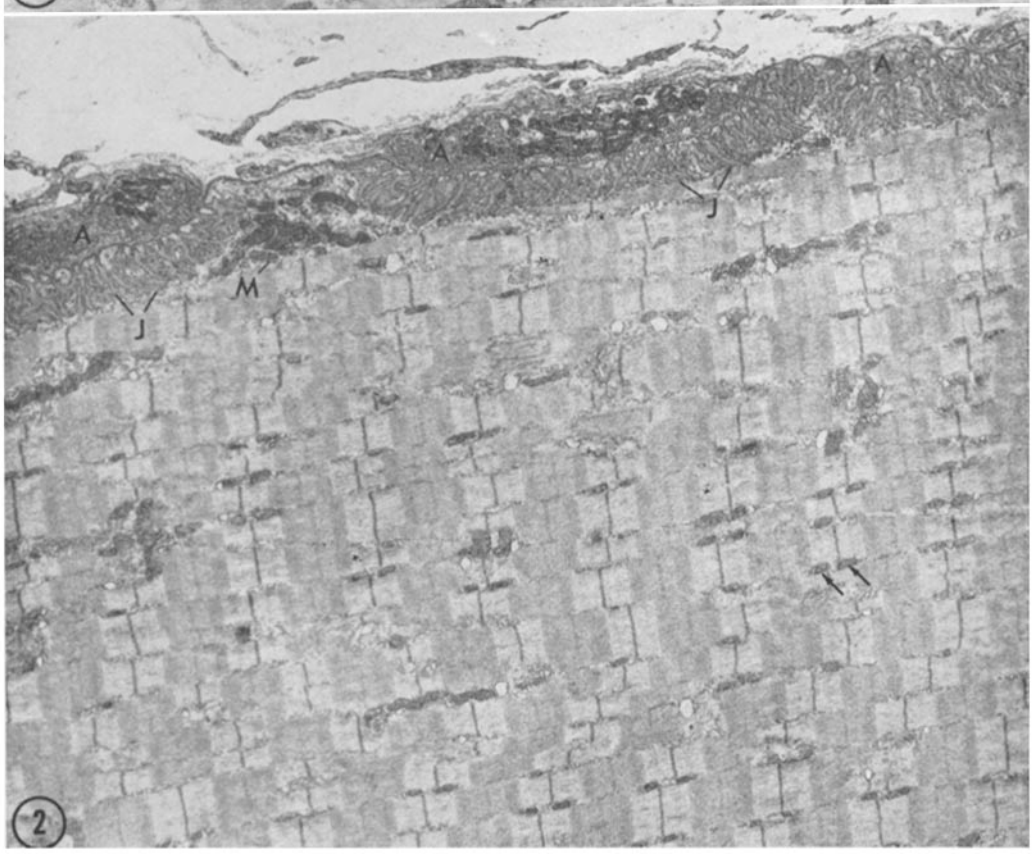
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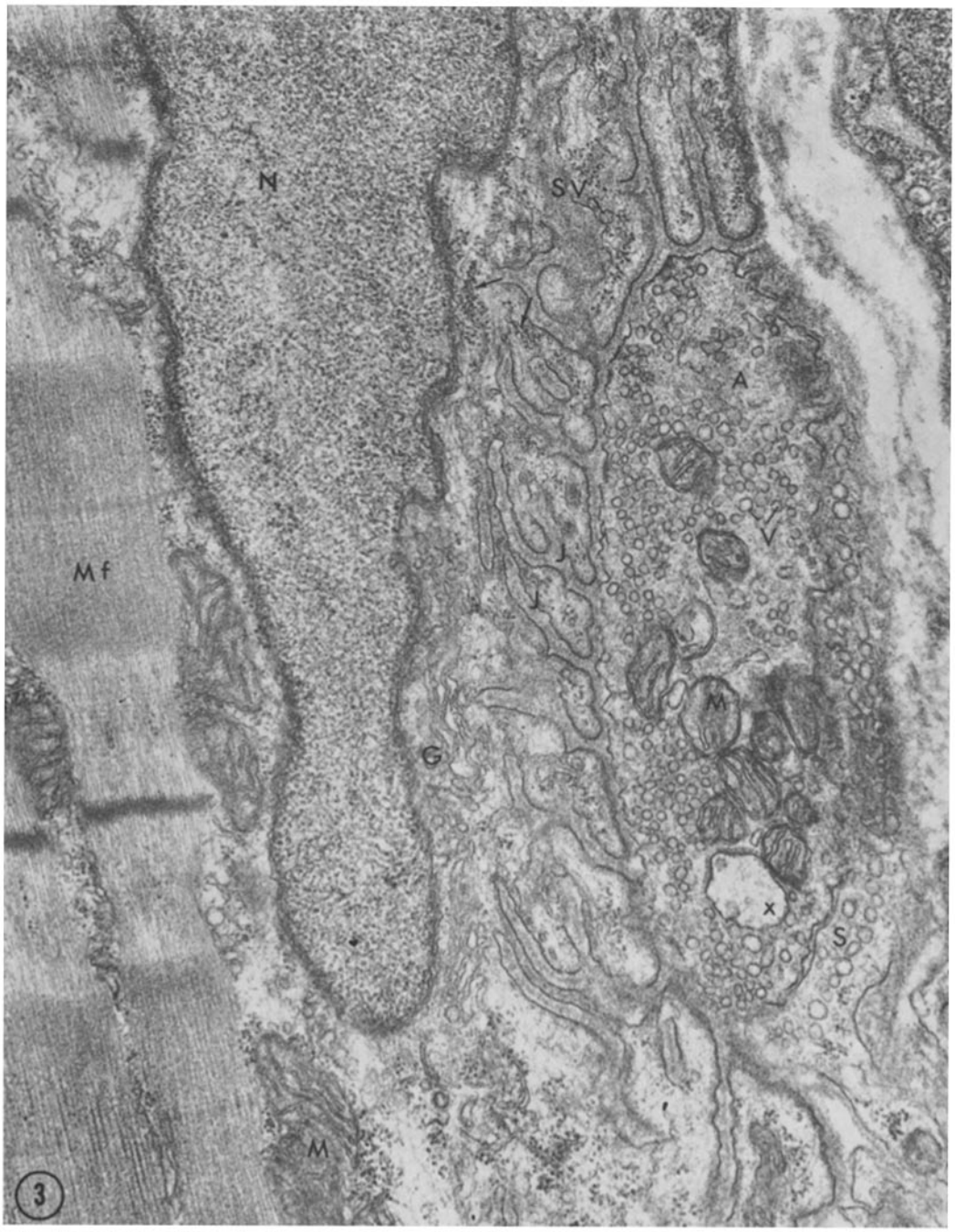


FIGURE 3 Neuromuscular junction, red fiber. A single elliptical axonal ending (*A*) lies in a depression of the sarcoplasmic surface and is covered by a thin lid of Schwann cell cytoplasm (*S*) which contains rows of smooth-surfaced vesicles. In the terminal axoplasm, moderate numbers of vesicles (*V*) and mitochondria (*M*) occur along with a single vacuole (*x*). The axoplasmic vesicles are relatively sparse when compared with those associated with the white fiber (Fig. 4). This neuromuscular junction is located near the surface of the nucleus (*N*) of the muscle cell. Junctional folds (*J*) are relatively sparse, short, and branching. In the sarcoplasm, between the junctional folds and nuclear surface, a portion of a Golgi apparatus (*G*), cisternal forms of granular endoplasmic reticulum (arrows), and free ribosomes occur. Sarcoplasmic vesicles (*SV*) are sparse. Compare the wide Z line of the red fiber with the narrow one of the white fiber (Fig. 4); this is an important criterion for the ultrastructural classification of fiber types. Mf, myofibril. × 30,000.

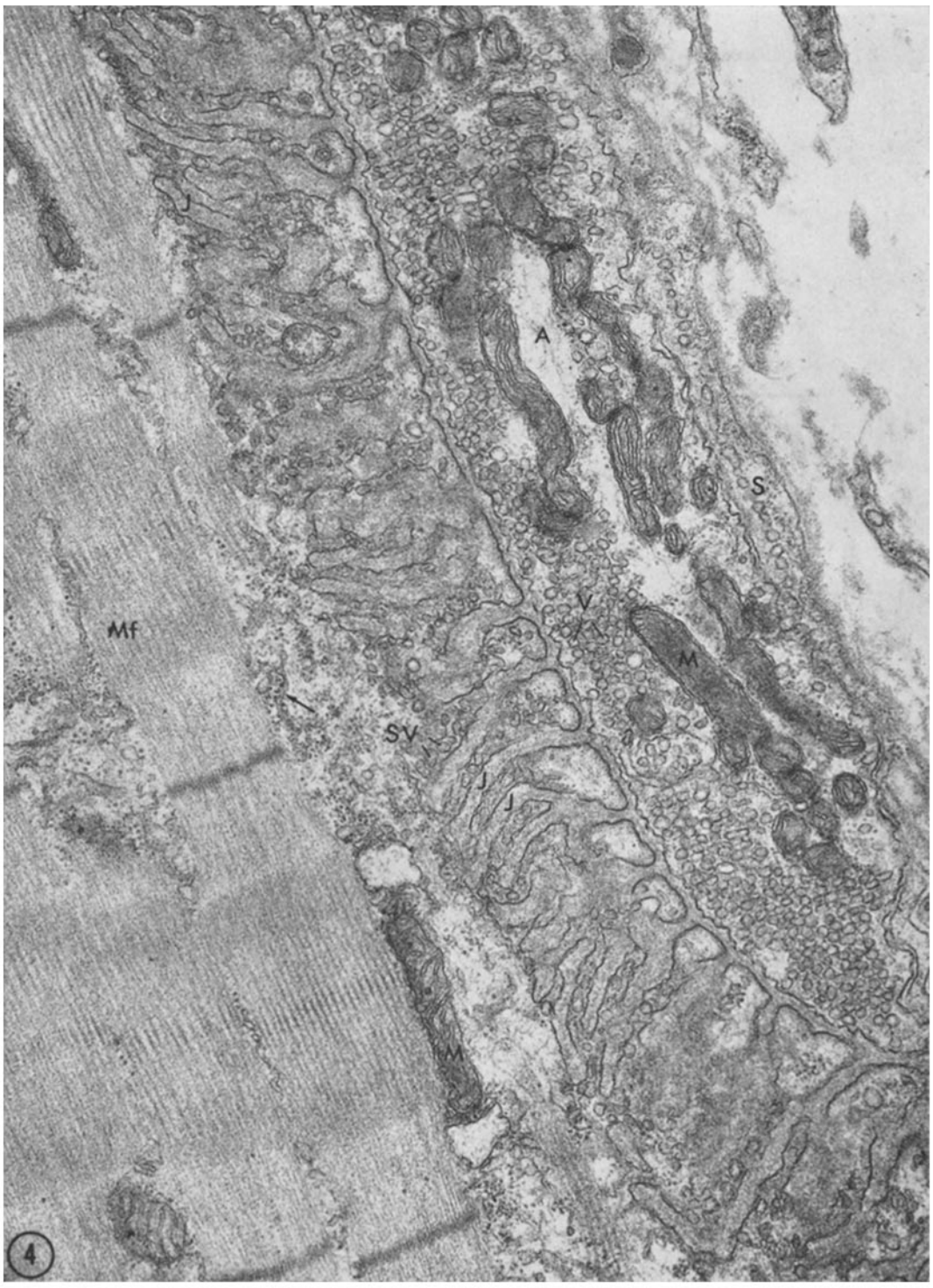


FIGURE 4 Neuromuscular junction, white fiber. The axonal ending (*A*) is flatter and broader than that associated with the red fiber. Vesicles (*V*) in terminal axoplasm are more closely packed than in the red fiber (Fig. 3). Mitochondrial cristae are longitudinal and lamellar. The nerve ending is capped by a thin layer of Schwann cell cytoplasm (*S*). The branching junctional folds (*J*) are numerous and closely packed. They extend deep into the sarcoplasm and nearly abut on the most superficial myofibrils (*Mf*). These junctional folds divide the junctional sarcoplasm into slender compartments that contain numerous sarcoplasmic vesicles (postsynaptic vesicles) (*SV*). A few profiles of sarcoplasmic mitochondria and rough surfaced endoplasmic reticulum (arrow) are evident. Compare with the red fiber in Fig. 3 which is at the same magnification, especially to establish differences in the width of Z lines. M, mitochondrion. × 30,000.

The axoplasmic vesicles of the nerve terminal on the red fiber (Fig. 3) are less closely packed than in terminals associated with the white fiber (Fig. 4). Terminal axoplasmic mitochondria are filamentous, the cristae are lamellar and longitudinal, and intramitochondrial granules are rare.

The blind end of each junctional fold is separated by intervening sarcoplasmic matrix from actual contact with myofibrils, Golgi apparatus, nucleus, and mitochondria. The sarcoplasm between the folds is relatively free of organelles, but contains free ribosomes and a few coated vesicles and smooth-surfaced vesicles (Fig. 3). Smooth-surfaced vesicles ("postsynaptic vesicles"), though few in number, occur in close proximity to the sarcolemma at the deepest portions of the junctional fold and closely resemble, in size and shape, the axoplasmic vesicles.

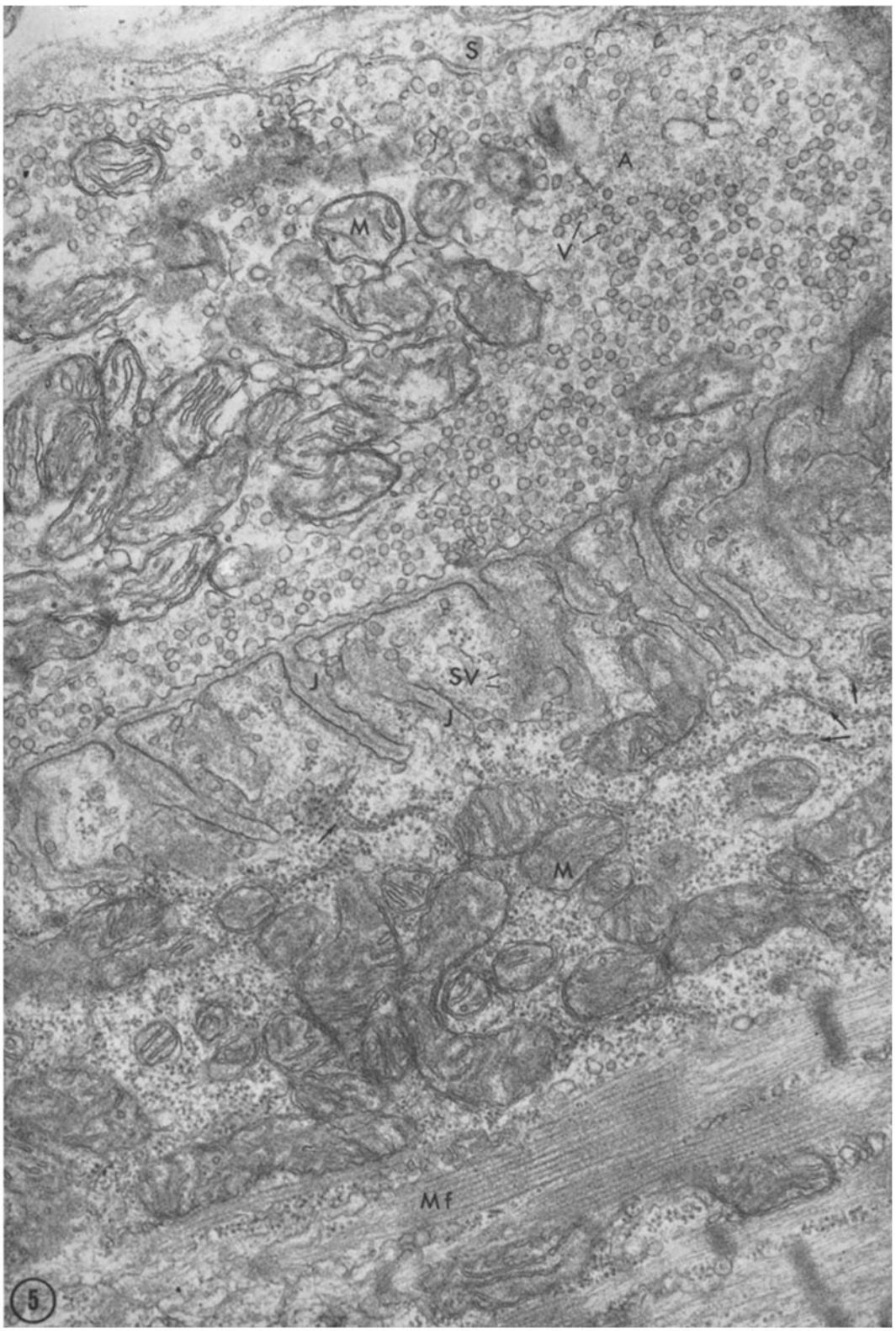
Sarcoplasmic mitochondria are numerous in the junctional area (Fig. 1), but it is difficult to determine whether these are special accumulations or whether they are part of the usual abundant complement of subsarcolemmal mitochondria of the red fiber. Most of the sarcoplasmic mitochondria are large and filamentous, but small ones are also present. Lamellar cristae are closely packed, and intramitochondrial granules are rare.

WHITE FIBER: The axonal processes terminating on the white fiber (Fig. 2) are longer and flatter (*circa* 4.6–8.4 μ by 1 μ) than those associated with red fibers. The axonal endings are distributed intermittently, but the junctional folds of one neuromuscular contact seem to merge with those originating at adjacent contacts. Thus, the junctional folds occupy a more continuous and widespread area of the sarcoplasmic surface than in the neuromuscular region of the red fiber. The shallow sarcoplasmic depression conforms to the shape of the axonal ending, and its lining sarcolemma and associated extracellular coat are thrown into numerous junctional folds which are uniformly long (*circa* 1 μ) and more regularly arranged than in the red fiber. The folds are more closely packed than in either the red or intermediate fiber; the space between adjacent folds is approximately 0.23 μ. Thus, the junctional folds of the white fiber are longer, straighter, and more closely packed than those of the red fiber, creating thereby a vast sarcoplasmic surface.

The axoplasmic vesicles of the nerve terminal (Fig. 4) are numerous, tightly packed, and occasionally have a linear arrangement. Terminal axoplasmic mitochondria are more slender than those associated with the red fiber. The cristae of these mitochondria are lamellar and are oriented longitudinally as in the red fiber. Intramitochondrial granules are relatively common.

Typically the blind end of each junctional fold lies close to the outermost myofibrils, and is thus separated from the contractile substance by only a thin layer of sarcoplasm containing an occasional single mitochondrion or ergastoplasmic components (Fig. 4). This is in sharp contrast to the neuromuscular junction of the red fiber which is embedded in a cushion of sarcoplasm rich in mitochondria. It should be pointed out, however,

FIGURE 5 Intermediate fiber. The identity of this fiber was established by comparison of the width of the Z line and mitochondrial content with adjacent red and white fibers. Two major findings are illustrated, the ultrastructure of the neuromuscular junctions of the intermediate fiber, and the prevalence of cisternal forms of granular endoplasmic reticulum (arrows). The latter is characteristic of the junctional sarcoplasm of all three fiber types. The axonal terminal (*A*) is both long and deep, and the junctional folds (*J*) are relatively long, unbranched, straight, but are widely spaced. The terminal axoplasm resembles that associated with the red fiber in that the vesicles (*V*) are loosely packed and the mitochondrial profiles (*M*) are fairly large. Numerous mitochondria (*M*) are present in the junctional sarcoplasm, but, as in the red fiber, this may also reflect the fact that the superficial cytoplasm throughout the intermediate fiber is rich in mitochondria. At least six profiles of flat cisternal granular endoplasmic reticulum occur here, three of which form a parallel array (arrows). Free ribosomes are scattered throughout the junctional sarcoplasm. The mitochondria and ergastoplasmic components in the three fiber types tend to be excluded from the immediate region of the junctional folds. There are, however, sarcoplasmic vesicles (*SV*) and a few ribosomes. *S*, Schwann cell cytoplasm. *Mf*, myofibrils. Approximately × 30,000.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that, although the white fiber has a sparse subsarcolemmal population of slender mitochondria throughout its length, there is some aggregation of mitochondria, especially in the sarcoplasm that intervenes between areas of neuromuscular contact. These aggregations are, however, less conspicuous than in the red fiber. They most likely represent definite differentiations of the neuromuscular association of the white fiber since they are not present in the remainder of the fiber. These sarcoplasmic mitochondria are entirely slender and filamentous, and possess lamellar cristae with relatively numerous intramitochondrial granules.

The sarcoplasm among the folds is compartmentalized into thin layers approximately 0.23 μ wide which contain numerous sarcoplasmic vesicles (postsynaptic vesicles) which resemble closely the axoplasmic vesicles. These sarcoplasmic vesicles, confined to the thin cytoplasmic compartments, are often in linear arrangement. They occur primarily near the deeper portions of the folds as in the red fiber. Occasional coated vesicles occur in these sarcoplasmic compartments.

INTERMEDIATE FIBER: Our measurements suggest that the axonal endings (Fig. 5) are longer and deeper (*circa* 4.2–18 μ by 2.1–2.8 μ) than those of either the red or white fiber. The axoplasmic vesicles are not so closely packed as in the endings on the white fiber. The junctional folds throughout the primary cleft extend deeper (1.2 μ) into the sarcoplasm than the majority of those of the other two fiber types, and the average distance between folds is greater than in either (0.5 μ). In addition, these folds are relatively straight and unbranched. Thus, large axonal terminals associated with long but widely spaced junctional folds are characteristic of the intermediate fiber.

As in the red fiber, the sarcoplasmic mitochondria are aggregated immediately beneath the zone of junctional folds (Fig. 5), but, since the intermediate fiber tends, as does the red fiber, to have numerous subsarcolemmal mitochondria along its length, it is not certain that this is a myoneural specialization. As in the white fiber, sarcoplasmic vesicles are conspicuous near the sarcolemma of the deeper portions of the junctional folds. Thus, the intermediate fiber shares some qualities of both red and white fibers. The individuality of the fiber depends on the distinct difference in Z line width, in mitochondrial struc-

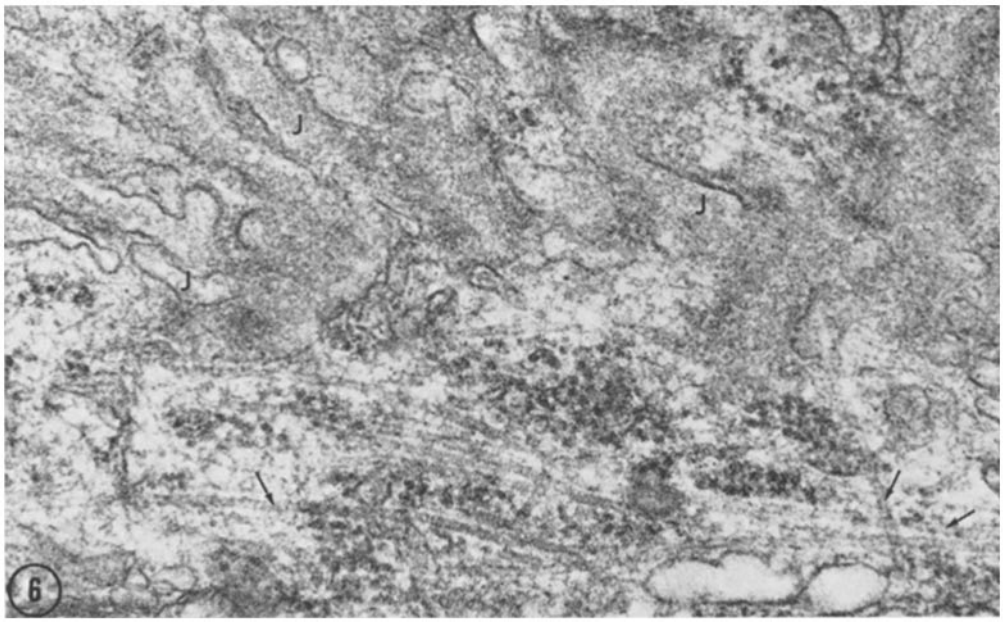

FIGURE 6 Microtubules in the junctional sarcoplasm of a red fiber. Microtubules (arrows) are abundant in the junctional sarcoplasm. They form, in some cases, parallel arrays, but the arrangement is, for the most part, random. Junctional folds (*J*) occur in the upper part of this field. × 65,000.

ture and distribution, and now also in neuromuscular ultrastructure, especially in the form and spacing of the junctional folds.

Additional Cytological Features Common to the Three Types of Neuromuscular Junctions

The junctional sarcoplasm of all three types of fibers is rich in free ribosomes and cisternal forms of granular endoplasmic reticulum (Figs. 3 and 5). Flat cisternae occur singly or are stacked in, more or less, parallel small arrays immediately below the zone of junctional folds.

As is well-known, nuclei are aggregated in the sarcoplasm of the neuromuscular junction (fundamental nuclei). The nuclear surface is invaginated into deep clefts which face toward the junctional folds, and thus greater nuclear surface area is directed toward this specialized region of neuromuscular interaction. Another constant feature is the occurrence of a paranuclear Golgi apparatus with typical cisternal and vesicular components (Fig. 3). Portions of the Golgi apparatus are often enclosed by invaginations of the nuclear surface.

Coated vesicles occur in small numers in the axoplasm and sarcoplasm. Specimens preserved in glutaraldehyde reveal the presence of microtubules (Fig. 6), which are abundantly distributed throughout the junctional sarcoplasm, including compartments between junctional folds, and extending toward the peripheral myofibrils. The arrangment of microtubules appears to be random.

DISCUSSION

Evidence of Heterogeneity at the Mammalian Neuromuscular Junction

MORPHOLOGICAL EVIDENCE: Numerous histochemical studies of mammalian skeletal muscle demonstrate, with striking illustrations, a complex pattern of heterogeneity of the component fibers, yet relatively little attention has been given to the possibility that differences may exist at the neuromuscular junctions. At the light microscopic level, the diaphragm and hind limb muscles are composed of twitch fibers, which possess *terminaison en plaque*, according to the nomenclature of Couteaux (1960) for vertebrate muscle. The terminations of the motor nerve form discrete oval or rounded disc-like configurations. Cole (1957) reported, however, that some structural variation occurs within the *en plaque* pattern among different muscles of the rat. He found the end plates of the diaphragm to be "most irregular" and "difficult to classify". Several different investigators (Cöers and Wolff, 1959; Anzenbacher and Zenker, 1963; Gruber, 1966) observed that muscle fibers of large diameter tend to have large motor end plates. In addition, Gruber indicated that the length of a motor end plate was directly related to the diameter of its motor nerve fiber.

At the ultrastructural level, it has been assumed that twitch muscles, including the diaphragm, are composed of a uniform population of muscle fibers, and this assumption has resulted in descriptions which do not distinguish neuromuscular junctions (Nickel, 1966; Miledi and Slater, 1968; Nickel and Waser, 1968; Teräväinen, 1968 *b*). The rat diaphragm has been a favorite muscle used for ultrastructural localizations of acetylcholinesterase (Barrnett, 1962; Csillik, 1967; Teräväinen, 1969); and in such cytochemical investigations also a homogeneous population of fibers has been assumed. Thus, the present description of three kinds of neuromuscular junctions in red, white, and intermediate fibers extends and challenges previous morphological and cytochemical findings. Cognizance must be taken of the intrinsic heterogeneity of mammalian skeletal muscle in future ultrastructural studies, especially those involving experimental or pathological changes.

PHYSIOLOGICAL EVIDENCE: Various kinds of physiological evidence have pointed to the possibility that differences exist at the neuromuscular junctions of fast vs. slow muscles or within a single mammalian muscle. Much of the experimental work related to this possibility has been performed on the cat soleus and gastrocnemius muscles, as examples of slow red and fast white muscles, respectively.

Evidence indicates that the motoneurons themselves are, to some extent, heterogeneous in that they may vary in size and functional properties. Generally it has been assumed that the diameter of the axon is directly related to the size of the motoneuron (see Henneman et al., 1965). In measuring deafferented nerves to the hind limb muscles of the cat, Eccles and Sherrington (1930) found that the distribution of sizes of the motor fibers formed two peaks indicating at least two populations, small (*circa* 6 μ) and large (*circa* 15 μ) diameter fibers. Even though two sizes pre-

dominated, there was a wide range of diameters for each nerve measured, which suggests an even greater heterogeneity in the population of motor fibers.

Size differences in motoneurons suggest differences in such properties as rate of discharge and conduction velocity. It is generally accepted that the conduction velocity is directly related to fiber diameter and to internodal distance (Hursh, 1939; Gasser and Grundfest, 1939). The motor fibers (α-motoneurons) to the slow soleus muscle have a mean diameter which is about 78% of the diameter of those occurring in the fast gastrocnemius (Eccles and Sherrington, 1930). Also, the α-motoneurons to the soleus discharge more slowly (10–20/second) than those supplying fast muscles (30–60/second) (Eccles, Eccles, and Lundberg, 1958). It would be logical to expect that the heterogeneity of the motoneurons might be reflected in the size and speed of motor units. A heterogeneous pattern of motor units has been demonstrated in certain hind limb muscles (Wuerker et al., 1965; Olson and Swett, 1966; Burke, 1967; and Close, 1967). In fact, three distinct types of motor units which vary in speed and size have been identified in the soleus and extensor digitorum longus muscles of the rat (Close, 1967).

From these various physiological consideration, it seems reasonable to think that differing form and function at the neuromuscular junctions would be related to the kind of motoneurons and the type of skeletal muscle fibers which interact. In the rat diaphragm, differences in the size and form of the terminal axons at the junctions in red, white, and intermediate muscle fibers may be, to some extent, a manifestation of differences in size of the motor fibers of the phrenic nerve. It is probable also that the size of the muscle fiber influences the neuromuscular relationship. In frog muscle, for example, the diameter (or "input conductance") of the muscle fiber is related to the amplitude of the miniature end plate potentials (Katz and Thesleff, 1957). In the rat the heterogeneity of the fibers of the diaphragm as well as other muscles might be related to the specific connections with different motoneurons. This hypothesis could be tested by repeating the cross-innervation experiments on fast and slow muscles (Buller, Eccles, and Eccles, 1960; Romanul and Van Der Meulen, 1967; Yellin, 1967) and then examining the ultrastructure of the cross-innervated fibers and their neuromuscular junctions. Furthermore, it seems logical to predict that three different types of motor units are present in the rat diaphragm and that physiological differences in end plate potentials among the three muscle fiber types should be demonstrable. Recently, the distribution of muscle fibers comprising individual motor units was identified histochemically in the anterior tibialis muscle of the rat, by demonstrating that one muscle fiber type responds after stimulation of a given motor nerve fiber (Edström and Kugelberg, 1968).

Implications of the Ultrastructural Differences among Neuromuscular Junctions of the Three Fiber Types

A major difference among the three neuromuscular junctions resides in the amount of surface area of the sarcoplasmic plasma membrane (postjunctional membrane), and this is undoubtedly an important factor in imparting distinctive functional properties to red, white, and intermediate fibers. This difference is manifested in the form and distribution of junctional folds, which are invaginations of the muscle plasma membrane and its accompanying extracellular coat. In the white fiber, the merging of the junctional folds at one axonal terminal with those of adjacent ones creates a widespread, relatively continuous area of invaginated cell surface (Figs. 2 and 4). This is in sharp contrast to the design in the red fiber, where the sparse junctional folds at one terminal are isolated from nearby ones by a noninvaginated cell surface (Figs. 1 and 3). Thus, the white fiber presents the largest sarcoplasmic surface at the contact areas in the form of extensive as well as long and closely spaced junctional folds. Although a quantitative analysis of surface area has not been made, our electron micrographs indicate that the red and intermediate fibers present considerably less sarcoplasmic surface at the neuromuscular junction than does the white fiber. A distinctive cytochemical property of the sarcoplasmic surface is the presence of acetylcholinesterase (Barnett, 1962; Davis and Koelle, 1967; Salpeter, 1967 and 1969; Csillik and Knyihár, 1968), and thus it is possible that the amount of membrane surface might reflect differences in this enzymic activity among the three fiber types.

Various ultrastructural features suggest that

transmission in the white fiber might differ significantly from that in the other two fibers. In addition to a highly invaginated sarcoplasmic membrane (Fig. 4), there is a relatively large axoplasmic surface in the form of a flat, broad axonal ending. The abundance of axoplasmic vesicles in the white fiber might reflect a potentially greater supply of acetylcholine or some trophic factor, since the axoplasm is packed with such vesicles. Although the function of the sarcoplasmic vesicles (postsynaptic vesicles) is unknown, it may be significant that they are most abundant in the white fiber (Fig. 4). In addition, intramitochondrial granules occur most frequently in both the axoplasmic and sarcoplasmic mitochondria of the white fiber. Since these intramitochondrial granules are known to be sites of concentrated cations (Peachey, 1964; Thomas and Greenawalt, 1968), the possibility exists that greater amounts of cations, e.g. calcium, may be stored at the neuromuscular junction of the white fiber. However, definite statements about the relative speed of contraction of the three fiber types cannot be made until electrophysiological data concerning threshold, contraction time, relaxation time, and endplate potentials have become available. Recent speculations concerning the functions of the red, white, and intermediate fibers are reviewed by Gauthier (1970).

An ultrastructural feature of all three fiber types which should be emphasized is the regular occurrence in the junctional sarcoplasm of cisternal forms of granular endoplasmic reticulum and of free ribosomes (Fig. 5). The presence of cisternae of granular endoplasmic reticulum was previously noted by Andersson-Cedergren (1959) in the neuromuscular regions of mouse intercostal muscles, but this important observation has received little attention. The local concentration of cellular machinery indicates that the neuromuscular junction is a site of protein synthesis. A distinctive protein on the sarcoplasmic side of the junction is acetylcholinesterase, which is localized along the postjunctional membrane (Barrnett, 1962; Csillik, 1967; Davis and Koelle, 1967; Salpeter, 1967 and 1969). This enzyme or other proteins required for transmission might be produced locally by this cellular machinery. For example, it has been postulated that the acetylcholine receptor at synaptic sites is a macromolecule, possibly a protein or lipoprotein complex (Nachmansohn, 1955; Durell et al., 1969). The presence of coated vesicles in the junctional sarcoplasm (Nickel et al., 1967) may be interpreted as an indication of macromolecular transfer at the neuromuscular junction. Experiments with horseradish peroxidase (Zacks and Saito, 1969) demonstrate that this exogenous protein is taken up by coated vesicles of the neuromuscular junction.

Microtubules in the junctional sarcoplasm (Fig. 6) might reflect a gelation of the protoplasmic areas at the site of nerve-muscle contact (see Porter, 1965). It is possible, for example, that, during muscular contraction, the neuromuscular junction remains firm while the myofibrils contract. The occurrence of cellular machinery for protein synthesis, coated vesicles for the uptake of macromolecules, and microtubules reemphasize that a high degree of metabolic and structural specialization exists at the junction. Upon these and other common features, there is superimposed the additional differential specializations evident in the neuromuscular junctions of red, white, and intermediate fibers which have been reported here.

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