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A Quantitative Ultrastructural Study of Isolated Canine Tracheal Smooth Muscle and its Innervation

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Dedicated to

My Mother, Father, Maryanne, and Ernie

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ABSTRACT

The present study was performed in an attempt to qualitatively and quantitatively describe the innervation and certain features of the morphology of the canine tracheal smooth muscle cell. These features were observed through the use of isolated canine tracheal smooth muscle strips.

A) Innervation - Qualitative

Qualitative observations were made through the examination of micrographs of both longitudinal and cross-sectioned fibers taken from muscle which was prepared by conventional electron microscopic techniques. Nerve terminals, i.e. Schwann cells and their associated axons were found alongwith connective tissue septa in the extracellular space between smooth muscle bundles. These terminals did not penetrate within the muscle bundles. No close nerve-muscle relationships were observed, and terminals were found in general to lie 1 - 3 microns away from the muscle. These observations suggested that transmitters released from nerve terminals would have to travel large distances before reaching the muscle fibers. The terminals often demonstrated the presence of axons which were partially "unsheathed" by the Schwann cell. These axons were also often found to contain synaptic vesicles.

B) Innervation Quantitative

Quantitative studies were performed using cross sections of muscle fibers taken from four randomly selected animals. Thin sections

of muscle fibers were placed on 300 mesh copper grids of which randomly chosen squares were used for quantification purposes. In each square chosen, the number of fully visible muscle cells and nerve axons were counted. The vesicular content of the axons was also noted. Small agranular vesicles 30 - 50 nm. in size were termed cholinergic, and large dense vesicles 80 - 120 nm. which demonstrated a halo were termed adrenergic. The study revealed a ratio of 10 : 1 muscle cells per nerve axon; 50% of the axons observed contained cholinergic vesicles. Approximately 5% of the axons contained adrenergic vesicles. Small dense core vesicles, and large clear vesicles were noted. However, they occurred with very low frequency, were often difficult to identify, and could not be classified.

C) Muscle Cell Morphology - Qualitative

Qualitative assessment of the muscle revealed morphological features similar to most smooth muscles. Both longitudinal and cross-sections were used. The fibers were arranged in parallel, and in bundles, which were separated by the connective tissue septa. Measurements of the cell length indicated cells approximately 850 microns in length. Fibroblasts, mast cells, and nerve terminals were found in the extracellular space and were occassionally seen in close proximity to each other. Collagen, elastin and elastic fibers were frequently observed in close association. The basement membrane of the muscle fibers was also found to be associated with elastin and elastic fibers. The collagen fibers were often found to run at right angles to the long axis of the muscle cells, with elastic fibers running parallel to the long axis of the cell.

The muscle fibers appeared as long, narrow structures with a centrally located nucleus when viewed in longitudinal sections. Numerous cytoplasmic constituents such as mitochondria, glycogen, Golgi apparatus, rough and smooth endoplasmic reticulum were found in the cytoplasm at the poles of the nucleus. These regions were devoid of myofilaments, although myofilaments were found to run parallel to the cell's long axis around the nucleus and at its poles. The remaining portions of the cytoplasm of the cells were filled almost entirely with myofilaments. Towards the most distal part of the cells the cytoplasm appeared extremely dense. Cross-sections of these regions revealed that the ends of the cells subdivide and branch, and interdigitate with other cells. The basement membrane in these regions appeared especially thick and was intimately associated with elastin. The elastin in turn was found to be associated with elastic fibers and collagen.

The sarcolemma demonstrated the presence of numerous pinocytotic vesicles or caveolae. The caveolae appeared to be most abundant at middle regions (at the level of the nucleus) of the cell. Sarcoplasmic reticulum (SR) was often found directly below and occassionally in contact with the caveolae. Mitochondria were also found in these regions associated with the SR and caveolae. However, the majority of the mitochondria appeared to be located in the midline of the cell amidst the myofilaments, extending from either pole of the nucleus. The caveolae in general appeared in groups and alternated with membrane specializations known as dense bands. The dense bands were identified as an incrustation of electron dense material at the cytoplasmic side of the membrane. Filaments were seen to leave and

enter the dense bands, however their exact identity was not ascertained. The cytoplasmic counterpart of the membrane dense bands increased from the middle of the cell towards either end, whereas caveolae were found to decrease in frequency in these areas. The most distal portions of the cell appeared to have membrane that was entirely comprised of dense band material.

Gap junction and intermediate junction were observed between smooth muscle fibers. Branched ends of cells were also observed to interdigitate between ends of neighbouring cells and form junctional structures.

D) Muscle Cell Morphology - Quantitative

This portion of the study was carried out by analysing cross sections of randomly chosen cells sectioned at different points along their length. The circumference of each cell, and each region along the membrane that was involved in either dense bands, caveolae, or intermediate junctions were measured. A ratio of the total dense band regions divided by the cell circumference was calculated for each cell. Cells with high dense band to circumference ratios were termed as cells sectioned near their ends, while others with low ratios were considered as cells sectioned near their middle. This grouping allowed the analysis of the caveolae and intermediate junctions and their relation to dense band regions at different points along the length of the cell. Through the use of these quantitative procedures and statistical techniques dense band regions of the cell were found to increase toward the ends of the cells as suspected through qualitative observations. Caveolae regions however, were found to decrease, and were at a maximum

at the level of the nucleus. Portions of the membrane involved in intermediate junctions were found to increase toward the cell ends. However, the ratio of intermediate junctional area to dense band area decreased towards the cell ends.

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INTRODUCTION

Smooth muscle research has been receiving greater attention in the basic medical sciences during the past number of years. Much emphasis has been placed on airway smooth muscle in particular as efforts to control and understand asthma have increased. As a result tracheal smooth muscle is becoming the focus of much interest, since this muscle is commonly used as a model for airway smooth muscle in general. The present study deals specifically with canine tracheal smooth muscle. It has become apparent that there exists significant differences in smooth muscles taken from different species, and each will eventually have to be considered separately. The use of a canine allergic model of asthma (Kepron et al. 1977) has stressed the importance of a more complete understanding of normal canine tracheal smooth muscle.

Tracheal smooth muscle has shown itself to be unique with respect to other smooth muscles as it exhibits properties akin to both single unit and multiunit smooth muscle. Classification of the muscle into a single unit or multiunit muscle has generally dealt with functional properties of the muscle, however, it is now believed that classification of smooth muscles in to either type will also rely on an accurate assessment of the nature of the autonomic innervation to these muscles (Burnstock, 1970). Therefore, the purpose of this project has been in part, to help establish a quantitative estimate of the nervous input to this muscle.

With regards to muscle contraction itself, much of the attention within the past few years has been directed toward the contractile

proteins at a molecular level, specifically dealing with the biochemical mechanisms involved in their activation and deactivation. However, very little is actually known of the arrangement of these elements and the transmission of force from one muscle cell to the next. Cooke (1976) proposed a model for myofilament organization involving a cytoskeleton, which has proved to be helpful in explaining force transmission within the cell itself. However, little is known about the transmission of force between cells and the role of the connective tissue elements that surround it. The presence of intermediate junctions and their role in mechanical transmission between cells has also been obscure. The pattern of these junctions and their association with the membrane specializations known as dense bands, their involvement with the myofilaments and the cytoskeleton will have to be studied in order to further understand mechanical coupling between cells. Therefore, this study has attempted to quantify these structures and also to bring forth observations from a qualitative standpoint. It is hoped that the information found may prove useful in defining a clearer and more quantified picture of the structural substrate subserving contraction in airway smooth muscle, using the canine trachealis as a model.

II. REVIEW OF LITERATURE - ULTRASTRUCTURE AND PHYSIOLOGY

A) Some General Physiological Properties of Tracheal Smooth Muscle

Due to the diversity of smooth muscle types, smooth muscle cells between themselves exhibit an entire spectrum of physiological properties, often species specific. Smooth muscles have generally been categorized into two types, namely single unit and multiunit muscles. Single unit muscles have been classically defined as having the characteristics that they develop spontaneous electrical oscillations or action potentials which are coordinated in time and space by good electrical coupling between cells (Bozler, 1948; Prosser et al. 1960). In contrast, multiunit muscle have been defined as those which do not generate spontaneous electrical activity and/or cannot transmit such activity between cells (Daniel et al. 1980). However, certain muscles demonstrate properties which belong to single unit as well as multiunit muscle have occasionally been termed as intermediate type muscles.

In the past, the function and regulation of tracheal smooth muscle has been poorly understood, however, recent investigations have proven it to be an active and responsive tissue (Widdicombe, 1966; Olsen et al. 1967; Stephens et al. 1968). Tracheal smooth muscle (TSM) is a muscle that is normally quiescent in vitro and shows no rhythmic or spontaneous contractions, as seen in smooth muscles of the single unit type. Tracheal smooth muscle belongs to the category of smooth muscles that do not readily produce action potentials. In fact, neither direct electrical stimulation nor nerve stimulation can evoke action potentials; they do however, cause a graded depolarization of the muscle membrane. It is believed

that smooth muscles which do not normally exhibit action potentials, fail to do so not becuase they lack an ionic channel admitting an inward current, but because the operation of this channel is masked or obliterated by the premature opening of the channel carrying an outward potassium current (Stephens and Kroeger, 1980). Suzuki et al. (1976) demonstrated a substantial rectification of the membrane which attenuates the action potential.

Studies pertaining to TSM regulation have shown that this muscle is controlled primarily by the nerves of the autonomic nervous system. Neural regulation is brought about by adrenergic sympathetic nerves (Miller, 1947), which cause relaxation, and parasympathetic through the vagus (releasing acetylcholine), resulting in constriction (Loofbourrow et al., 1957; Widdicombe, 1966). However, the parasympathetic system has been shown to predominate both physiologically and anatomically (Cabezas et al., 1971). In contrast to single unit muscles, such as those of the gut, studies of tracheal smooth muscle show that these neural elements play a large role in the control of the muscle. Recent evidence has brought to light the existence of non-cholinergic, non-adrenergic relaxant nerve fibers. These fibers have been named purinergic nerves, since it has been suggested that purine nucleotides are their putative transmitters. This type of innervation was first described in the gastrointestinal tract (Burnstock, 1972), but recently has been shown to be present in the smooth muscle of the airways of the guinea pig (Coburn and Tomita, 1973; Coleman and Levy, 1974; Richardson and Bouchard, 1975). Richardson and Beland (1976), were also able to demonstrate the presence of purinergic nerve fibers in human airway smooth muscle.

These investigators suggest that the purinergic innervation of the airways may contribute to a much greater degree to the control of the muscle than do the adrenergics. Indeed, the purinergic system in the gastrointestinal tract has shown to exhibit an extremely powerful inhibitory effect upon the smooth muscle cells. In fact, it has been demonstrated by Burnstock (1979) that stimulation of purinergic nerves with single pulses of short duration (less than 0.3 ms.), produces transient hyperpolarizations of inhibitory post-synaptic potential's (IPSP's) of up to 25 mV in single smooth muscle cells of the gut. It is also known that when this system is absent from the gastrointestinal tract, the smooth muscle develops an uncontrolled myogenic activity that produces a functional spasm of the tract (Frigo et al. 1973; Wood, 1973; Richardson 1975). This condition is commonly known as Hirschsprung's disease. A recent publication by Richardson and Ferguson (1979) has suggested an interesting analogy to this situation that may have implications in airway smooth muscle. They suggest that a condition similar to that seen in Hirschsprung's disease may exist in the airways. This loss of inhibitory control may perhaps account for, or at least contribute in part towards the spasm of the airways seen in a variety of diseased states. However, canine TSM does not demonstrate the presence of purinergic innervation (Suzuki et al., 1976; Kannan and Daniel, 1980). This constitutes an important difference from human airway smooth muscle.

Trachealis in general shows a similarity to multi-unit smooth muscle, which is believed to have individually innervated muscle cells.

It has been shown that norepinephrine and high potassium solutions (Keatinge, 1966) which convert intermediate type arterial smooth muscle into single unit type preparations, causing these muscles to exhibit phasic activity and the production of a myogenic response to stretch, are unable to do so in canine TSM (Stephens et al. 1975). However, TSM has been shown to exhibit properties similar to single unit muscle under the effect of various agents and drugs. Kirkpatrick in 1975 was able to induce depolarization of bovine tracheal smooth muscle due to slow oscillations in the membrane potential with histamine. These depolarizations corresponded to rhythmic fluctuations in contractile activity, and showed some dependency on extracellular calcium. In another experiment, tracheal strip preparations from sensitized guinea pigs were shown to exhibit spontaneous mechanical activity, before as well as after a histamine stimulus or antigen challenge (Souhrada and Dickey, 1976).

Tetraethylammonium (TEA), is another substance that has been shown to change the physiological nature of TSM. Investigations by Stephens et al. (1975), demonstrated the induction of spontaneous electrical and mechanical activity in airway smooth muscle. Application of TEA gave rise to membrane depolarization as well as trains of small decrementally conducted action potentials. The presence of these action potentials was believed to be produced as a result of a decrease in potassium permeability of the membrane. This decrease in permeability inhibits the potassium conductance of the membrane potential that normally takes place upon the opening of sodium channels, resulting in the formation of spike potentials seen in this preparation. In addition to the production of spontaneous

activity, TEA has also been shown to induce the muscle to show a myogenic response to quick stretch, a phenomena not elicited in normal TSM. Findings such as these suggest that although TSM under normal conditions demonstrates characteristics of multi-unit smooth muscles, it has also the potential for single unit behavior. These alterations in activity may perhaps contribute to the abnormalities seen in a number of disease states (important among which is asthma) found in airway smooth muscle, and will require further investigation.

B) Structural Features

1. General Morphology

The smooth muscle of the trachea or musculus transversus trachea consists of a unified layer of thick bundles of smooth muscle cells which branch little and are transverse in direction. In the canine trachea, the sheet of muscle is approximately 30 cells thick. The muscle bundles are fastened by elastic tendons to the external perichondrium of the tracheal cartilage (towards the dorsal ends of the cartilage) and the annular ligaments. Luschka (1963) has reported that bundles of muscles extend from the trachealis and insert into the ventral wall of the esophagus. The smooth muscle bundles have been shown to be separated by wide interstitial spaces, which contain collagen and interstitial cells (Kannan and Daniel, 1980). The individual cells are extremely long in comparison to their width, with their length exceeding 1 mm. and their width being approximately 3.3 ± 0.5 um. S.D. as reported by Suzuki et al., (1976). These cells have a centrally located nucleus and have been confirmed by light and electron microscopy to run parallel to each other.

2. Nucleus

Tracheal smooth muscle cells demonstrate a prominent centrally located nucleus. In contrast, skeletal muscle cells are multi-nucleated with the nuclei located directly below the sarcolemma. Mitochondria and Golgi bodies are generally seen close to the nucleus, however, the mitochondria are usually situated at the nuclear poles. The presence of a nuclear membrane can be seen and pores in this membrane are occassionally observed. In resting muscle, the nuclear outline appears smooth, but becomes convoluted when the muscle fibers are shortened markedly (Stephens and Kroeger,1980).

3. Cytoplasmic Organelles

i. Golgi bodies and lysosomes

Golgi bodies, in common with the mitochondria, are usually observed at the poles of the nucleus. Lysosomes are also observed in smooth muscle cells but not with regular frequency (Burnstock, 1970). De Duve (1974) has shown that active smooth muscle cells can be transformed into foam cells, and suggested that this is a result of a relative inefficiency of some lysosomal enzymes. A study using piglet aortic smooth muscle cells exposed to hypoxia for 5 days demonstrated the development of a considerable number of lysosomes and phagosomes, alongwith membrane swirls or myelin figures (Paul et al., 1976). Studies pertaining to the effect of hypoxia on smooth muscle contractility, have suggested that part of the mechanical impairment observed in this state may be related to the activation of lysosomal enzymes (Stephens and Kroeger, 1970; Kroeger and Stephens, 1971).

ii. Mitochondria

Several mitochondria are seen throughout the cytoplasm in airway smooth muscle although primarily at the nuclear poles. They are sometimes observed in rows, and are often closely associated with glycogen granules. Stephens and Wrogemann (1970), have studies the oxidative phosphorylation parameters for canine tracheal smooth muscle and have reported that the ADP : O and respiratory control ratios are the same as that for mitochondria of skeletal muscle. However, the quantity of mitochondrial protein present per gram wet weight of muscle is much less than in skeletal muscle. From these findings they calculated that the ATP synthesized oxidatively in smooth muscle is only 10% of that reported for skeletal muscle.

The role of mitochondria in excitation - contraction coupling in smooth muscle is under question. It is tentatively believed that mitochondria act as a sink for calcium, and can sequester calcium during or after mechanical relaxation of the smooth muscle cell. In fact, Verbeke et al. (1977) have suggested that mitochondria appear to be the major calcium sink among smooth muscle cellular components, however, the in vitro studies in which this was based was done at abnormally high calcium concentrations.

iii. Sarcotubular system and rough endoplasmic reticulum

The sarcoplasmic reticulum in airway smooth muscle appears to be poorly developed, although a quantitative study of this aspect has not been conducted. The sarcoplasmic reticular structures are very variable and distorted in their distribution. However, a fairly organized longitudinal smooth sarcoplasmic reticulum system

in intestinal smooth muscle has been reported (Yamauchi, 1964). Rogers (1964) has also claimed to be able to demonstrate the presence of transverse tubular structures (similar to the T-tubule system in skeletal muscle) in guinea-pig ureter. However, an organized T-tubule system or sarcoplasmic systems has not been clearly identified in airway smooth muscle (Stephens and Kroeger, 1980). Somlyo et al. (1977) have suggested that these structures play a role in the intracellular translocation of calcium. It should be noted that since smooth muscle cells are extremely long and narrow, diffusion distances are small, and the surface areas of the cells are large in comparison to cell volume, therefore, extracellular calcium could be sufficient to provide the calcium needed for contraction. In addition, the pumps in the sarcolemma could be adequate to enable relaxation (Stephens and Kroeger 1980).

Rough endoplasmic reticulum is seen in tracheal smooth muscle although in relatively few occurrences in comparison to connective tissue cells such as fibroblasts. However, Yamauchi and Burnstock (1969) have shown that this structure is particularly prominent in developing smooth muscle.

4. Caveolae

The presence of invaginations or inpocketings of the cell membrane known as caveolae is one of themore distinct ultrastructural characteristics of smooth muscle cells. They are nonetheless present also in a variety of other cell types. Caveolae are often termed as pinocytotic vesicles (as referred to in the observations and in the legends to figures of this thesis) and as micropinocytotic vesicles, although they have not been shown conclusively to have pinocytotic

activity. However, this is not true for all micropinocytotic vesicles, as those observed in endothelial cells do in fact have micropinocytotic activity and are involved in active transport across the endothelium, (Palade, 1960, Jennings et al., 1962; Bruns and Palade, 1968). For this reason and others which will be mentioned below, it is believed that the caveolae found in different tissues often play different roles, and are responsible for different functions. For example, caveolae of cardiac muscle cells are less numerous than those in smooth muscle or endothelial cells. They are larger in size, often varying in shape, closely related to sarcoplasmic reticulum, and are not surrounded by intramembranous particles near the neck, as is seen with smooth muscle caveolae (Gabella, 1978). Similarly, caveolae of skeletal muscle cells more closely resemble those of cardiac cells than those of smooth muscle.

A thorough morphological study has not been done for all smooth muscles, however, a few fairly detailed studies have been carried out. Most caveolae, when observed with conventional electron microscopic techniques are seen to be in communication with the extracellular space through a narrow neck. This has been demonstrated through the use of tracers such as colloidal lanthanum, peroxidase and ferritin (Devine et al., 1972), which enter into the caveolae through the neck. The pinocytotic vesicles themselves show the same membranous structure as the sarcolemma. The question as to the presence of basement membrane material within the caveolae was examined in early studies by Gabella (1971; 1973) and led to the conclusion that the cell coat does not enter into the caveolae. In a more recent study using a silver-methenamine procedure for the demonstration of the

carbohydrate - containing "cell coat" (Forbes et al., 1979) also could not detect the presence of basement membrane within the caveolae. The inner portions of the vesicles are often seen to be in close association with the sarcoplasmic reticulum. This has suggested that caveolae may represent the smooth muscle counterpart of the T-tubule system known to exist in skeletal muscle, and may therefore aid in excitation - contraction coupling (Somlyo and Somlyo, 1975). Perhaps the most obvious function served by caveolae is in increasing the area of the cell membrane, owing to the fact that caveolae are basically saccules which are continuous with the plasma membrane. Gabella (1976) has shown that taenia coli smooth muscle cells of the guinea-pig contain approximately 170,000 caveolae per cell, producing an increase in the cell membrane area by roughly 70%. It has been suggested that this increase in membrane area would provide more membrane to be available for the exchange of nutrients, ions, and fluid across the cell membrane. In fact, this large surface area - to -volume ratio presents problems for the maintainence of the intracellular ionic composition, since all the sarcoplasm is relatively close to the extracellular fluid, and there is no large bulk to buffer changes in intracellular ion concentrations. This problem may be especially acute in smooth muscle as they are frequently found in areas of the body that may be sugjected to relatively large changes in the composition of the extracellular fluid. As a result of this situation, it has been suggested that smooth muscle membrane must possess well developed mechanisms for transporting ions and for maintaining a constant intracellular environment, despite continuous

fluctuations in the extracellular fluid, (Brading, 1979).

From a structural view point, early transmission electron microscopy (Pease and Molinari, 1960; Prosser et al., 1960) demonstrated that caveolae are not uniformly distributed over the cell surface. Freeze-fracture studies (Devine et al., 1971; Wells and Wolwyk, 1971; Muggli and Baumgartner, 1972; Orci and Perrelet, 1973) further clarified this point. Freeze-fracture splits the cell membrane along its hydrophobic component and exposes two artificial surfaces from inside the membrane (Branton, 1966; Chalcroft and Bullivant, 1970). These two surfaces are known as the P-face (or protoplasmic face) and E-face (or extracellular, exoplasmic face) (Branton et al. 1975). This technique cleaves apart large areas of the cell membrane enabling an accurate visualization of the arrangement of the cell membrane's pinocytotic vesicles or caveolae. The caveolae were shown to be fractured at the level of their necks appearing as rising structures on the E-face and as shallow depressions on the P-face. In addition to the distribution of the caveolae along the cell surface, the question arose as to whether their number remained constant. Dulhunty and Franzini-Armstrong (1975) using skeletal muscle had shown that during extreme stretch, caveolae open up and provide the membrane required for the increase in surface area. They found that in frog skeletal muscle fibers, the number of caveolae remains constant during passive stretch up to a sarcomere length of roughly 3 um., with the membrane lengths need for the increase in surface area being provided by the opening of folds of the sarcolemma. At longer sarcomere lengths, membrane is provided by the opening of caveolae and at a

sarcomere length of about 8 um., all the caveolae are opened up. In a study using smooth muscle of aplysia (Prescott and Brightman, 1976), fibers stretched to three times their resting length showed caveolae that had opened up and become level with the cell surface. These investigators suggested that caveolae may serve as miniature stretch receptors within the membrane of the cells. However, Gabella and Blundell (1978) demonstrated that the packing density of smooth muscle caveolae is not significantly altered in strips isotonically contracted with carbachol or stretched and released in a calcium-free solution, under a range of loads varying from 1 to 15 grams. They also found that the diameter of the fractured necks (which measure about 40 - 50 nm. in diameter from freeze-fracture preparations) showed no change in diameter under the same experimental conditions. These results indicated that there is no change in number and no partial opening of caveolae due to stretch. However, it is still possible that passive stretch and contraction affect other properties of the caveolae.

Freeze-fracture studies have also elicited the presence of intramembranous particles associated with the membrane in close proximity to the caveolae. The particles are found to be 9 nm. in diameter on the fractured cell membrane and are approximately three times more numerous on the P-face than on the E-face (Gabella, 1978). According to Devine and Rayns (1975), in smooth muscle cells of taenia coli, the P and E faces of the fractured membrane show 450 and 300 particles respectively Wells and Wolowyk, (1971) observed that the particles are generally fewer in number in the regions between the rows of caveolae (281 per um^2.) than between the caveolae themselves (1105

per um^2.) and are consistently present near the neck of each caveolae, forming a complete ring around it. The caveolar membrane itself appears smooth or with few intramembranous particles (Prescott and Brightman, 1976). Gabella (1978) has reported similar finding, along with the observation that the particles found on the caveolar membrane itself are a third to a half size smaller than the 9 nm. diameter particles seen in the surrounding sarcolemmal membrane. In contrast, the membrane of the sarcoplasmic reticulum shows numerous particles of 9 nm. diameter. Flat sacs of sarcoplasmic reticulum are seen to lie beneath the cell membrane or directly below groups of caveolae. The significance of the characteristic distribution of intramembranous particles in relation to the caveolae remains obscure. They may in fact play a dramatic role in smooth muscle homeostasis, since it is known that the intramembranous particles shown by freeze-fracture of cell membranes correspond to proteins and/or membrane bound enzymes which in turn may be responsible for membrane transport mechanisms.

During an isotonic contraction against a light load, the cell membrane is thrown into a number of folds, the caveolae are generally found on the folds themselves and not in the groves between them, although on occassion they are present there as well. The study by Gabella and Blundell (1978) also demonstrated that caveolae are not randomly distributed throughout the cell membrane but do in fact occur in rows one to five caveolae in width, which run approximately parallel to the cell length. The rows do however vary in width along their length and are often seen to split and merge with other rows. Some caveolae are also seen as isolated or belonging to a separate group. Between the rows themselves however, are areas

occuppied by membrane specializations known as dense bands. The dense bands are attachment sites for actin filaments, and the filaments that make up the cytoskeleton of the cell, (which will be discussed in more detail in the following sections). It has been suggested that the extensive attachment of filaments to the cell membrane, is the reason for arrangement of caveolae into rows. It would therefore appear that the arrangement of caveolae is perhaps a result of a necessaty arrangement or pattern of dense bands which is required for contractile function. On the other hand, caveolae of endothelial cells appear to be randomly distributed. This situation is seen in skeletal as well as cardiac muscle, where the distribution of caveolae form no specific pattern, and are not related to any part of the sarcomere (Dulhunty and Franzini-Armstrong, 1975; Smith et al. 1975; Gabella, 1978). It should be noted, as mentioned earlier, that caveolae have different functions in different types of cells, and that comparisons made between caveolae from different sources in an attempt to assign specific functions or roles to them may perhaps be misleading.

5. The Contractile Apparatus

The ability of smooth muscle cells to contract isometrically, isotonically, or auxotonically is a property of the contractile machinery within the cells. The contractile components consists of the myofilaments and their association with the membrane specializations known as dense bands. The myofilaments themselves are made up of thick filaments, thin filaments, and the 10 nm. intermediate filaments.

From investigations using x-ray diffraction and electron-

microscopy, it has now become fairly well established that the thick and thin filaments are orientated in a manner more or less parallel to the long axis of the cell, (for a review, see Small, 1977). These findings as well as those from mechanical studies have suggested that contraction is brought about via a process similar to that seen in skeletal muscle where there is sliding between thick and thin filaments and cross-bridge formation. However, the means by which the sliding of filaments eventually results in contraction is still quite obscure. There is much disagreement over the organization of the thick filaments and also with the thin filaments regarding their sites of anchorage and force transmission (Small, 1977b).

i. The thick filaments

The thick filaments, which are in fact the filamentous form of the myosin molecule, were found to be difficult to observe using conventional transmission electron microscopic techniques. It was believed that myosin could not be seen in its organized form due to inadequate fixation and preparatory procedures. Studies performed by Somlyo et al., (1973) and Ashton et al., (1975) determined that the in vivo form of myosin could be demonstrated but that it required both the fixation of muscles under "physiological conditions" and an independent means of controlling the state of the contractile apparatus during fixation.

The thick filament or myosin-containing filament is seen to be approximately 15.0 to 18.0 mm. in diameter in vertebrate smooth muscle and has been shown to have a relatively regular lateral spacing (Rice et al., 1971; Somlyo et al., 1971; Somlyo et al., 1973).

The lengths of these filaments however, is presently under debate between different investigators. The filaments are observed in both relaxed and contracted muscle (Devine and Somlyo, 1971). However, dedifferentiated smooth muscle, like skeletal myoblasts, and non-muscle cells, may contain a type of myosin that is not organized into thick filaments (Pollard and Weihing, 1974).

As mentioned previously, the major unanswered question remaining about the ultrastructure of the myosin filaments seen in ventebrate smooth muscle is their exact molecular assembly and organization of cross-bridges. This problem exists primarily because of the observation that smooth muscle does not exhibit regular sarcomeric patterns as in skeletal muscles, and therefore no definable contractile unit can be seen. Nevertheless, striking similarities between the contractile proteins, and parallels in mechanical activities between the two types of muscle are known to exist. This fact has suggested that perhaps a similar sarcomere-like structure or arrangement of filaments and similar myosin cross-bridge interactions are present in smooth muscle. The presence of a 14.3 nm meridional x-ray reflection suggestive of cross-bridge repeat has been demonstrated under certain conditions by two groups of investigators (Lowy et al., 1970; Shoenberg and Haselgrove, 1974). Smooth muscle myosin filaments however, in intact muscle cells, do not show a definite bare zone (i.e. an area void of cross-bridge projections radiating from the filament) within the central aspect of the filament (Ashton et al., 1975). which is seen in both skeletal and cardiac thick filaments. In vitro assembly of smooth muscle filaments have also shown no central bare zone (Sobieszek, 1972; Wachsberger and Pepe, 1974; Craig and Megerman,

1977). The central bare zone alongwith a protein which is believed to be of major importance regarding the lateral spacing of thick filaments in skeletal muscle, comprises what has been termed the M-line of the sarcomere. The lack of a central bare zone in smooth muscle thick filaments suggests that the filament arrangement may be fundamentally different from that of a sarcomere (Small, 1977).

The use of isolated cells to obtain a more rapid fixation may allow for better preservation of the fine structure of the myosin filaments (Small, 1977b; Somlyo et al., 1977). Unfortunately, this type or approach involves the use of proteolytic enzymes (used for the isolation of single cells), which potentially may distort the fine structure of myosin: trypsin and/or collagenase treated smooth muscle cells often contain tactoids, rather than filaments of myosin (Rosenbluth, 1971; Gabella and Raeymaekers, 1976). Thick filaments showing cross-bridges having some helical symmetry have been observed in fragments of isolated cells (Somlyo et al.,1977), and in some well preserved single cells, considerable fine structure has been demonstrated (Small, 1977b). However, Somlyo and Somlyo (1977) have observed that grossly dissimilar filament structures are often seen in adjacent cells. Cryoultramicrotomy is another method now being explored, as it avoids the use of the fixative osmium, which tends to be destructive. In negatively stained transverse sections of rabbit portal anterior mesenteric vein smooth muscle, rosettes consisting of a central thick and surrounding thin (actin) filaments are present (Somlyo and Somlyo, 1977).

ii. <u>The thin filaments</u>

The thin filaments of vertebrate smooth muscle lack any troponin-like components and are composed only of actin and tropymyosin (Sobieszek and Bremel, 1975; Sobieszek and Small, 1976). In all vertebrate smooth muscles, actin has been shown to be the major protein component. Actin : myosin mass ratios vary considerably in different smooth muscles. However, when correlated with filament counts from cross-sections of smooth muscle fibers, taenia-coli shows an actin to myosin filament ratio of 10 to 13 : 1 (Small, 1977), and vascular smooth muscle a ratio of approximately 15 : 1 (Somlyo et al., 1973). Whalen et al., (1976) and Rubenstein and Spudich (1977) distinguished three forms of actin designated as α, β and γ, on the basis of their isoelectric points. The report that the amino acid sequences of skeletal and cardiac muscle actin are not identical (Elzinga and Lu, 1976) and that differences in sequences occur between actin from chicken-gizzard, rabbit fast skeletal muscle and mammalian cytoplasmic actin (Vanderkerckhove and Weber, 1978), have suggested that each muscle or non-muscle contractile cell contains a form of actin specific for the cell type.

Tropomyosin can be extracted from smooth muscle in greater quantities than can be extracted in skeletal muscle. Its orientation with respect to actin in thin filaments is believed to be analogous to that in skeletal muscle. This has been suggested by x-ray diffraction sutdies performed on mulluscan smooth muscle (anterior byssus retractor of mytilus) (Lowy and Vibert, 1972), and guinea pig taenia-coli (Vibert et al., 1972) eliciting patterns similar to those seen in skeletal muscle. It has been observed that changes in the

intensities of these reflections occur upon contraction, as also seen in skeletal preparations. This finding is of interest, since if smooth muscle contraction is myosin regulated, as it is presently believed, then the tropomyosin would be expected to lie nearer the center of the groove in the actin filament, even in a relaxed muscle (Huxley, 1977). This paradox has yet to be resolved.

There is no direct information available on the length of the thin filament in smooth muscle. In addition, it's exact longitudinal organization in relation to the thick filaments remains unclear. It is generally believed that the intracellular dense bodies seen in smooth muscle, or at least a certain proportion of them act as pseudo Z-lines, producing a sarcomere-like structure within the cell, (Heumann, 1971; Ashton et al., 1975). However, some investigators are skeptical as to the attachment of thin filaments to these dense bodies, and believe that only the 10.0 nm filaments are involved (Small, 1977b). Some investigators doubt whether the thin filaments are in fact attached to the dense bands on the sarcolemma, although others are fairly convinced of their being anchored to it (Panner and Honig, 1967; Ashton et al., 1975; Nonomura and Ebashi, 1975 ; Somlyo and Somlyo, 1977; Gabella, 1979).

iii. The 10 nm. or 100 A filaments

The intracellular structures commonly known as intermediate, 100 A, or 10 nm. filaments were first recognized as unique structures in immature striated muscle cells and in certain non-muscle cells (Ishikawa et al., 1968), and have since been observed as components of virtually all types of animal cells (Gaskin and Shelanski, 1976).

It is now generally believed that the 100 A filaments in smooth muscle form a complex network throughout the smooth muscle cell which has connections to the cytoplasmic dense bodies and to the plaques or dense bands on the sarcolemma, (Cooke and Fay, 1972; Cooke, 1976; Small and Sobieszek, 1977). Other roles have been suggested for these filaments, including intracellular transport (Goldman and Knipe, 1973), interconnection of contractile filaments (Lazarides and Hubbard, 1976), and positioning of the cell nucleus (Small and Celis, 1978).

The major polypeptide component isolated from smooth muscle intermediate filaments has been called desmin (Lazarides and Hubbard, 1976) and skeletin (Small and Sobieszek, 1977), it has a molecular weight of approximately 55,000 daltons, and an amino acid composition similar to intermediate filaments isolated from other types of cells. It has recently been suggested that an actin-like polypeptide may also be an integral component of the intermediate filament in smooth muscle as well as other types of tissue (Lazarides and Balzer, 1977; Starger and Goldman, 1977; Buckley et al., 1978). Johnson and Yun (1980) have suggested that it is possible that the actin of the intermediate filaments is functionally and genetically different from thin filament actin, since polymorphic forms of actin have been recognized in a single cell type (Whalen et al., 1976; Izant and Lazarides, 1977; Vandekerhove and Weber, 1978). Nevertheless, the precise in vivo function of the actin of intermediate filaments is unknown, and its presence as a constituent in the native form of the filament is not universally accepted (Hubbard and Lazarides, 1979).

Buckley et al., (1978) have provided evidence that this actin-like polypeptide in intermediate filaments of connective tissue cells can interact with heavy meromyosin after partial proteolysis of the filament. Interaction between heavy meromyosin and undigested filaments was not observed, suggesting that the 55,000 dalton component shields the actin component. An interpretation of this result is that perhaps the 55,000 dalton polypeptide forms a polymeric structure which dictates the gross external morphology of the filament, with the actin component occupying an interior location within the filament. Earlier ultrastructural studies have in fact commented on the apparent subunit structure of intermediate filaments seen in cross-sections, which could be indicative of more than one protein being involved (Small and Squire, 1972; Rice and Brady, 1973). In contrast to these findings, however, a study by Huiatt et al., (1980) has demonstrated that purified desmin, free from actin is capable of self-assembly into 10 nm. filaments at near physiological conditions of pH and ionic strength that are similar in morphology to native 10 nm. filaments.

The 100 A filament network, forming a cytoskeleton in smooth muscle is most clearly shown following repeated extraction of actomyosin from isolated smooth muscle cells or smooth muscle strips (Small and Sobieszek, 1977). Isolated cells treated in this way appear as "ghost cells" which still retain the general cell form and therefore support the belief that the 100 A filaments perform a cytoskeleton role in smooth muscle. Although these filaments are resistant to extraction in solutions of high ionic strength, they are nonetheless readily degraded by proteolytic enzymes and can be

removed from smooth muscle cells without affecting the general integrity of the actin and myosin filaments. This has been shown in triton-extracted smooth muscle cells in rigor, (Small, 1977b). In this state, the actin and myosin filaments tend to form groups within the cells which can be identified with the birefringent fibrils recognized using light microscopy (Small, 1974, and Small, 1977a). The 100 A filaments are seen between these groups, either as being free or associated with the dense bands. These cells, if then treated with collagenase, will result in the dissappearance of the 100 A filaments and eventually the dense bodies as well, although the general organization of the actin and myosin filaments appear to remain intact. An interesting finding to note at this point is that these cells will still contract, although more slowly than normal, in the presence of Mg-ATP (Small and Sobieszek, 1977). This suggests the independence of the 100A filament-dense body network from the contractile elements. It was also observed by these investigators that cells lacking the 100 A filaments tend to lose their nucleus rather quickly, and are more easily dissociated into myofilaments under relaxing conditions. Both of these observations represent changes which might be expected from cells suffering from the loss of an intracellular cytoskeleton.

6. Dense Bodies and Dense Bands

Prominent features of most smooth muscle cells are the so-called electron-opaque "dense bodies" that are seen either in the cytoplasm or attached to the plasma membrane. Generally speaking, these structures when found attached to the cell membrane are referred to as "dense bands", and althtough the term "dense bodies" refers to both the membrane bound and free cytoplasmic structures, it is more

precisely the term given to the cytoplasmic structure. In transverse sections, the dense bodies are roughly 2.4 - 3.8 um. long (Ashton et al., 1975). They are seen to occupy 11% of the mitochondria and nucleus-free region of the cell in rabbit mesenteric portal vein smooth muscle (Somylo et al., 1977).

The dense band is formed by an area of the cell membrane, on the cytoplasmic side, where there is an incrustation of electron-dense material. This material is firmly attached to the cell membrane, although it has been observed as being separated from it by a thin (15 nm.) electron-lucent layer (Gabella, 1979). As mentioned in previous sections, there is much disagreement as to the exact function of the dense bands and dense bodies, especially with respect to the attachment of filaments to these structures. Gabella (1979) has shown electron micrographs demonstrating actin filaments penetrating into the dense material of the membrane dense bands, as well as the association of the 100 A filaments with these structures. Freeze-fracture preparations show that the size and distribution of the intramembranous particles of the cell membrane at the level of the dense bands are not different from those of the remaining cell membrane. The particles would differ however, if the filament did actually penetrate into the membrane or cross it, as has been suggested in desmosomes (macula adherens) and hemidesmosomes of epithelia (McNutt and Weinstein, 1973; Kelly and Shienvold, 1976). In fact, the intramembranous particles in smooth muscle membranes at the level of the dense bands are less numerous than in adjacent membrane areas which are predominently occupied by caveolae, (Wells and Wolwyk, 1971; Devine and Rayns, 1975). Therefore, it appears

that thin filaments and intermediate filaments penetrate into the electron-dense material, which is in turn cemented to the cell membrane. The filaments do not reach the membrane itself. However, it is not known whether they terminate inside the dense material or simply re-emerge after looping within it, as seen with filaments involved with desmosmes (Kelly, 1966). Tilney and Mooseker (1976) have shown the anchorage of thin filaments to the cell membrane (through a portion of electron dense material), without actual penetration into the membrane, and with a reduced number of intramembranous particles, at the tips of intestinal microvilli. More recently however, McNutt (1978) has shown in choroid microvilli that microfilaments may be present inside the membrane.

The exact composition of the electron-dense material in dense bands is not knwon. Schollmeyer et al., (1976) have shown the bindingof anti-alpha-actinin to dense bands. Alpha-actinin is a protein with a molecular weight of approximately 100,000 daltons and is also believed to be concerned with the attachment of thin filaments to them (Goll et al., 1969). These findings stress the similarity between dense bands and Z-lines (Pease and Molinari, 1960). In addition, alpha-actinin has been shown to be present in non-muscle tissue (Schollmeyer et al., 1974).

It can be seen that portions of the membrane containing dense bands are thrown into folds, indicating that these structures are not completely rigid. In vascular smooth muscle, dense bands extend deep into the cell, providing attachement sites for a large number of filaments (Rhodin, 1967; Bussow and Wolfhekel, 1972). Gabella

(1977) has reported in transversally sectioned muscle cells, that the dense bands appear as patches, which sometimes alternate regularly along the cell profile with areas rich in caveolae and sarcoplasmic reticulum. It was also observed in this study that the amount of cell membrane profile occupied by dense bands was roughly 50% (range 40 - 60%) at the level of the cell nucleus. However, in the tapering parts of the cell, towards either end, the percentage of area occupied by dense bands increased, up to 100% in the smallest of profiles. It should be noted here that there have been no quantitative studies to date, which have examined the pattern or change in pattern of these dense bands at different cell levels, which may give insight into the arrangement of filament attachment within the cell.

7. Cell to Cell Junctions

i. Nexuses or gap junctions

The nexus or gap junction is an area of close apposition of the membrane of two cells. The gap between the cells is approximately 2 - 3 nm. and can be penetrated when small tracers such as lanthanum are introduced into the extracellular space (Revel et al., 1967; Uehara and Burnstock, 1970). In freeze-fracture preparations, the nexus is identified as a characteristic cluster of intramembranous particles (on the external aspect of the cytoplasmic leaflet of the cell membrane: the P-Face) and pits (on the inner aspect of the outer leaflet of the cell membrane: the E-Face) inside each of the two membranes, (Griepp and Revel, 1977). The fine structure of gap junctions has been studied extensively (for reviews see McNutt and Weinstein, 1973; Staehelin, 1974), although primarily in nervous and epithelial tissue.

In smooth muscle, nexuses illustrate the same ultrastructural morphology as observed in other tissues. The nexus is believed to allow a private communication between two cells with a passage of ions and small molecules (Gilula et al. 1972). The demonstration of good cable properties exhibited by several smooth muscles (reviewed by Tomita, 1970), had led to the belief that the nexus is the site of electrical coupling (Dewey and Barr, 1962; Barr et al., 1968; Bennett, 1973; Caspar et al. 1977). However, the number of nexuses varies considerably in different types of smooth muscles. The sphincter pupillae of the guinea-pig has a large number of nexuses when observed in either sectioned (Gabella, 1974) or in freeze-fractured (Fry et al., 1977) preparations. It should be noted perhaps that these junctions in the above mentioned tissue were also found to be associated with the sarcoplasmic reticulum. Gap junctions are also quite numerous and occupy roughly 0.2 % of the cell surface in guinea-pig ileum (Gabella and Blundell, 1978). However, they are not found in the longitudinal muscle of the dog duodenum (Henderson et al., 1971) or in the longitudinal muscle of the guinea-pig ileum, the vas deferens or rectum (Gabella and Blundell, 1979).

The lack of a consistently good correlation between the degree of coupling and the presence of nexuses has suggested the existence of other structural mechanisms of electrical coupling, in addition to the nexus. This discrepancy in the number of gap junctions has stressed the importance of obtaining accurate quantitative estimates of their numbers. This information may be of special importance in tracheal smooth muscle, due to its multi-unit behavior. Multi-unit

smooth muscles in general, were initially believed to lack gap junctions, being entirely dependent on excitation by nerves. However, Kroeger and Stephens (1975) reported a space constant of 1.6 mm. in control tissue, which is almost twice the length of a smooth muscle cell (Burnstock, 1970). Kannan and Daniel (1978) have observed the presence of gap junctions in canine smooth muscle, (accounting for approximately 0.05% of the total membrane area of the cells) and have suggested that they may provide the basis for cell to cell coupling. Koreger and Stephens (1975) had also reported an increase in space constant in TEA-treated tissue. It was suggested that the increase could be a consequence of either an increase in the transmembrane resistance, or a decrease in the junctional membrane area or both. Kannan and Daniel (1978) have in addition reported an increase in the number of gap junctions in tissues treated with TEA and 4-AP (4-aminopyridine, which is also a potassium conductance blocker, such as TEA), and attribute the possible decrease in conductance to the increase in junctional membrane area.

At present, therefore, the evidence that gap junctions provide the low resistance pathways of current flow between cells is somewhat questionable. There have been no direct measurements of the resistance of these junctions. Nevertheless, indirect estimates of the specific resistance of the nexus at the intercalated disk in the canine myocardium, (a structure which is similar to gap junctions seen in smooth muscle cells), support their role as low resistance contacts (Spira, 1971). Direct measurement of junctional resistance by means of intercellular current injection techniques is impossible due to the spreading of current in three dimensions through

interconnection between smooth muscle cells (Tomita, 1970). Passive membrane studies have therefore required extracellular polarization and intercellular measurement of electrotonic potentials. This technique has helped to demonstrate electrical coupling between smooth muscles. However, Daniel et al. (1976) have observed good electrical coupling in smooth muscle in vitro, before the appearance of gap junctions, which makes it difficult to support the notion that gap junctions are sites of low impedance, intercellular communications.

At present, there is an increasing amount of evidence that there exists a dynamic system of gap junction formation and regulation within the cell. The development of gap junctions between smooth muscle cells of the myometrium of pregnant animals and humans during delivery has been observed (Garfield et al., 1978; Garfield et al., 1980a). Loewenstein and Rose (1978) have shown that increased intracellular calcium concentration in the vicinity of the gap junction leads to functional uncoupling with no apparent changes in gap junction morphology. Other studies have also indicated that there are conditions which cause the channels in the gap junctions to maintain either closed or open positions (Unwin and Yampigh, 1980). Johnson et al., (1974) have proposed that cAMP regulates the size of gap junctions in hepatoma cells. Griepp and Revel (1977) have suggested that gap junctions may control growth, differentiation, and metabolism of cells by allowing exchange of some control messenger between cells. The total functional and formational control of gap junctions may involve several factors, which at present have not been identified. Garfield et al. (1980) have proposed that gap junctions are dynamic structures regulated by steroid hormones,

and controlled by protein synthesis, prostaglandins, calcium and a degradation process. They suggest that the steroid hormones regulate gap junctions in the myometrium by controlling protein synthesis. Connexins, (proteins intimately associated with gap junctions), are synthesized, and then inserted into the plasma membrane to interact with others via disulfide bridges or by cross-linking by prostaglandins to form aggregates. The aggregates are suggested to be associated with adenylate cyclase activity or a calcium transport or binding mechanism. The intercellular channels created by the aggregate may be open or closed depending upon the activity of the functional proteins. Finally, when the aggregates have grown to sufficient size or become superfluous, they are, perhaps, incorporated into one of the connected cells by an endocytotic mechanism where they are digested by a lysosomal process. It should be noted that although this scheme was proposed by Garfield et al. (1980) in order to explain the control of gap junctions of the myometrium during pregnancy, similar mechanisms may exist for the formation and regulation of gap junctions in other tissues.

ii. Intermediate junctions

Intermediate junctions alongwith gap junctions form the two best-known types of cell to cell connections in smooth muscle cells (for a review see Henderson, 1975). The intermediate junctions have been referred to in the past as zonula adherens type junctions. Both of these junctions appear symetrical in the two cells involved. They are characterized by the presence of electron-dense material on the cytoplasmic side of both membranes. The actual intercellular gap is approximate 30 - 40 nm. or more in width, and exhibits a

band of dense material which is continuous with the basal lamina and has an ill-defined periodicity. These junctions appear to be formed by two dense bands from adjacent cells, which exactly match each other in appearance and are held together by an intercellular cement. As seen in individual dense bands, these junctions can extend for 1 - 2 um. along the cell membrane, and intermediate and thin filaments are associated with them as well. In freeze-fracture preparations, the portions of the membrane containing these junctions reveal no noticable specializations, but do show a reduced number of intramembranous particles. The implications and significance of this observation remain unclear.

The intermediate junctions seen in smooth muscle have several features in common with the intermediate junctions of epithelium as as described by Sjostrand and Elfvin (1962), and Farquhar and Palade, (1963). Although these junctions have been known to exist for a number of years in other tissues and in smooth muscle, there has not been to date an accurate quantitative study of these structures in smooth muscle. A study surveying the relationship between these junctions and the dense bands has not been carried out. Information on this subject would perhaps lead to a better understanding of not only the transmission of force between muscle cells, but also of the internal development of force within the cell itself. This latter point owes its existence to the observation that the thin and the 100 A filaments are attached to the junctions as well, via the dense bands.

iii. Other junctional structures

It can be seen that some cells have tapering cylindrical

ends where the sarcolemma is involved with extensive and practically continuous dense bands. In addition, in many cells, the sectioned profile of the terminal portions of the cell is star shaped or highly convoluted. In this process, the cell sectional area is reduced, but the surface area is greatly increased, by the means of laminar projections. Around these projections, the basal lamina appears, exceptionally thick, and has been suggested by Gabella (1977) to form an apparatus designed to join the cell's end to the stroma.

More complex junctional structures are seen between muscles cells involving the interdigitation of the ends of two cells, each having divided into numerous laminar and cylindrical process (Gabella, 1977). The space (50 -100 nm. in width), between these processes are filled with microfibrils and a material which appears to be similar to basal lamina. Aside from these regularly observed junctions, isolated cell processes and invaginations of the cell surface which partially penetrate into a neighbouring cell are frequently seen in smooth muscle.

C. Mechanical Coupling Between Smooth Muscle Cells

The process of how the forces generated by the individual smooth muscle cells combine to produce the overall contraction of the muscle is poorly understood. Gabella (1977a) has attempted to study this problem using guinea-pig taenia coli. In taenai-coli, as in other smooth muscles, a large portion of the membrane is involved with the dense bands, up to 50% in the middle portions of the cell and up to 100% in certain distal profiles. Due to the intimate association of the dense bands with the myofilaments, it has been suggested (Gabella

1976a) that this arrangement may account for the remarkably large tension developed by this muscle per unit of transverse sectioned area. If this large tension development is due in part to the presence of "lateral" attachments to the membrane, it is also important that the dense bands be capable of transmitting forward (i.e along the length of the muscle) the tension developed inside the cell by these myofilaments. This is achieved where dense bands match similar structures in a neighbouring muscle cell, at intermediate junctions (zonula adherens type) or at levels of more complex cell to cell anchorings. However, because many dense bands do not match each other and are often facing wide intercellular spaces, it is tentatively believed that the sarcolemma itself can transmit some of the muscular tension. This has been suggested as one of the roles played by the sarcolemma of skeletal muscle fibers. Fields (1970) found that the sarcolemma of the frog semitendinosus muscle is anistotropic, being much stiffer in the longitudinal direction. In addition, Schmalbruch (1974) has observed that the orientation of collagen fibrils of the sarcolemma with respect to the fiber axis changes at different sarcomere lengths. It is therefore possible, that even in skeletal muscle (where tension is primarily transmitted from the ends of the fibers through a long series of sarcomeres) the sarcolemma at the "lateral" sides of the fiber plays some role in the transmission of force, particularly during isotonic contraction.

Direct evidence of a mechanical link between the cell membrane and basal lamina from the muscle cells and the collagen fibril network is lacking. However, a few collagen fibrils can be

seen to come in close contact with the basal lamina, and a microfilamentous network can be seen between collagen fibrils and basal lamina. Indirect evidence for this collagen to cell connection has been demonstrated using isotonically contracted smooth muscle cells (Gabella, 1977a). In this preparation, the collagen fibers were observed to follow parallel and in close proximity to the dense bands which were situated in deep groves at the cell surface and ran obliquely to the long axis of the cell.

Further evidence of mechanical links between smooth muscle cells and material in the intercellular space is believed to be provided by finger-like invaginations of the cell membrane at the cell's end. These structures have been described previously in smooth muscle cells of the guinea-pig vas deferens (Merrillees et al., 1963), and as prominent features of the muscle - tendon junctions in skeletal fibers (Gelber et al., 1960; Schwarzacher, 1960; Ishikawa, 1965; Mackay et al., 1969; Hanak and Bock, 1971). Using serial sections, Gabella (1977) has shown that these finger-like invaginations and divisions of the cell ends also interdigitate between other cells, forming end-to-end and end-to-side junctions. This form of coupling was suggested to be a major form of cell-to-cell mechanical transmission.

Gabella (1977a) has described the septa of connective tissue present between the muscle cells of taenia coli in the guinea - pig, and has commented on its role in mechanical transmission in smooth muscle. Previously it had been suggested that smooth muscles (taenia-coli in praticular) are composed of "bundles" formed by the connective tissue septa, and that perhaps these bundles, more than the individual cells comprised the functional units of the muscle.

However, it was observed that the "bundles" of smooth muscle cells were found to lose their individuality within tens of microns of their length, splitting into or merging with other "bundles" (Gabella 1976). Nevertheless, each connective tissue septa can be visualized as giving attachment to several arrays of muscle cells pulling in opposite directions. This arrangement and role of the septa may not be true for all smooth muscles.

D. Innervation

Canine tracheal smooth muscle has been described as a multi-unit smooth muscle alongwith other smooth muscles which do not show spontaneous rhythmical activity, although it is known to develop a high degres of non-rhythmical tone under in vitro experimental conditions (Koreger and Stephens, 1975). The mechanical activity of multiunit smooth muscle is generally believed to be initiated by the diffusion of transmitter from nerve terminals. In fact, the density of innervation of some multiunit smooth muscles has been shown to be very high, with close nerve-muscle relationships (Bennett and Merilees, 1966; Gabella, 1974). In contrast, single unit muscles are believed to require a much lower density of innervation, since the nerves appear to modulate rather than initiate activity. However, this rigid classification of smooth muscle into single and multi-unit types has recently been extended to include muscles that demonstrate features of both types, based on the nature of their autonomic innervation (Burnstock, 1970).

With the exception of a study performed by Kannan and Daniel (1980), the ultrastructural studies pertaining to canine tracheal smooth muscle innervation have dealt with the types of innervation

to this muscle from a qualitative stand point and have not taken into consideration the relationship between smooth muscle cells and the nerves, i.e. the density of innervation and the closeness of nerve-muscle contacts. In other studies, ultrastructural and histochemical investigations have been made of mammalian airway smooth muscles from several species (Altnahr, 1965; Blumke, 1968; Mann, 1971; Rikimaru and Sodoh 1971; Silva and Ross, 1974; Suzuki et al., 1976). The description and pattern of innervation was found to vary considerably between species.

The recent studies of bovine (Cameron and Kirkpatrick, 1977) and canine (Kannan and Daniel, 1980) trachealis have provided some quantitative description of nerve profiles, their density, and their relationship to these types of smooth muscle cells. The innervation in these two species was observed to be relatively sparse, predominantly cholinergic and appeared to have a distribution pattern characteristic of that of single unit smooth muscle.

Guinea pig tracheal smooth muscle is also classified with other smooth muscles which do not show spontaneous rhythmic myogenic activity (i.e. multi-unit muscles), but are known to develop a high degree of non-rhythmical tone under in vitro conditions. However in a recent investigation performed by Jones et al., (1980), guinea pig trachealis was shown to exhibit different features in innervation to those seen in canine and bovine trachealis (Kannan and Daniel, 1980; Cameron and Kirkpatrick, 1977). In the guinea pig trachealis, nerve varicosities were seen beside, although some distance from smooth muscle bundles (as in canine and bovine trachealis) but occasionally were also observed to penetrate deep into the space within a muscle

bundle and make close contacts with smooth muscle cells (less than 20 nm.). In addition the number of varicosities reported (13 - 19 per 100 smooth muscle cells), is significantly greater then the 1.0 - 1.5 varicosities per 100 smooth muscle cells reported for canine and bovine trachealis (Cameron and Kirkpatrick, 1977; Kannan and Daniel, 1980). Jones et al. (1980) suggested that the pattern of innervation seen in guinea pig trachealis indicates that transmitter release from terminals in response to nervous activity could directly activate innervated cells. They also suggested that the spread of activity from these cells to other adjacent cells could be by way of electrotonic spread. However, there have been no electrophysiological studies to determine the extent of coupling in this smooth muscle to date.

In canine tracheal smooth muscle, Kannan and Daniel (1980) observed that the nerves were seen as bundles of axons and axonal varicosities with synaptic vesicles. Most of the nerves were found to lie in the large clefts between bundles of smooth muscle cells. Nerves that were found near blood vessels were seen to possess a medial layer. In some regions it was reported that the axons and their varicosities were not fully enclosed within and were sometimes devoid of the Schwann cell sheath. Occassionally, several axons and varicosities were observed within muscle bundles. The smallest distance seen between a nerve varicosity and a smooth muscle cell was approximately 140 nm. In fact, most were more than several micrometers in distance from the smooth muscle cells. They also observed two types of axonal varicosities, which were differentiated on the basis of vesicular content. They reported the observation of three types of vesicles, ranging from the small agranular vesicles (SAV) 40-70nm, to

small dense vesicles (SDV) 40-70 nm, and to large dense core vesicles (LGV) 80-100 nm in diameter. However, the large dense vesicles were not encountered in significant numbers and were not classified as belonging to a specific nerve type. The varicosities were therefore classed into two types based on the nature of the synaptic vesicles, using criteria defined by Daniel et al. (1977). The small agranular vesicles were designated as cholinergic, the small dense vesicles as adrenergic, and the large granular vesicles were believed to be neither cholinergic nor adrenergic. The varicosities containing predominently small agranular vesicles, considered to be cholinergic were the most abundant. Such a predominance of cholinergic nerves is consistent with other studies of airway smooth muscles i.e. histochemical, mechanical, and ultrastructural (El-Bermani, 1973a; 1973b; El-Bermani and Grant, 1975; Cameron and Kirkpatrick, 1977; Stephens, 1977). The small granular vesicles, termed as adrenergic were found in varicosities located near blood vessels and also within the muscle, although their incidence was low. They suggested that the mode of fixation used in their experiment does not always preserve the dense cores of vesicles in adrenergic neurons, and therefore the number of adrenergic nerves innervating the muscle may have been underestimated.

It is important to mention at this point that Kannan and Daniel (1980) did not observe the presence of the large opaque vesicles similar to those seen by Richardson and Ferguson (1979) in human trachealis. These large opaque vesicles were believed to be representative of a non-adrenergic inhibitory system.

This system was first described in the gastrointestinal tract (Burnstock, 1972), and has more recently been demonstrated in guinea pig trachealis (Coburn and Tomita, 1973; Coleman and Levy, 1974;

Richardson and Boucher, 1975), as well as in the primary bronchus of the chicken (Bhatla et al., 1979). The presence and role of this system in airway smooth muscle, which at the present is rather obscure, is of much interest.

E. Intercellular Components

a. Collagen fibers

By far the most prominent component of the extracellular space between smooth muscle cells are the collagen fibers. These fibers which occur in fibrils measuring 30 - 35 nm. in diameter exhibit the usual cross-striation with a period of 64 nm. They differ however, from collagen fibers found in connective tissue proper, such as those in the adventitia of blood vessels of the intestinal mucosa, where most fibrils measure from upwards of 50 nm.

The ratio of collagen to muscle tissue varies with different smooth muscles. Smooth muscles as a whole however, have a higher content of collagen than that of striated muscles, and can generally be said to fall between those of striated muscles and tendons. Estimates of collagen content can be determined by measuring the concentration of the amino acid hydroxyproline. Collagen can be synthesized and laid down by the smooth muscle cells themselves, and has been demonstrated to do so in tissue culture (Ross, 1971; Layman and Titus, 1975). This capability of the smooth muscle cells has also been shown to occur in developing blood vessels (Ross and Klebanoff, 1971), and in adult muscles during hypertrophy (Gabella and Yamey, 1977).

The structural and functional role of collagen fibrils in smooth muscle is not as yet clear. Gabella (1977a) using guinea-pig taenia

coli has shown that in minimally stretched muscles, the majority of the collagen fibrils run parallel to the longitudinal axis of the muscle cells. However, in isotonically contracted muscles, the collagen fibrils are found to run diagonally to the cell's longitudinal axis and appear wound in low pitch spirals around the muscle cells. The mechanical significance of this rearrangement in orientation of the fibrils is not known. The oblique arrangement of collagen fibrils and the dense bands of the muscle suggests that the cell undergoes a certain amount of torsion during shortening. It has also been observed that the myofilaments, in contrast to the collagen fibrils, remain close to the cell's longitudinal axis in both contracted as well as shortened cells.

b. Basal lamina

The basal lamina is an ill-defined yet abundant component of smooth muscle, coating the entire surface. Due to the small size of the cells, the amount of cellular surface per unit volume of tissue is considerable. In the guinea-pig taenia coli, each gram of tissue contains nearly 10,000 cm^2 of cell membrane, and also an equal amount of basal lamina (Gabella, 1979).

The presence of basal lamina of smooth muscle in the tunica media of muscular arteries was demonstrated by Pease and Molinari (1960), it appears lacy in smooth muscle and as two layers in heart muscle. The role of the basal lamina is still obscure although early studies had reported its involvement in the maintenance of membrane potential in frog skin (Ottoson et al., 1953), and its function as a microskeleton for young axons and their Schwann cells in nervous tissues Nathaniel and Pease, 1963; Nathaniel and Nathaniel, 1973). At present, the basal lamina is believed to consist of a

protein-polysaccharide material (Bloom and Fawcett, 1975).

c. Elastic fibers

In a study using guinea-pig taenia coli, Gabella (1977a) observed that elastic fibers, microfibrils and other ill-defined materials are present between the muscle cells. Elastic fibers have been shown to consist of two distinct structural components; a microfibrillar glycoprotein and an amorphous, extensively cross-linked protein identified as elastin (Ross and Bornstein, 1969). Microfibrils similar in appearance to those which are part of the elastic fibers are known to be associated with the basal lamina of smooth muscle cells (Haust, 1965). In addition, cross-banded structures with a period of 86 - 87 nm. were occassionally found between muscle cells in the guinea-pig taenia coli (Gabella, 1977a). These structures, the significance of which is still obscure, are similar to those described in other connective tissues (Phillai, 1964; Carvioto and Lockwood, 1968; Sun and White, 1975).

d. Fibroblasts

Fibroblasts are another common feature of the extracellular space between smooth muscle cells. They have been shown to be responsible for the production of collagen and an amorphous ground substance. They are generally spindle shaped or stellate, and have an oval nucleus. Within the cytoplasm, clusters of ribosomes and short cisternae of rough endoplasmic reticulum are often seen. Cytoplasmic tonofilaments are abundant, and a few lysosomes are also present. Collagen synthesis and secretion follows the same basic pattern observed in other secretory cells, with the exception that

there is no extensive storage of the synthesized product in granules. Proline, which is a major component of collagen, enters the cell and is hydroxylated to hydroxyproline. Hydroxyproline and other amino acids are then assembled into peptides on the ribosomes of the endoplasmic reticulum and are released into the cisternae. These peptides are then incorporated into three alpha chains that coil together in a helical fashion to form the tropocollagen molecule. Tropocollagen, which is the basic molecular unit of collagen, is roughtly 280 nm. long and 1.4 nm. wide. It is then segragated in vacuoles in the Golgi complex, and finally released. Tropocollagen molecules polymerize extracellularly to produce the collagen fibrils. The typical cross banding pattern seen in collagen at 64 nm. is produced by the overlap of about one quarter of the length of the laterally associated tropocollagen molecules. The mucopolysaccharides of ground substance secreted by the fibroblasts may be synthesized in the Golgi complex(Lenze, 1971).

e. Mast cells

Mast cells, like fibroblasts are also seen amongst connective tissue in the extracellular space between smooth muscle bundles. These cells are round or void with the central nucleus conforming to the shape of the cell. The nucleus is generally small in size in comparison to the surrounding cytoplasm. Most of the cytoplasmic space in mast cells are occupied by large membrane - bound secretory granules. The granules are seen to be extremely dense. However, immature granules have a less dense matrix within which are embedded irregular aggregates or strands of very dense material. Organelles are generally few in number and are compressed in the small amounts

of cytoplasm between the granules. Some short microvilli are seen to extend from the cell surface (Lenze, 1971). The granules contain a variety of low molecular weight chemical mediators including histamine, SRS-A(Slow Reacting Substance of Anaphylaxis), ECF-A (Eosinophil Chemotactic Factor of Anaphylaxis), prostaglandins, thromboxanes, various kinins, and other factors (Wilson and Galant, 1974). The secretory process is believed to involve a calcium dependent phase in which a diisopropylfluorophosphate sensitive esterase is activited, followed by a glycolytic energy dependent stage. This stage is in turn required for the activation of micro-tubular contractile elements whch are responsible for extrusion of the mediatros. Exocytosis of the chemical containing granules occurs in the final, calcium dependent phase (Gold, 1976). It should be mentioned that changes in the cyclic nucleotides have been implicated in the modulation of the release of these mediators, in particular histamine and SRS-A (Orange et al., 1971).

III. Formulation of Hypothesis

As indicated in the review of literature, there has been only one study to date that has dealt with the innervation of tracheal smooth muscle from a quantitative standpoint. Previous investigations had merely dealt with determining the type of innervation seen and had not examined adequately the nerve muscle relationship. Unfortunately, the quantitative study performed by Kannan and Daniel (1980) used some rather questionable criteria for the identification of different neurons. These investigators did not consider the large dense core vesicles as being representative of adrenergic innervation, whereas this has been somewhat generally accepted in the past (Pellegrino

de Iraldi, et al., 1963; Aghajanian and Bloom, 1966; De Robertis, 1967). These vesicles have also been shown to increase in density after loading with 5-hydroxy-dopamine (Tranzer et al., 1969; Chiba, 1973). Therefore it is possible that the method of classification used in their study may have given rise to inaccuracies in the quantitative values presented. Because of this it was decided that another quantitative account of the innervation to the muscle was in order. A good understanding of the type and degree of innervation to a particular smooth muscle is vital for determining the regularatory influence of these nerves upon the muscle cell. Such findings, in addition to enabling a more suitable classification of the muscle, would help in predicting the requirements within the muscle for cell to cell electrical coupling.

With regards to mechanical aspects of contraction, little is knwon about mechanical transmission between smooth muscle cells. The mechanisms of force development within the muscle cells by the myofilaments is also equally vague. Henderson et al. (1971) had demonstrated mechanical coupling between cells by intermediate junctions which were comprised of identical electron dense areas of two adjacent cells. These electron dense regions of the cell membrane or dense bands have been implicated as sites of anchorage of myofilaments. Gabella (1977) had stated that in cross sections of intestinal smooth muscle cells dense bands occupied roughly 50% of the membrane at the level of the nucleus, and up to 100% of the membrane at the narrow distal portions of the cell. In consideration of this finding and the involvement of dense bands in intermediate junctions, it became apparent that a quantitative

assessment of both the dense bands regions of the cell membrane and the intermediate junctions would be necessary for a better understanding of the role of these structures in force development within individual cells and force transmission between muscle cells. This portion of the study was to be carried out by analysing cross sections of randomly choosen cells sectioned at different points along their length. The circumference of each cell, and each region along the membrane that was involved in either dense bands, caveolae, or intermediate junctions would be measured. A ratio of the total dense bands regions divided by the cell circumference would be calculated for each cell. Cells with high dense band to circumference ratios were considered as cells sectioned near their ends, while others with low ratios were considered as cells sectioned near their middle. This grouping would allow the analysis of the caveolae and intermediate junctions and their relation to dense band regions at different points along the length of the cell.

MATERIALS AND METHODS

A. Removal of Tissue

Normal control dogs were anesthetized by intraveneous injection with sodium pentobarbital (Nembutal, Abbott) at a concentration of 30 mg/kg. prior to the removal of the trachea. An intracardiac injection of saturated potassium chloride solution was used to sacrifice the animal after the trachea had been removed. The entire procedure was carried out within five minutes of the administration of the anesthetic. Upon removal of the trachea, the tissue was immediately immersed in oxygenated Krebs-Henseleit solution kept at $4^{o}C$. The composition of the Krebs-Henseleit solution is given in Table 1. This was done to prevent the muscle tissue from becoming hypoxic.

The trachealis muscle itself, known as the muscular transversus tracheae, composes only the most dorsal layer of the paries membraneceus, which connects the incomplete dorsal ends of the cartilaginous canine tracheal rings (Fig. 1). Single muscle strips could be obtained by removing single tracheal rings by cutting the trachea parallel to the radial axis of the rings. The tunica fibrosa (connective tissue segment), was then removed from the trachealis muscle. This could be done easily, by bisecting the anterior portion of the trachealis ring, and then everting the two cartilaginous ends. Once the muscle strip was removed by cutting its attachment to the cartilaginous ring, it was cut into 2 mm. square pieces under a dissecting microscope, and then placed immediately in Karnovsky's fixative. It should be noted that this entire

procedure was done as quickly as possible (under 7 minutes), with ample additions of cold Krebs-Henseleit solution to insure no tissue damage. In addition, strips were hung isometrically in a muscle bath at resting length and then fixed at this length. As mentioned earlier, this was done in order to observe the muscle under the conditions normally used in the laboratory for mechanical studies.

B. Fixation and Embedding

Pieces of muscle strips, or whole strips fixed at resting length (L_o) were placed in Karnovsky's fixative for 2 - 3 hours at 4^oC. The tissues were then removed and rinsed repeatedly in 0.1 M Millonig's phosphate buffer, and post-fixed in 1% phosphate buffered osmium tetroxide for 1 - 1½ hours. With respect to entire muscle strips fixed at resting length, these tissues were quickly cut into 2 mm. squares after removal from Karnovsky's fixative, and then carried through the remaining fixation procedure. The tissues were subsequently processed and embedded Araldite 502, according to the procedure given below.

1) Rinse several times in phosphate buffer.
2) 50% alcohol - two changes for a total of five minutes.
3) 75% alcohol - two changes - five minutes each.
4) 95% alcohol - two changes - seven minutes each.
5) Rinse in 100% alcohol.
6) 100% alcohol - three changes - 10 minutes each.
7) Rinse in propylene oxide - once.
8) Propylene oxide - three changes - 10 minutes each.
9) Equal parts of propylene oxide and araldite. Leave on rotator overnight.

TABLE I. Composition of Krebs-Henseleit Solution

	mM	g/L
NaCl	115	6.72
$NaHCO_3$	25	2.10
NaH_2PO_4	1.38	0.167
KCl	2.51	0.187
$MgSO_4 \cdot 7H_2O$	2.46	0.296
$CaCl_2$	1.91	0.145
Dextrose	5.56	1.00
osmolarity = 304 mOsM		

10) Place in 75% araldite and 25% propylene oxide. Leave on rotator for one hour.

11) Transfer to pure Araldite. Leave on rotator for one hour.

12) Embed in pure araldite, in beem capsules, allow to stand overnight at room temperature (to allow bubbles to come to surface), and leave in incubator at 40 - 55°C. for three days.

For a complete explanation of fixative preparation, see the section on solution preparation.

C. Preparation of Solutions

1. To make 500 cc. of Millonig's phosphate buffer:

Solution A: 2.26% $NaH_2PO_4 \cdot H_2$)

10.17 grams in 450 ml of H_2O

Solution B: 2.52% NaOH

2.52 grams in 100 ml. of H_2O

Solution C: 5.40% glucose

2.70 grams in 50 ml. of H_2O

Solution D: 415 ml. of Solution A + 85 ml. of Solution B.

Final Buffer Solution: 50 ml. of Solution C + 450 ml. of Solution D.

Final pH = 7.3

2. To make 200 ml. of Karnovsky's fixative:

Eight grams of paraformaldehyde were dissolved in 100 ml. of distilled water by heating to 65°C. In order to clear the solution, 1 N sodium hydroxide was added drop by drop to the above solution. After the solution was brought to room temperature, 40 ml. of a 25% glutaraldehyde solution was added. Subsequently, the volume was made up to 200 ml. with Millonig's phosphate buffer and the pH

adjusted to 7.2.

D. Light Microscopy

For light microscopic observations, the araldite embedded tissues were trimmed and then sectioned at 0.5 microns, using glass knives on a Reichert ultramicrotome. The sections were mounted on glass slides, and stained with toluidine blue. The slides were photographed with an Olympus photomicroscope. Suitably fixed areas were then trimmed for ultramicrotomy.

E. Electron Microscopy

Thin sections of approximately 70 nm. thickness, of both longitudinal and cross-sectional areas of the muscle were cut on a Reichert ultramicrotome and mounted on 200 mess copper grids. The grids were then stained using a saturated solution of uranyl acetate for two hours, rinsed with double distilled water (dd H_2O) then stained with lead citrate (0.3%) for 7 - 9 minutes, and again rinsed with dd H_2O. Both the lead citrate and uranyl acetate were centrifuged at 4000 RPM for 20 minutes, before application to the grids to prevent the formation of precipitate. The stained sections were observed with a Philips 300 electron microscope.

F. Quantitative Morphology

i. Quantification of Nerve Ending Encounters

The density of innervation in the canine trachealis was determined in tissues from four randomly selected animals. Only cross-sectioned material was used. Sections cut from randomly chosen blocks from each animal were placed on 300 mesh copper grids. Each section was seen to cover roughly 20 - 30 squares per grid.

Randomly chosen squares were used for quantification purposes. In each square that was selected, all completely visible smooth muscle cell profiles were counted. Approximately 1000 smooth muscle cell profiles were counted per animal.

In each selected square, every axon profile was counted. In all cases, groups of axons were seen with each group ensheathed or partially ensheathed by a Schwann cell process. Each Schwann cell process, and its axons were termed collective and arbitrarily as a nerve terminal encounter. Every axon profile was counted, and those which contained synaptic vesicles were classified according to their vesicular content. Small agranular vesicles (30-50 nm. in diameter) were termed cholinergic, while large dense core vesicles (80 - 120 nm. in diameter) were termed adrenergic (Grillo and Palay, 1962; Peters et al., 1970). Other types of vesicles seen were not classified into distinct nerve types due to their lack of significant numbers and to their questionable identity, and were therefore grouped strictly according to their morphological appearance.

ii. Quantification of Muscle Cell Morphology

Cross-sections of tracheal smooth muscle fibers were used for quantification purposes. Montages of muscle cell profiles were made from each animal. The montages were taken from grid squares randomly selected in a manner identical to that used for the quantification of nerve profiles. The montages consisted of 13 to 15 film plates (3¼ x 4") per montage which were taken at magnification of 15,960 times. Print enlargements of 2.5 times that of the negative size were made and assembled into montages.

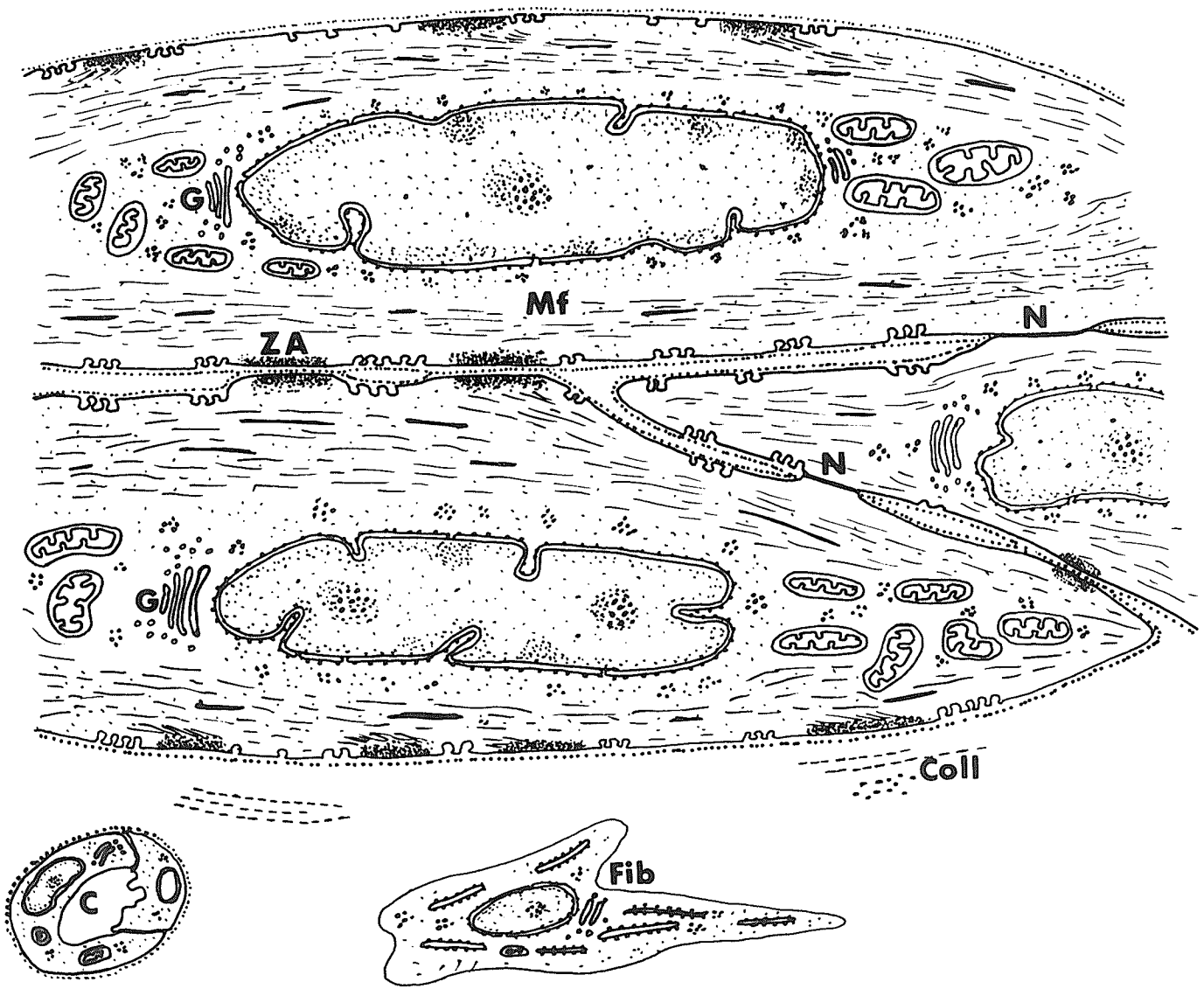
The cross-sectioned cell profiles from the montages were numbered as individual cells. Only complete cell profiles were used. Along the circumference of the cell, various portions of the cell membrane showed regions occupied by caveolae, and also dense band material (See Schematics 1 and 2). Each region of the membrane, whether it was an area involved with caveolae, or dense bands was designated as a separate segment and numbered. In addition, all intermediate (zonula adherens type) junctions were labelled similarily. It should be noted that since intermediate junctions are in fact comprised of dense bands from adjacent cells along with specialization of the basement membrane in the extracellular space between the two cells, the specific dense bands which were involved in each junction were recorded in all cases. This was done in order to allow for the calculation of the size of the junctions in relation to the size of the dense bands (See schematic 3). Intermediate junctions, gap junctions of nexuses between the cells were also counted and recorded.

All measurements were made using a Talos Systems graphic tablet digitizer with a point density of 1000 points per square inch. The micrographs of the cross-sectioned cells from each montage were measured by placing the micrographs on the digitizer surface. The cell circumference and lengths of the dense bands, caveolae, and intermediate junctions were measured by tracing the outline of the cell or the designated portions of themembrane with the digitizer cursor. The digitizer pen could have also been used, however, the cursor enabled a more accurate tracing of the cell membrane. The

lengths of the membrane for each measurement was portrayed on a monitor. Each measurement was converted to microns by multiplication with a conversion factor; this was performed internally by the digitizer. The measurements taken from each cell were dept on record in a computer file. Statistics performed on these measurements were carried out using the SPSS (Statistical Package for the Social Sciences) program. Linear regressions were performed on graphs 27B - D to determine linearity and slope. However, data from graph 27D were not linear and were eventually fit to a polynomial function.

Schematic 1

This is a schematic diagram of longitudinally sectioned smooth muscle fibers. Intermediate type or zonula adherens (ZA) junctions and gap junctions or nexuses (N) have been indicated. Myofilaments (Mf) and Golgi (G) are shown as well as collagen fibers (Co), a fibroblast (Fib) and capillary (C) are also included.

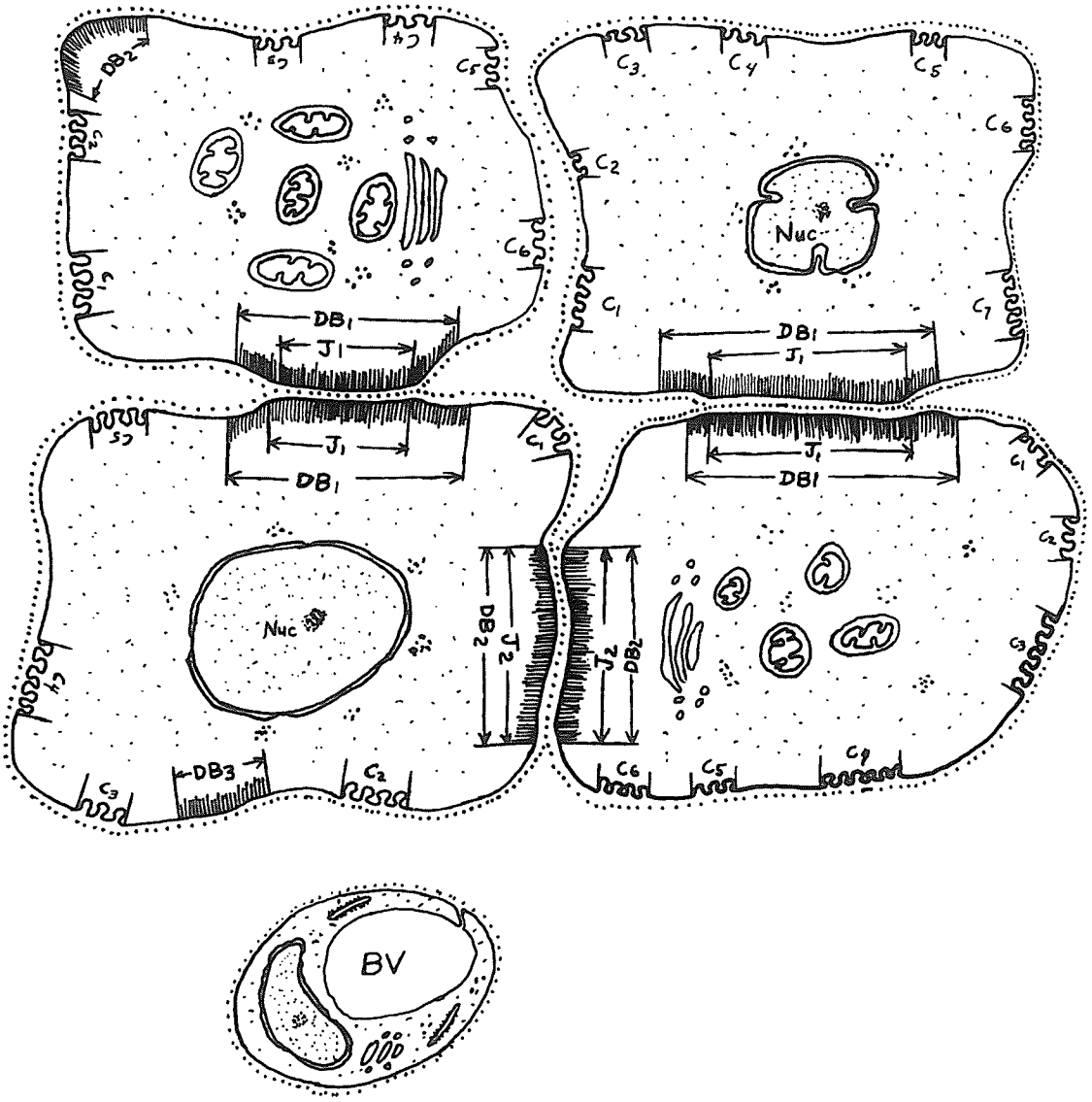

G
Mf
N
ZA
N
G
Coll
C
Fib

Schematic 2

This diagram illustrates the method in which the caveolae, dense bands, and intermediate junctions were measured for each cell. Note that the computation of the dense band/circumference ratio for a particular cell involved the sum of all the dense band regions for that cell, which was then divided by the circumference. Dense Band/ Circumference Ratio = $\frac{DB_1 + DB_2 + DB_3 \ldots}{\text{Circumference}}$

A similar procedure was performed for the Caveolae/Circumference Ratio and Junction/Circumference Ratio. The intermediate Junction/ Dense Band ratio for each cell involved the sum of all junctions divided by the sum of all dense band regions for a particular cell.

Intermediate Junction/Dense Band Ratio = $\frac{J_1 + J_2 + J_3 \ldots}{DB_1 + DB_2 + DB_3 \ldots}$


DB2
C5
C4
C3
C6
C1
DB1
J1
C3
C4
C5
C6
C2
Nuc
C1
C7
DB1
J1
J1
DB1
C5
C1
Nuc
DB2
J2
C4
DB3
C2
C3
J1
DB1
C1
C2
J2
DB2
C6
C5
C7
C3
BV

RESULTS

A. Muscle Morphology - Qualititative Observations

1. Arrangement and Shape of Muscle Fibers

Low power micrographs of longitudinally sectioned tracheal smooth muscle strips fixed at resting length illustrate the parallel arrangement of muscle fibers (Figs. 1 - 4). Large numbers of longitudinal sections have been examined previously, all of which demonstrated the parallel arrangement of the muscle fibers. From preliminary measurements (taken from longitudinal sections), the fibers were found to be roughly 700 - 850 um. in length, which is compatible with the findings of Suzuki et al. (1976) which reported fibers about 1000 um. long. Since serial sections were not involved in the present study, it is quite possible that the cells measured may have been slightly longer, as the ends of these cells may have left the plane of section and were therefore not included in the measurement. The fibers appeared to be grouped in bundles, separated by the connective tissue septa containing collagen fibers and extracellular components. The bundles varied in size and, although a quantitative light microscopic study was not performed, electron microscopic grids of cross-sectioned fibers revealed that the bundles contained 75 to 125 smooth muscle cells. It was not determined whether or not the bundles remained as discrete units or subdivided and joined adjacent bundles. Figure 1 also illustrates the finding that at least 75 to 85 per cent of the tissue is muslce, a property that enables tracheal smooth muscle to be a good model for mechanical and biochemical studies. An observation that has been reported for several smooth muscles is the presence of light and dark cells. This was also found in tracheal smooth muscle preparations (Fig. 5). It's significance

is not definitely known.

2. Basement Membrane

In practically all micrographs taken of tracheal smooth muscle fibers, a thick basement membrane was found, (Fig. 6). Figure 6 illustrates that the basement membrane usually appears to be especially thick when overlying areas of the cell membrane that contain dense bands. The basement membrane was also found to be abundant in areas of the cell membrane occupied by caveolae. Although the caveolae themselves are continuous with the cell membrane and open to the extracellular space, it could not be determined whether the basement membrane material did in fact enter the caveolae. It should be noted that many of the caveolae or pinocytotic vesicles appear to be filled with an amorphous substance of unknown origin. A careful study of figure 6 suggests that the basement membrane is somewhat separated from the cell membrane itself, at areas involved with caveolae, and does not appear to enter within them.

3. Nucleus

Low power micrographs revealed that the nucleus was located in the central portion of the muscle fiber (Fig. 7). In general the nucleus was seen as an elongated, cigar-shaped structure. In muscle fibers fixed at their resting length (Figs. 1 and 3), the nuclear outline appears smooth, however in contracted fibers the nucleus can be seen to undulate and fold according to the degree of shortening undergone by the fiber. The nucleus was seen to be surrounded by a well defined nuclear membrane as shown in figures 8 and 9. The presence of nuclear pores can also be identified in figure 9 as well as a

prominent nucleolus.

4. Cytoplasmic Organelles

The majority of the cytoplasmic organelles found in smooth muscle fibers such as mitochondria, smooth and rough endoplasmic reticulum, Golgi, were usually found located at the poles of the nucleus, (Figs. 7 and 10). This region of the cell (seen clearly in Fig. 11), in addition to being the major location for cellular organelles, is also an area that is devoid of myofilaments. Organelles were also occasionally seen lying alongside the entire length of the nucleus (Fig. 12).

The mitochondria were found to be present at the nuclear poles alongwith the other organelles as in figure 13 (although the nucleus is not present in the micrograph), large numbers of mitochondria were present within the center of the fiber, amidst the myofilaments, along the entire length of the fiber as demonstrated in figure 14. Figure 10 indicates that mitohcondria were often found in close association with the regions of the cell membrane involved with caveolae. This arrangement is also seen in figure 8, where mitochondria can be seen lying closely to protions of the sarcoplasmic reticulum network (SR) alongwith the caveolae. At regions near the end of the fibers, which were divided and interdigitated with other fiber ends, mitochondria were found to be randomly distributed.

Tough and smooth endoplasmic reticulum were generally located near the poles of the nucleus (Fig. 11). Golgi complexes were also seen situated in these regions of the cell (Figs. 11 and 15), although these structures as well as the rough and smooth endoplasmic reticulum were also present alongside the entire length of the nucleus, (Figs.

9 and 12).

Glycogen particles were seen both individually and in clusters through the cytoplasm (Fig. 15). At the level of the nuclear poles, glycogen particles were usually seen randomly distributed although a few clusters were also noticed (Fig. 11). In the remaining areas of the cytoplasm, glycogen was scattered throughout the cytoplasm but was also observed in large groups which were generally located near the cell surface (Figs. 16 and 17). Lysosomes were observed only rarely in tracheal smooth muscle preparations (Fig. 18).

5. Junctions

a. Nexuses or Gap Junctions

Gap junctions were found rather infrequently in our tracheal smooth muscle preparations. They were identified as being a close (2 - 3 nm.) apposition of two cells as indicated in figure 40 (see innervation - qualitative). They tended to vary in length, however in most cases they were 0.07 to 0.10 um in length.

b. Intermediate Junctions

As mentioned previously in the introduction, gap junctions and intermediate junctions are the best known junctional structures found in smooth muscle cells. Intermediate junctions or zonula adherens type junctions (marked as ZA in the micrographs) were seen with great frequency between smooth muscle fibers (Fig. 19). These junctions were found at all levels of the cell and were readily identified by their appearance. The junctions involved identical electron dense bands from adjacent cells separated by a roughly 30 - 40 nm. distance between the two opposing cells. In the extracellular space, a single

layer of basement membrane was seen in the center of the separation between the two cells, (Figs. 19 and 20). As a qualitative observation, the junctions were less prevalent at the level of the nucleus than that at the cell ends. The junctions themselves appeared to vary in size, at times involving large portions of the cell membrane.

c. Other Junctional Structures

At the cell ends, the muscle fibers separated and divided into numerous laminar projections as demonstrated in the left portion of figure 21. These projections connect between and around other projections from neighbouring cells. These junctions or cell end specializations were not quantified.

6. Caveolae

Tracheal smooth muscle cells were seen to possess large numbers of caveolae on their surface. The caveolae were seen as invaginations or inpocketings of the cell membrane. Figure 2 clearly shows that the caveolae appear to be open to the extracellular space through a narrow neck. Figures 3 and 22 illustrates that the caveolae are in close proximity to the sarcoplasmic reticulum and mitochondria. Figure 22 also illustrates the observation that the outpouchings or projections of the smooth muscle outline are often the areas associated with caveolae, whereas the dense areas lie towards the interior. Portions of the sarcoplasmic reticulum are found extremely near the caveolae and a few of the mitohcondria, although direct contact between the outer portion of the caveolae and sarcoplasmic reticulum was somewhat difficult to observe.

With regard to orientation, most caveolae appeared in groups which at the level of the nucleus, alternated with areas of the

membrane involved with the dense bands. Proceeding further down to either end of the cell, the caveolae become progressively less numerous until often at certain points near the ends of a cell, no caveolae can be seen.

7. Dense Bands, Dense Bodies, and Filaments

Dense bands were seen as an incrustation of electron dense material attached to the surface of the membrane of tracheal smooth muscle cells, (Fig. 16). The internal cytoplasmic counterpart of the sarcolemmal dense bands, the structures known as dense bodies were quite readily, and are illustrated in figure 19. The dense bodies which usually appeared as round or slightly irregularly shaped electron dense areas within the cytoplasm, were also seen as linearly shaped structures as well (Fig. 23).

Filaments could be found attached to both the dense bands as well as the dense bodies. Thin (actin) filaments, as seen in figure 2, were the most abundant. The 10 nm. filaments and the thick filaments were difficult to observe, especially the myosin filaments, as they may not have beenpreserved adequately during fixation. The presence of filament associated with the dense bands was observed, however the identity of the filaments was not discerned, and would require a relatively difficult and indepth study to establish this point.

The dense band regions of the membrane were found to alternate quite regularly with the regions of the membrane involved with caveolae, at the level of the cell nucleus. Proceeding towards the fiber ends, the dense band portions of the membrane increased insize. Near the fine finger-like projections at the cell ends, the entire membrane at this point appeared to be involved with dense

bands. It was also observed in these regions however, that the electron density of the dense material below the membrane was partially diminished and difficult to identify at some points. In addition, the myoplasm in these areas appeared especially dense.

Extracellular Components

1. Mast Cells

Mast cells were also observed within the connective tissue septa of the extracellular space surrounding the smooth muscle cells. However, they were not observed with the same frequency as that of the fibroblasts which were more numerous. Most of the mast cells seen were roughly ovoid or round in shape, displaying a nucleus within the center which conformed to the overall shape of the cells (Fig. 25a). The mast cells contained several extremely dense granules, although some of the granules seen were at an immature stage and therefore granules of reduced density were also seen in active cells. In general the mast cells were located a few microns away from the muscle fibers, although they were also seen in quite closs apposition to the muscle cells in several cases. In some instances the mast cells were found to be located near both nerve terminals and muscle cells fibers, (Figure 32, see section under Innervation - Qualitative).

2. Blood Vessels

Blood vessels were readily seen within the extracellular space lying between the smooth muscle bundles, (Fig. 24b and 25b). Occasionally, red blood cells could be seen inside the lumen of the capillaries, although in some cases the lumen had all but disappeared, during the process of fixation. In figure 25b, a pericyte is present outside

the endothelial lining of the capillary. The function of pericytes is poorly understood. It has been suggested that they are contractile and can play a role in regulation of the size of the vessel lumen. Pericytes are capable of phagocytosing certain materials and are considered to be a component of the reticuloendothelial system. In some micrographs, nerve terminals could be seen approximately 1 - 3 microns away from the blood vessels. It is important to note at this point, that although nerve terminals were seen in relatively close association with some of the blood vessel profiles, the number of nerve terminals actually involved comprised only a small fraction of the total number of nerve profiles seen lying between the smooth muscle bundles.

3. Elastin and Elastic Fibers

Elastin and elastic fibers were seen in abundance and closely associated with the smooth muscle cell basement membrane. In general, elastin was visualized as a homogeneous amorphous substance in the extracellular space with direct contact to both the basement membrane of the muscle cells and the elastic fibers (Fig. 14). The elastic fibers themselves were seen as small microfilament, which penetrated the elastin substance as well as the basement membrane material (Fig. 16). Elastic fibers were also found randomly dispersed in the extracellular space. Between muscle fibers (Fig. 14). As mentioned previously, collagen fibers were also found in association with the elastic fibers (Fig. 6a and 10), which may aid in the structural integrity of the connective tissue septa.

4. Collagen

Within the extracellular space between the muscle cell fibers, collagen fibers were seen to be the most prominent constituent (Fig. 2 - 4). In figure 4, a distinct repeating pattern was demonstrated by the collagen fibers. In longitudinally sectioned fibers, much of the collagen network appears to run parallel and slightly oblique to the longitudinal axis of the muscle fibers. However, a more striking finding, especially in slightly contracted muscle fibers was that large numbers of collagen fibrils were cut in cross-section, in longitudinally sectioned muscle fibers. The coolagen fibers therefore could possibly exist in spirals around the muscle fibers. This is illustrated in figures 21, 24a, 8 and 13. However, serial sections of the fibers would have to be made to demonstrate this with greater clarity.

Collagen fibers that are seen lying immediately adjacent to the muscle fibers, were often found to be associated with the elastic fibers and elastin as well as the basement membrane of the muscle fibers (Figures 24A, 13 and 6). Figure 19 demonstrates the alternating pattern of collagen and elastic fibers situated at right angles to each other, seen in longitudinally sectioned muscle.

5. Fibroblasts

Fibroblasts were seen regularly in the larger clefts between muscle bundles, (Fig. 5). However, they were also found lying close (within 2 - 3 microns) to the muscle fibers themselves on occasion (Fig. 6a). These cells could be easily identified due to their long processes extending for distances from their center, along with the great abundance of rough endoplasmic reticulum and vacuoles seen in the cytoplasm.

FIGURES 1 - 4.

(Figures 1 - 4 are micrographs taken of tracheal smooth muscle fixed at approximately L_{max}). The calibration bar when present corresponds to 1 micron in all micrographs.

FIGURE 1.

A low power micrograph of longitudinally sectioned muscle. Portions of the nucleus (Nu) of 2 cells can be seen. This micrograph demonstrates that the majority of the tissue (over 85%) is muscle, and also the k parallel arrangement of the fibers, both qualities being essential requirements for mechanical studies. Longitudinally sectioned mitochondria (Mit) and numerous pinocytotic vesicles (PV) are seen in the muscle fibers. Longitudinally oriented collage fibers (Co) showing characteristic periodicity are found in the extracellular space. (X 16,200).

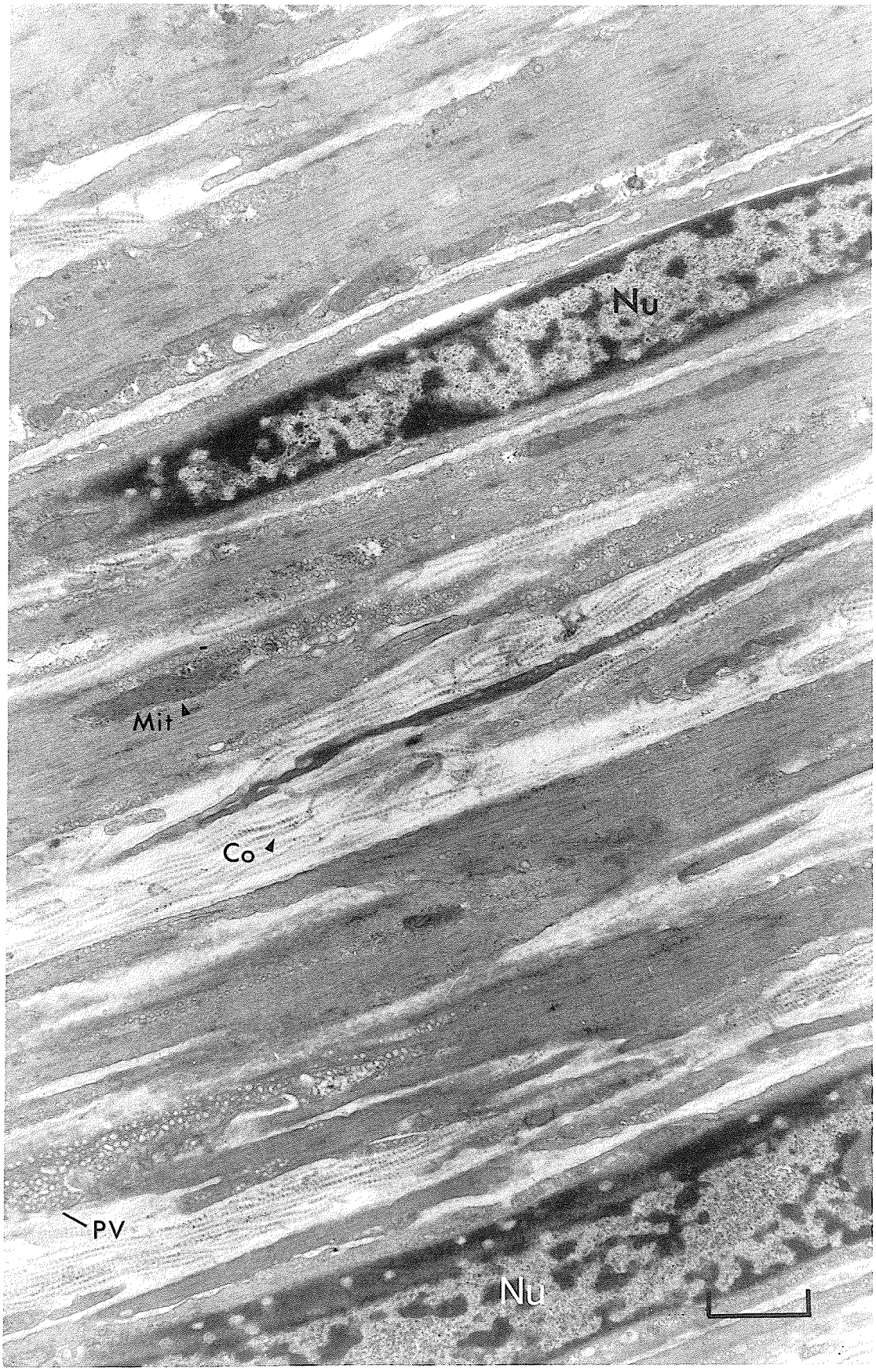

Nu
Mit
Co
PV
Nu

FIGURE 2.

This higher power micrograph shows many pinocytotic vesicles (PV) or caveolae. The vesicles marked on the bottom half of the picture clearly demonstrate that they are in communication with the extracellular space through narrow necks. Basement membrane material can also be seen to fill the vesicles to varying degrees. Many of the vesicles in the large cluster seen at the top have been sectioned slightly below the level of the membrane and therefore do not show the connecting neck of the caveoli. The clear areas in the center of some of the caveoli may represent the interior of the neck which has been transversely sectioned. Actin filaments (Ac) of the smooth muscle fibers are clearly visualized, the periodicity exhibited by collagen fibers (Co) is apparent. (X 49,760).

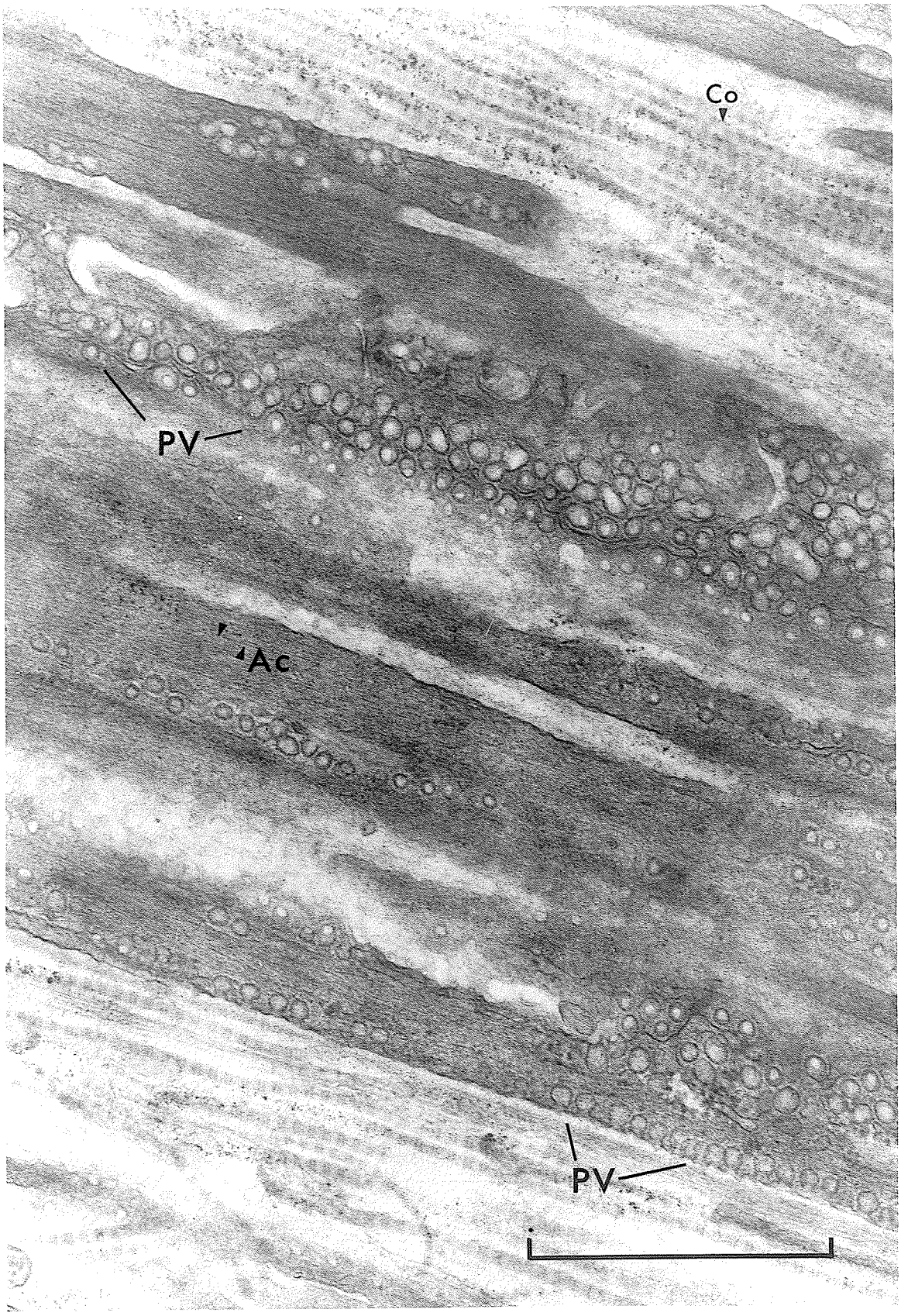

Co
PV
Ac
PV

FIGURE 3.

A micrograph demonstrating the close association of mitochondria (Mit) and sarcoplasmic reticulum (SR) with pinocytotic vesicles (PV). It should be noted that although mitochondria and other organelles generally lie at the poles of the nucleus (Nu), mitochondria are often seen near the cell membrane in more peripheral portions of the cell lying in close proximity to the sarcoplasmic reticulum and the caveolae. (X 38,700).

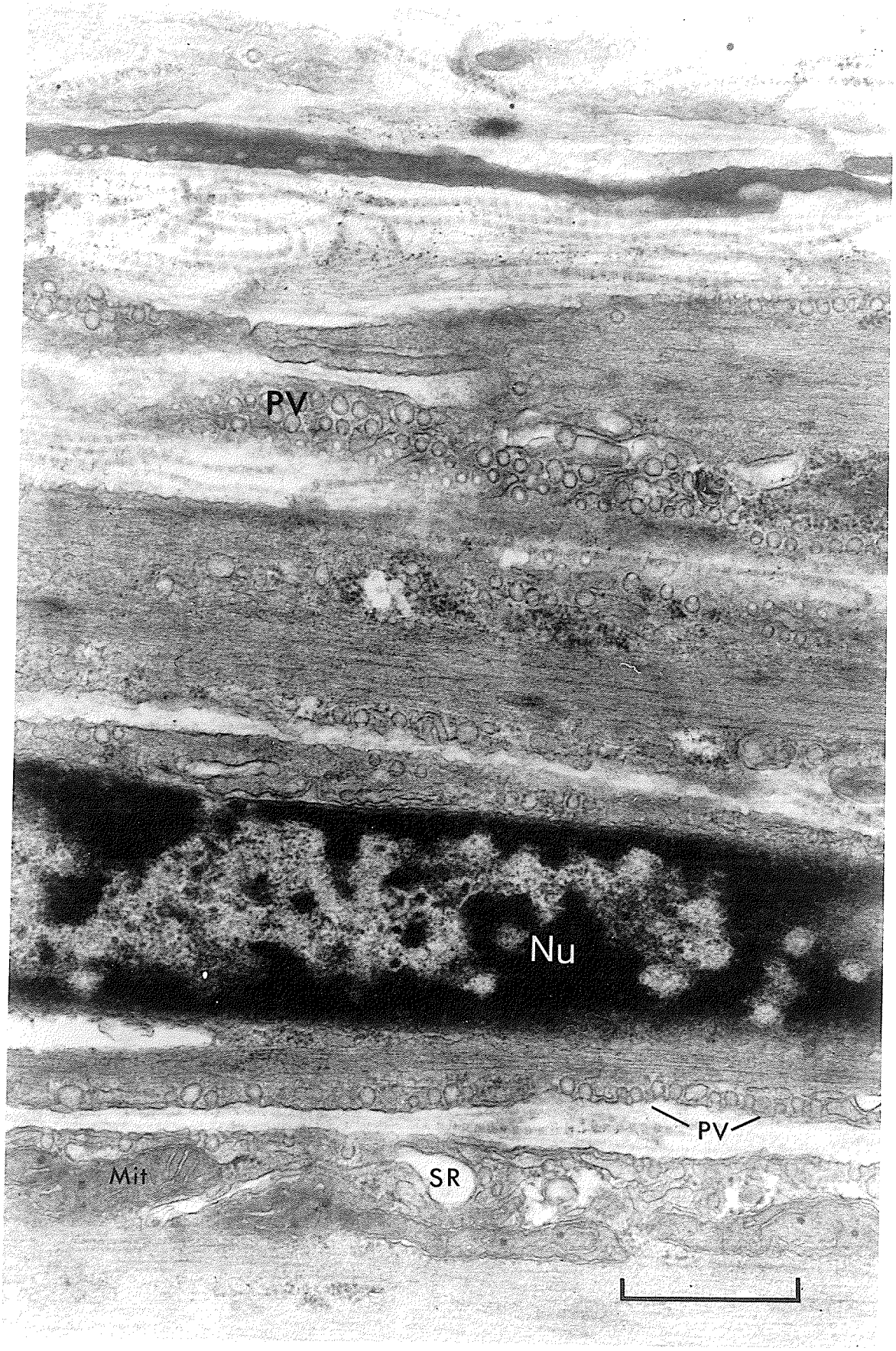

PV
Nu
PV
Mit
SR

FIGURE 4.

An example of the presence of collagen (Co) between the fibers. Although in slightly stretched muscle fiber as in this preparation, collagen fibrils run parallel to the longitudinal axis of the muscle fibers. It can also be seen that some groups of collagen fibers are also found obliquely and transversely positioned. Note the characteristic repeating pattern seen in the labelled fibers in the micrograph. Micrograph also illustrates a nucleus (Nu), numerous pinocytotic vesicles (PV) and mitochondria (Mit) in smooth muscle. (X 49,760).

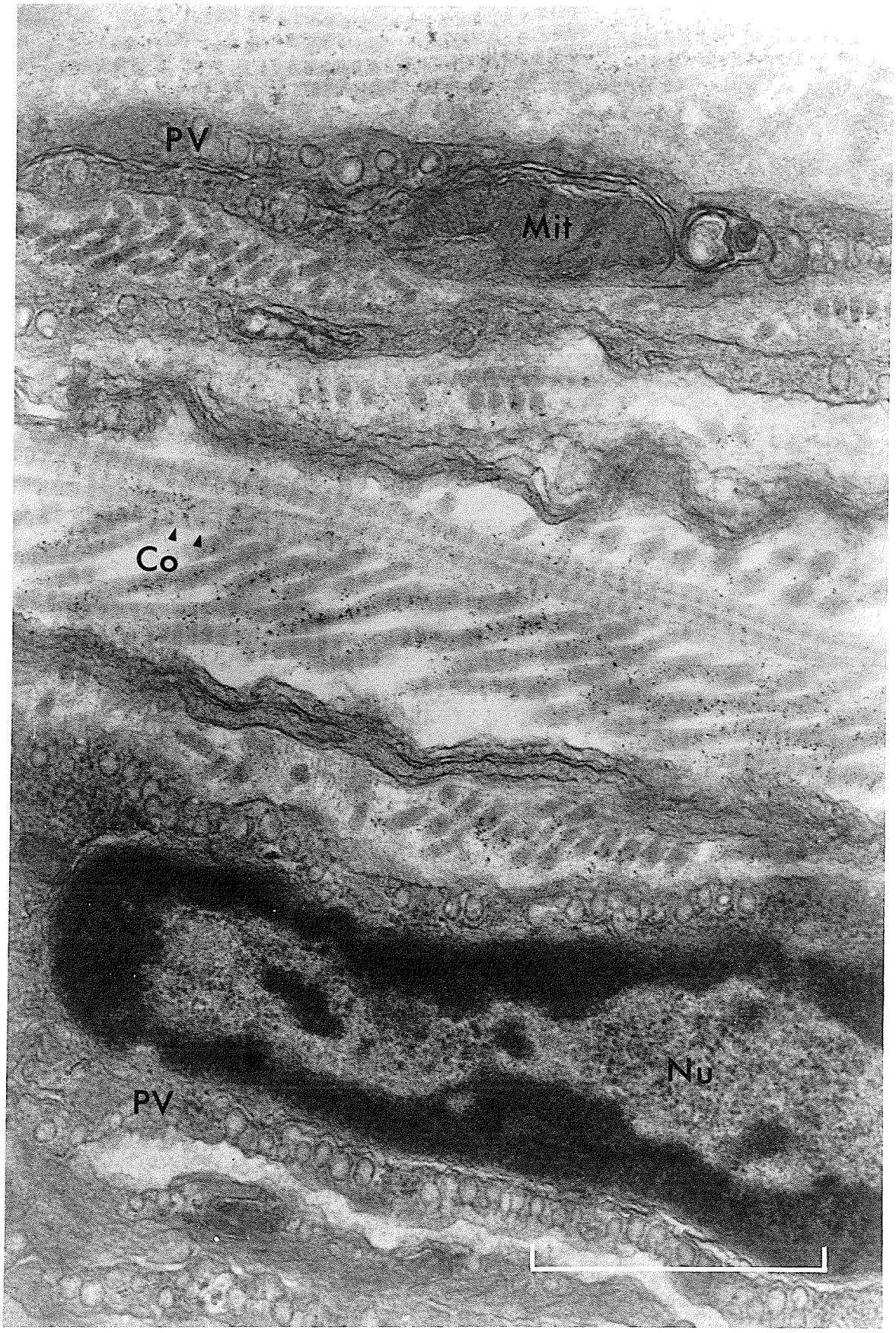

PV
Mit
Co
Nu
PV

FIGURE 5.

This micrograph demonstrates the phenomena of light and dark cells often seen in smooth muscle. An extremely light or perhaps even edematous cell is seen at the upper right hand corner of the plate. Note the collagen fibers appearing at right angles to the longitudinal axis of the cell. (X 12,540).

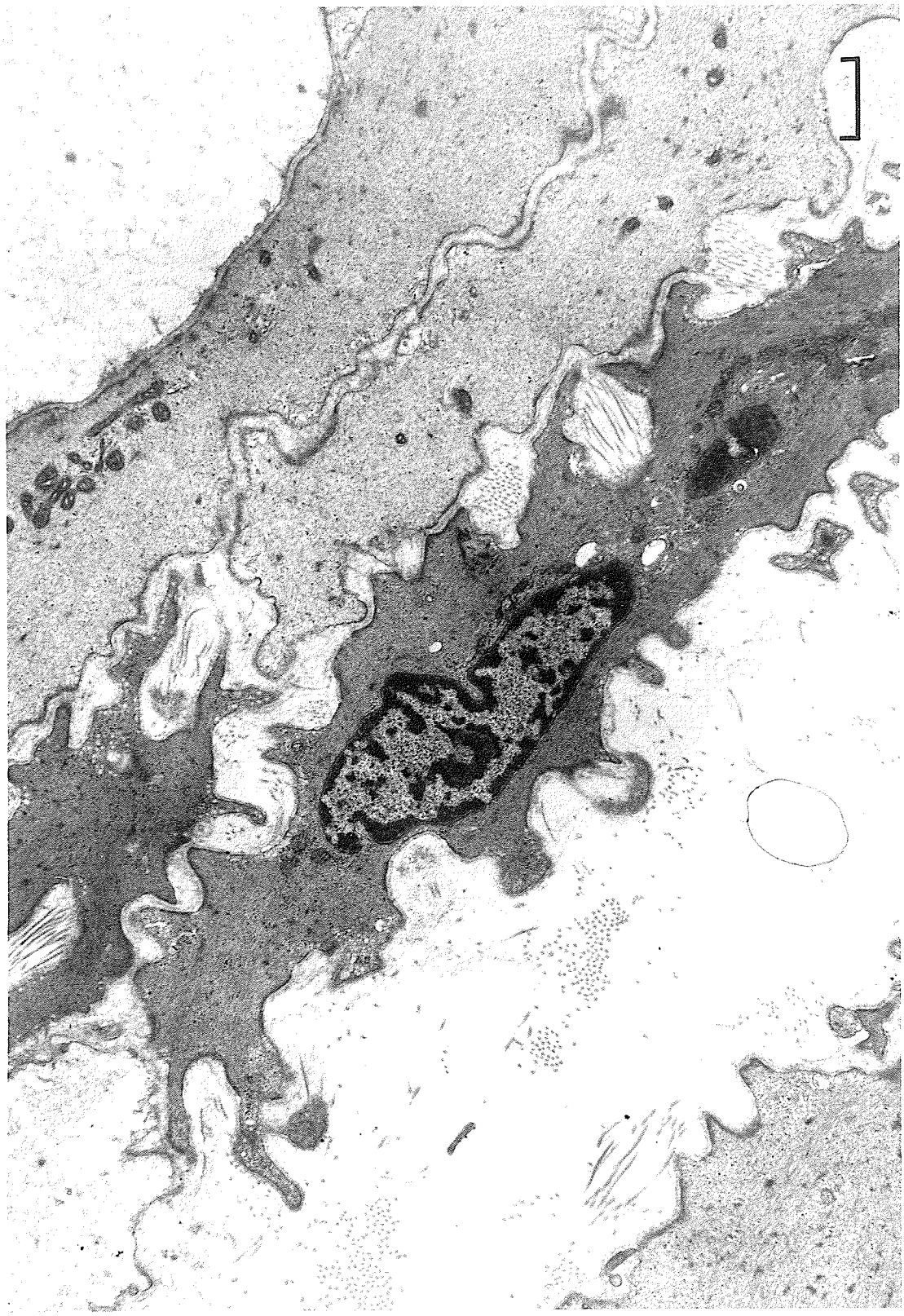


FIGURE 6.

A thick basement membrane (BM) is an evident feature in tracheal smooth muscle cells. Note that it is associated with microfilaments in the extracellular space which is in turn involved with collagen fibrils of the connective tissue septae. Several projections of out-pouchings of the muscle cell are observed, and can be seen to contain numerous pinocytotic vesicles (PV) which in turn are observed to be associated with sarcoplasmic reticulum (SR). Mitochondria, (Mit), which are often seen in rows or in groups and centrally located are found in the cell at the bottom portion of the micrograph. (X24,370).

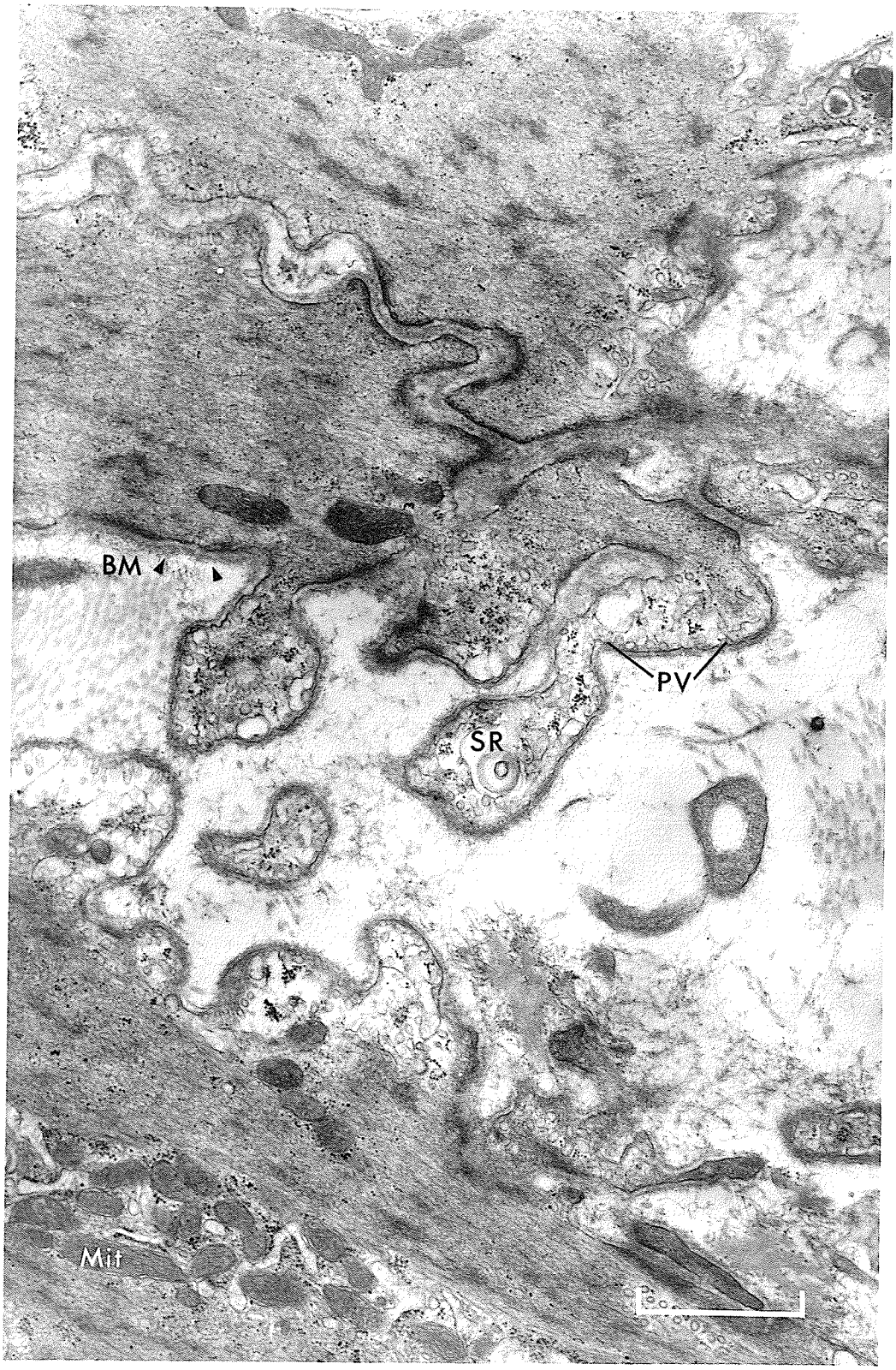

BM
PV
SR
Mit

FIGURE 7.

This low power micrograph of a longitudinal section of tracheal-smooth muscle shows the presence of numerous mitochondria (Mit) and other organelles at the poles of the nucleus. Note the presence of symmetrical dense band areas between adjacent cells. (X 9,920).

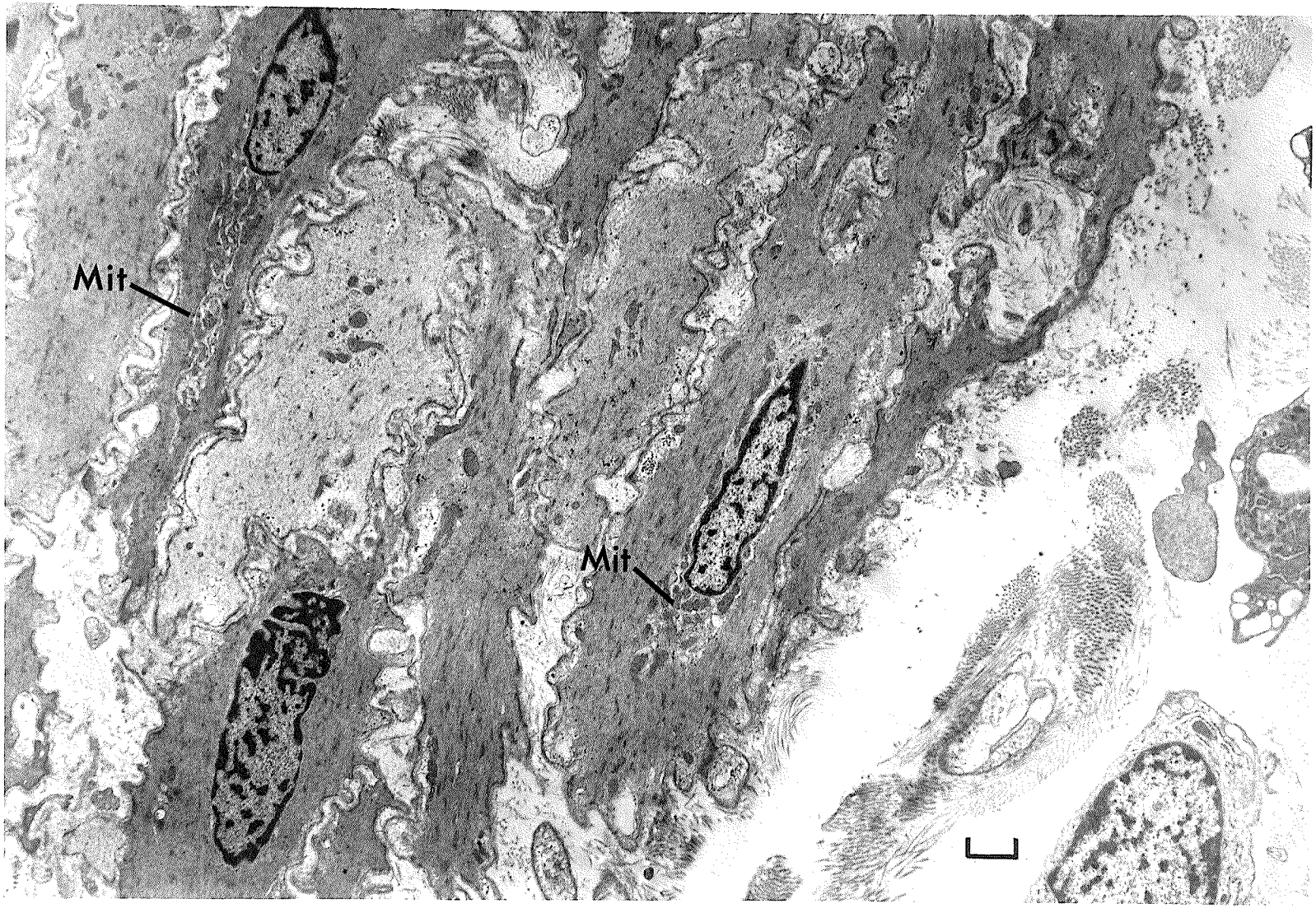

Mit
Mit

FIGURE 8.

This high power micrograph more clearly shows the relationship of the mitochondria (Mit) to the pinocytotic vesicles. The labelled mitochondria in the center of the plate is adjacent to a portion of sarcoplasmic reticulum. (X 53,870).

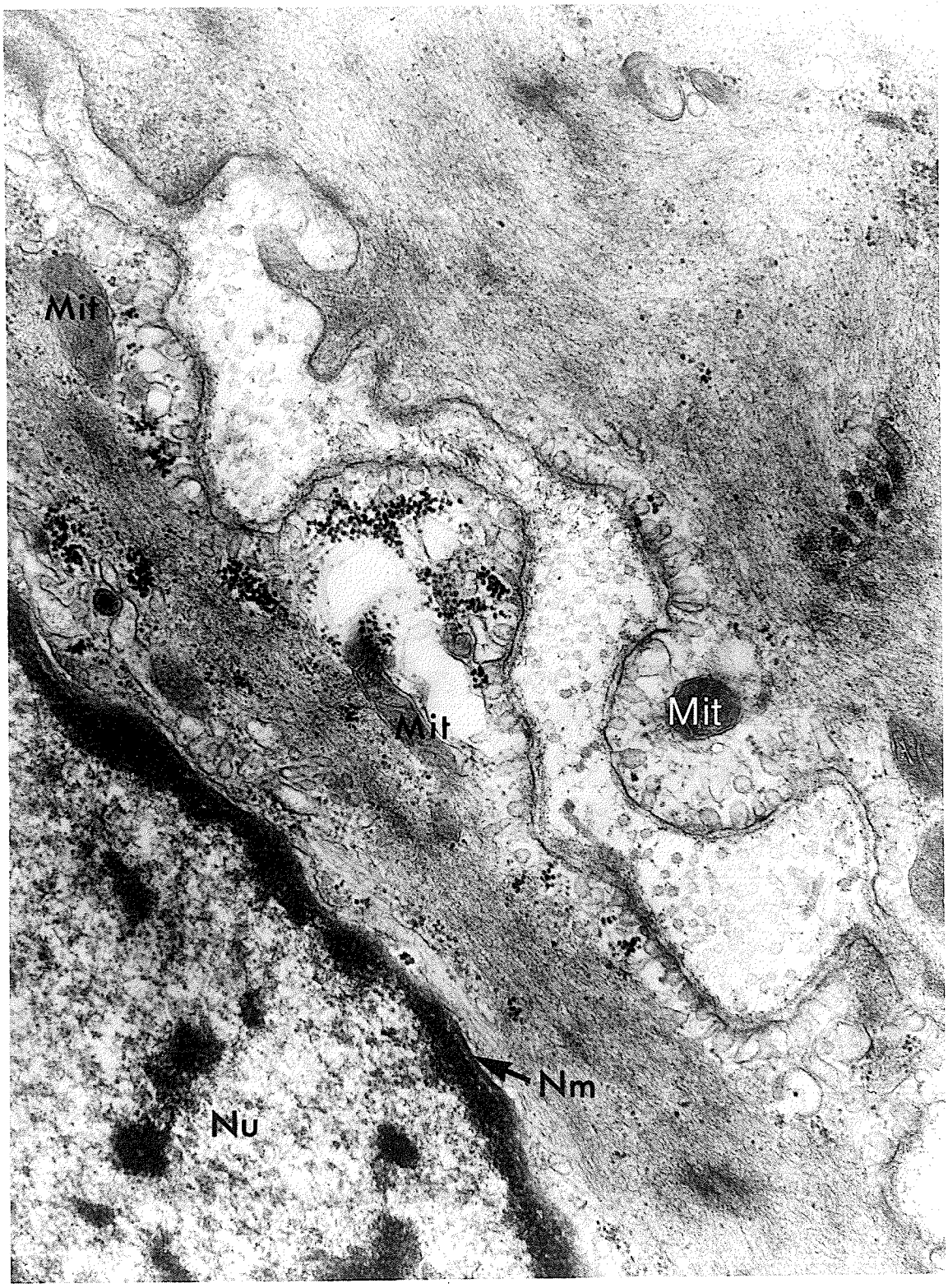

Mit
Mit
Mit
Nm
Nu

FIGURE 9.

A micrograph typifying the elongated, oval nucleus (Nu) seen in tracheal smooth muscle cells. Occasional mitochondria (Mit) are seen along side the nucleus, although the majority of them are usually observed at the nuclear poles. A well defined nuclear membrane can also be observed. Nuclear pores along the nuclear membrane can be seen; one directly above the top arrow and another roughly 1 cm below the lower arrow. (X 25,650).

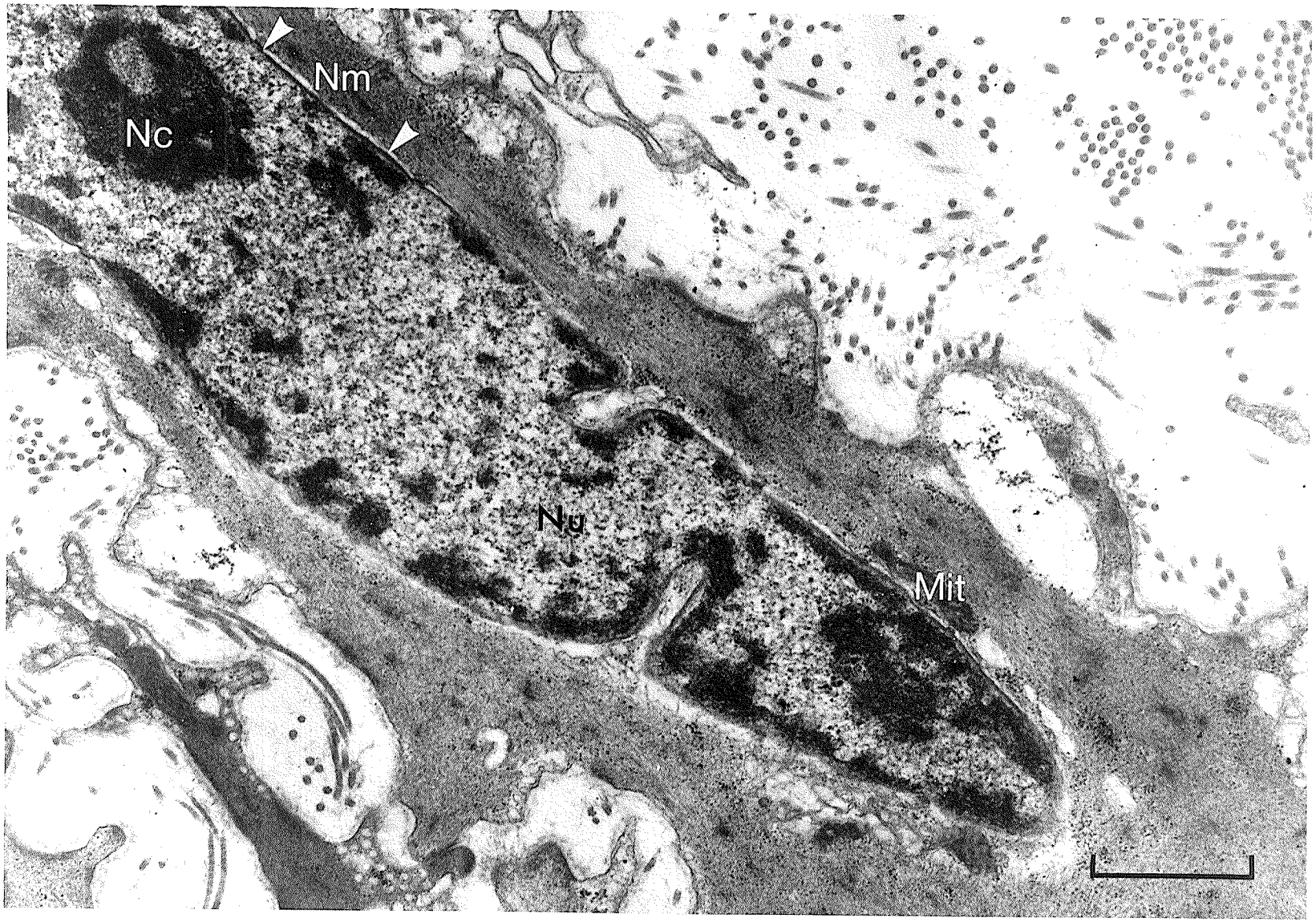

Nm
Nc
Nu
Mit

FIGURE 10

A micrograph demonstrating both the membrane dense bands (Db) and the cytoplasmic dense bodies (db). The dense bands (Db) are seen as incrustations of electron dense material on the cytoplasmic side of the cell membrane. They are seen to alternate with portions of the cell membrane that are involved with caveolae. Thin filaments can be observed penetrating into the dense bodies (db) in the cytoplasm. (X 44,630).

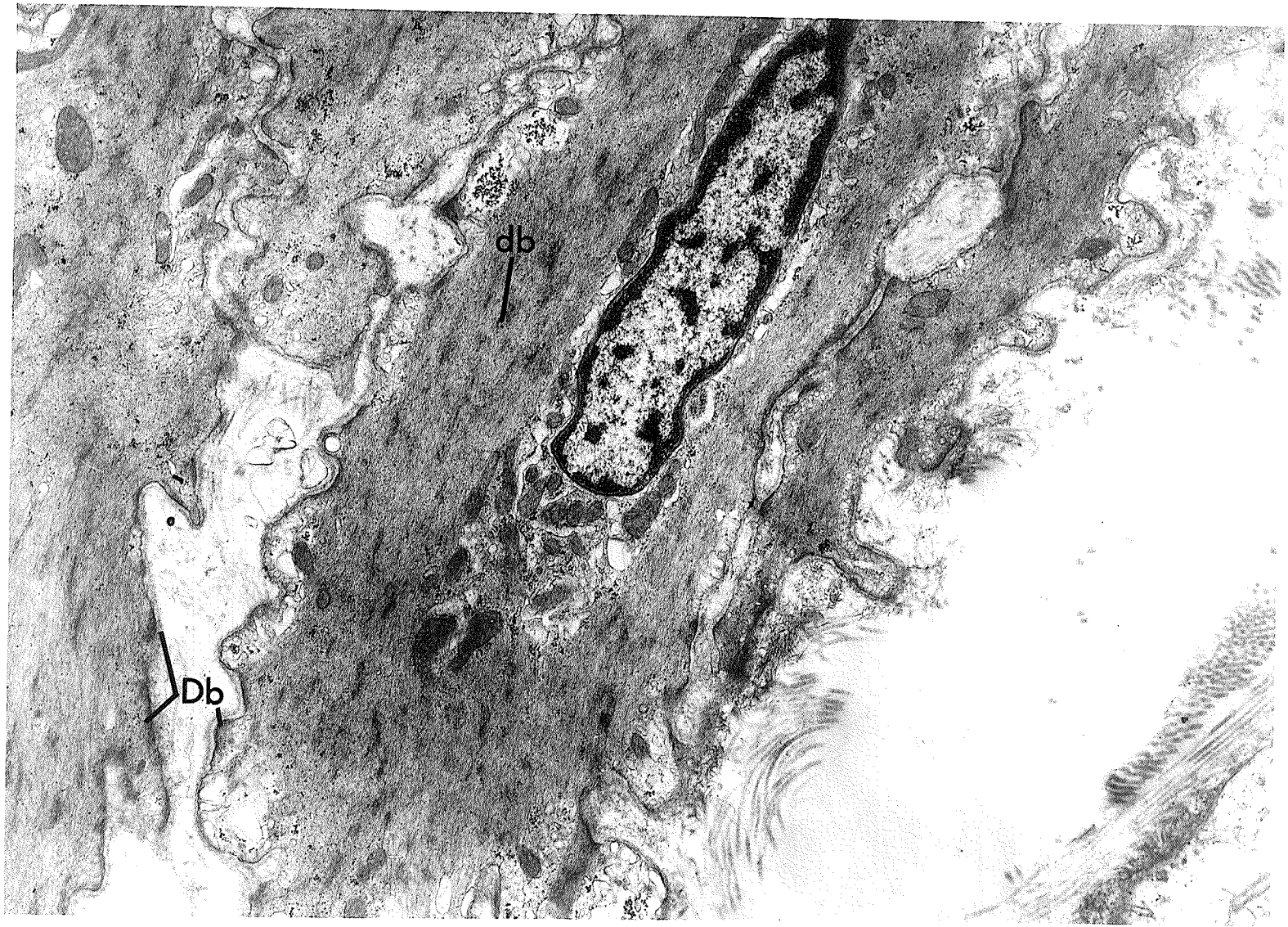

db
Db

FIGURE 11.

This high power micrograph demonstrated the complexity of the organelles situated at the pole of a nucleus: glycogen (Gly) particles in clusters; Golgi apparatus (Go), and clear and coated vesicles; smooth endoplasmic reticulum (SER). An intermediate junction between this cell and an adjacent cell can be seen at the right. (X 48,220).

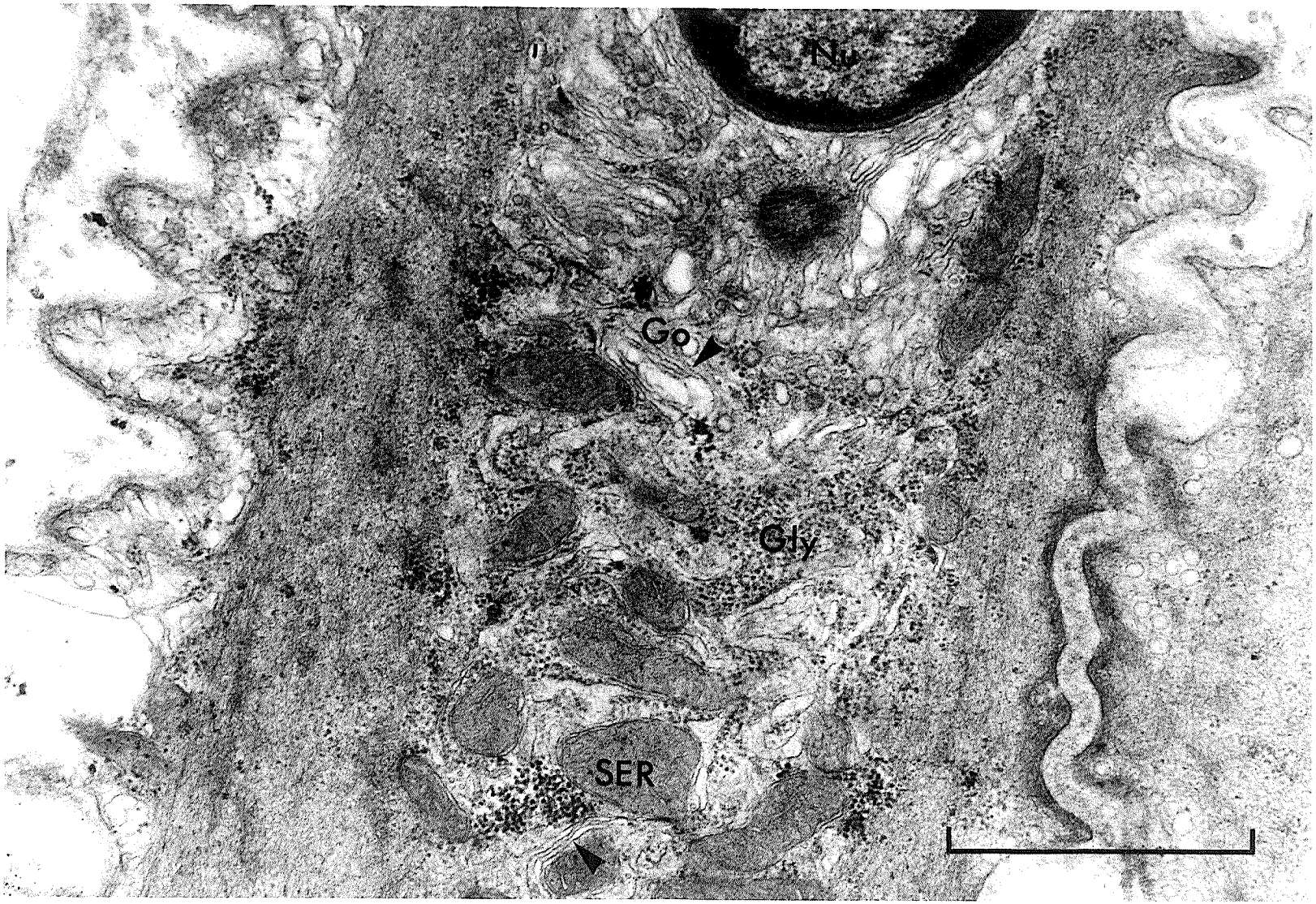

Nu
Go
Gly
SER

FIGURE 12

A higher power micrograph demonstrating the nucleus (Nu) and other cellular organelles in close association. Smooth endoplasmic reticulum (SER) and a mitochondria (Mit) are in near proximity to the nucleus, as well as a prominent Golgi apparatus (Go). Within the nucleus itself the highly condensed electron dense heterochromatin can be seen along with the dispersed less electron dense euchromatin. The nucleolus (Nc) is also shown in this plate. (X 48,220).

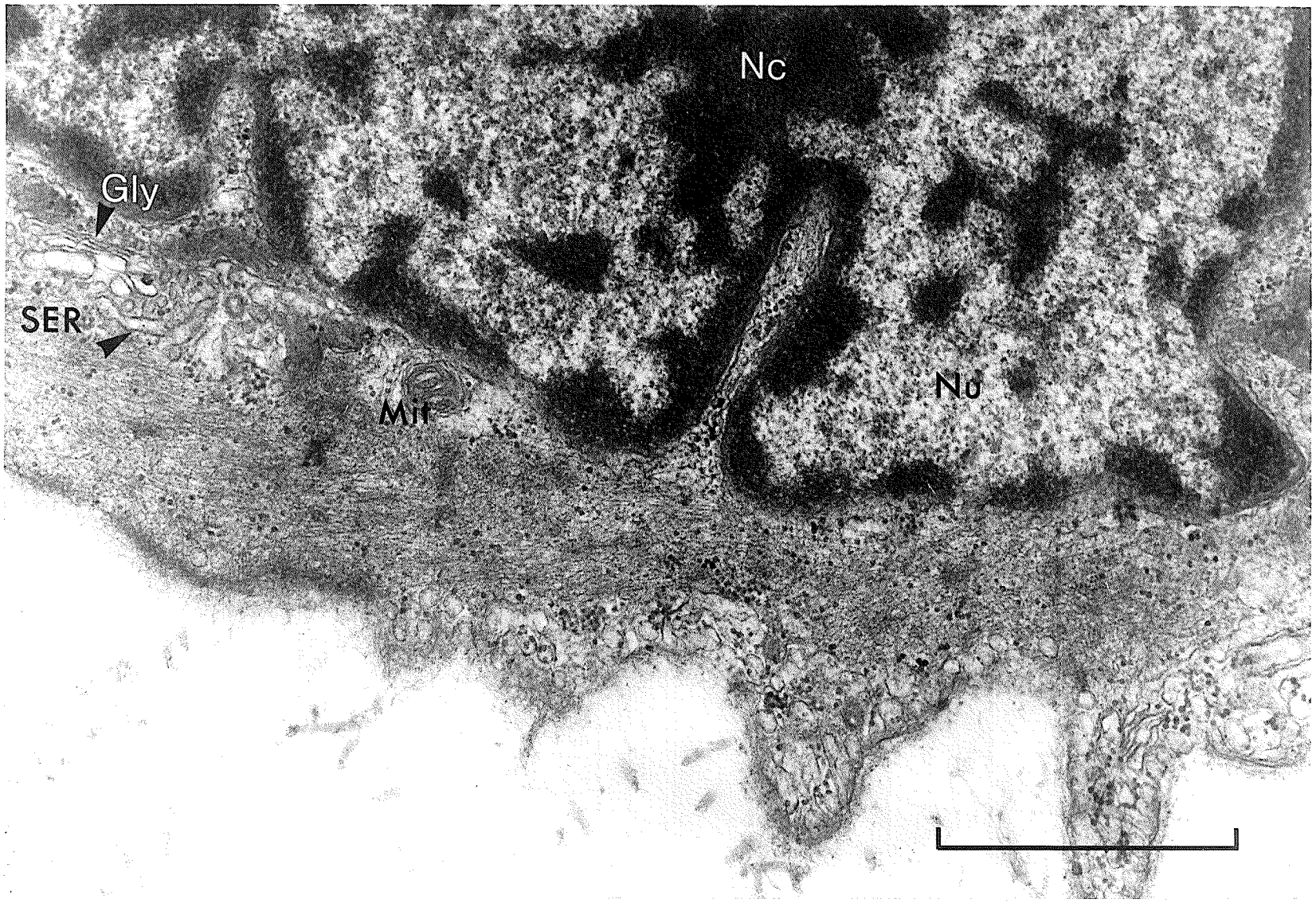

Nc
Gly
SER
Mit
Nu

FIGURE 13.

This micrograph illustrates the typical arrangement of mitochondris (Mit) seen in tracheal smooth muscle cells. Most mitochondria are located in the central aspect of the cell, near the nucleus along with the majority of the organelles. This general region of the cell is usually seen to be devoid of microfilaments. Mitochondria however are often seen near the cell membrane as demonstrated in this micrograph, in close association to the sarcoplasmic reticulum and pinocytotic vesicles (PV). (X 14,400).

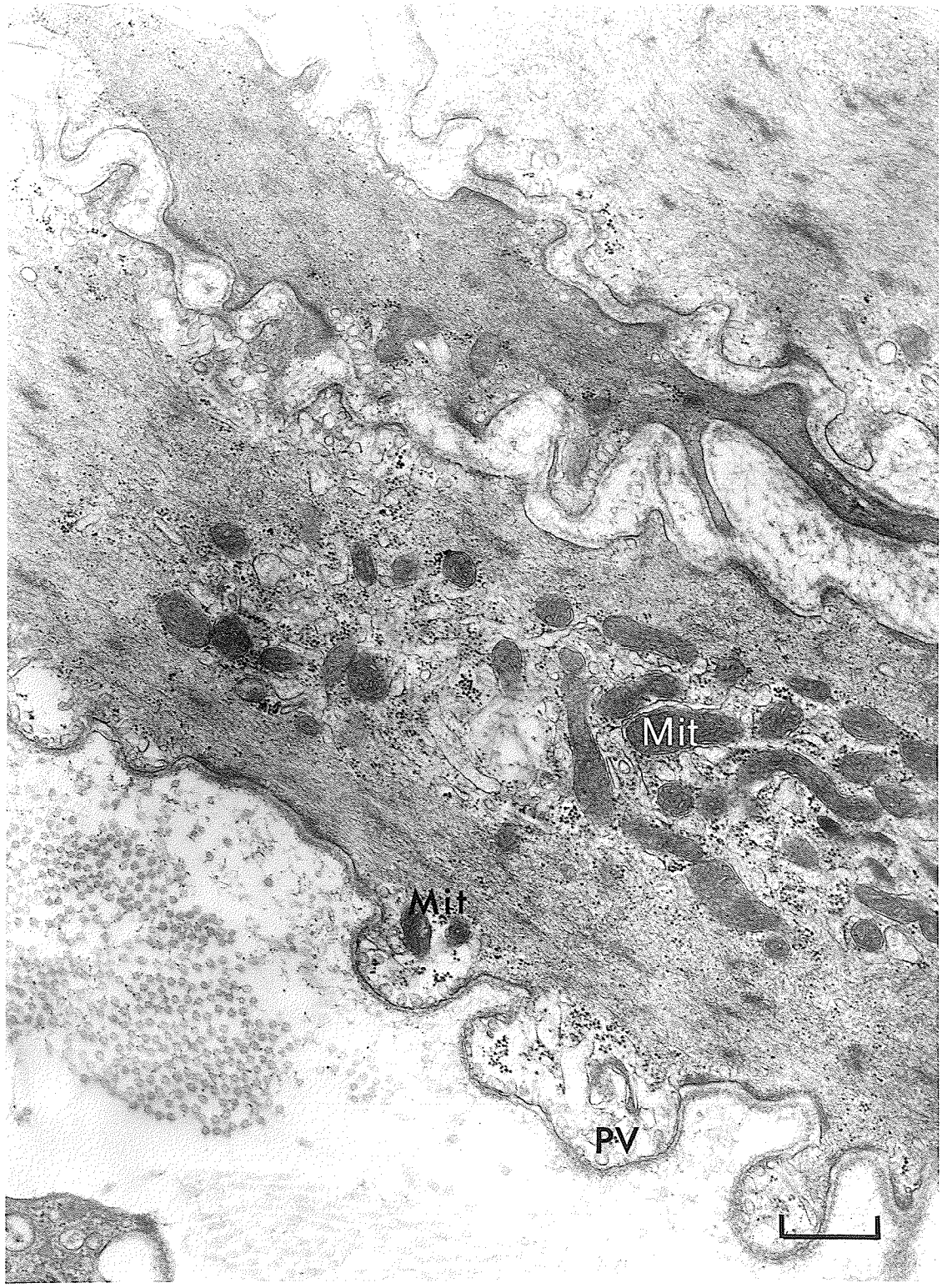

Mit
Mit
PV

FIGURE 14.

A longitudinal section of muscle demonstrating the abundance of mitochondria and their usual centrally located positions within the cell. Elastin (E) can be seen in close association with microfilaments and the basement membrane of the muscle cell. The muscle cell at the bottom displays well defined nucleus (Nu) with a prominent nucleolus (Nc) contained within. (X 28,570).

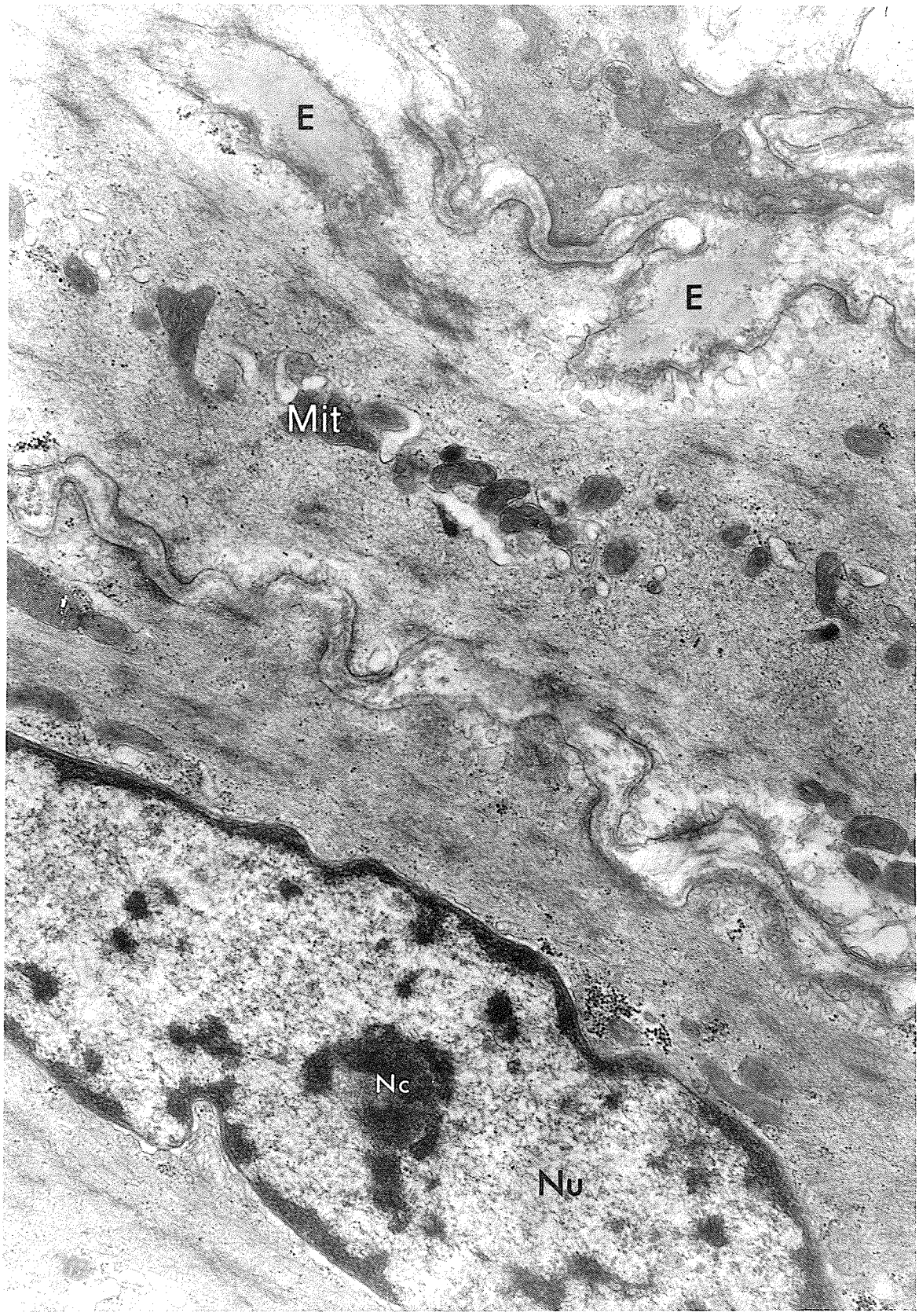

E
E
Mit
Nc
Nu

FIGURE 15.

Tracheal smooth muscle cells are often observed to contain glycogen (Gly) particles. They are seen to be aggregated in rosettes (alpha particles) and also dispersed (Beta particles) within the cytoplasm. (X 50,270).

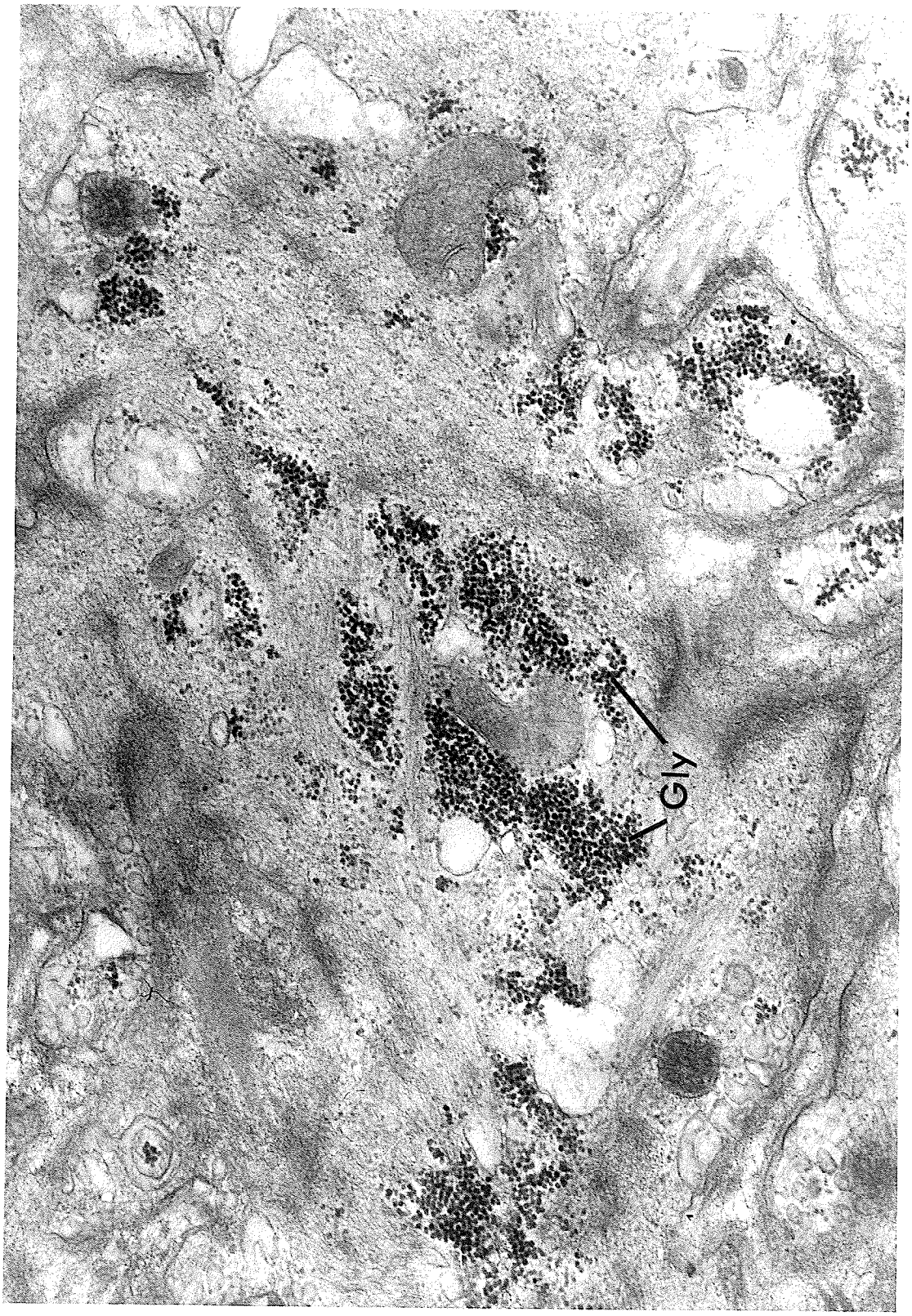

Gly

FIGURE 16.

Another example of the membrane dense bands (arrow) seen in smooth muscle. The basement membrane associated with these dense bands is often seen to be more prominent than that found in adjacent areas of the cell membrane. Dense bodies (db) are seen clearly in the cell at the lower left alongwith a portion of its nucleus. 100 A and thin filaments are found to penetrate the dense bodies. Elastin (E) associated with the basal lamina can be seen in the extracellular space. Note the presence of large clusters of glycogen granules (Gly) in the cytoplasm of the cells (X 35,100).

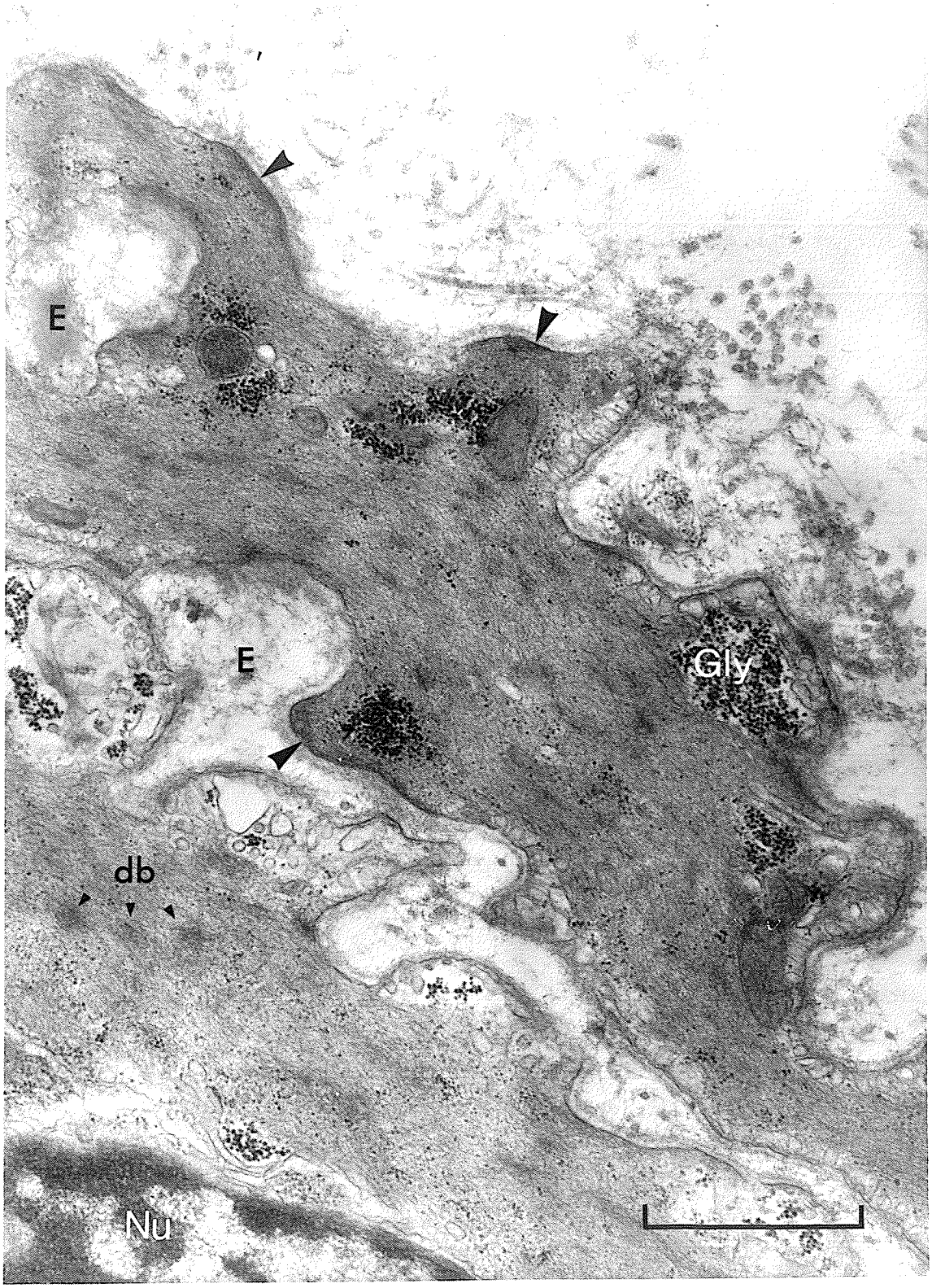

E
E
Gly
db
Nu

FIGURE 17.

A micrograph depicting the position of mitochondria and glycogen particles in the outer end regions of a muscle fiber. Several pinocytotic vesicles (PV) can be seen associated with mitochondria (Mit). (X 35,100).


Mit
PV
Gly
Mit
PV

FIGURE 18.

A rarely seen lysosome (Ly) in tracheal smooth muscle is found in this section. Sacs of dilated cisternae of the Golgi apparatus (Go) is also present. Innumerable glycogen particles can be seen both dispersed and as rosettes in the cytoplasm. (X 45,140).

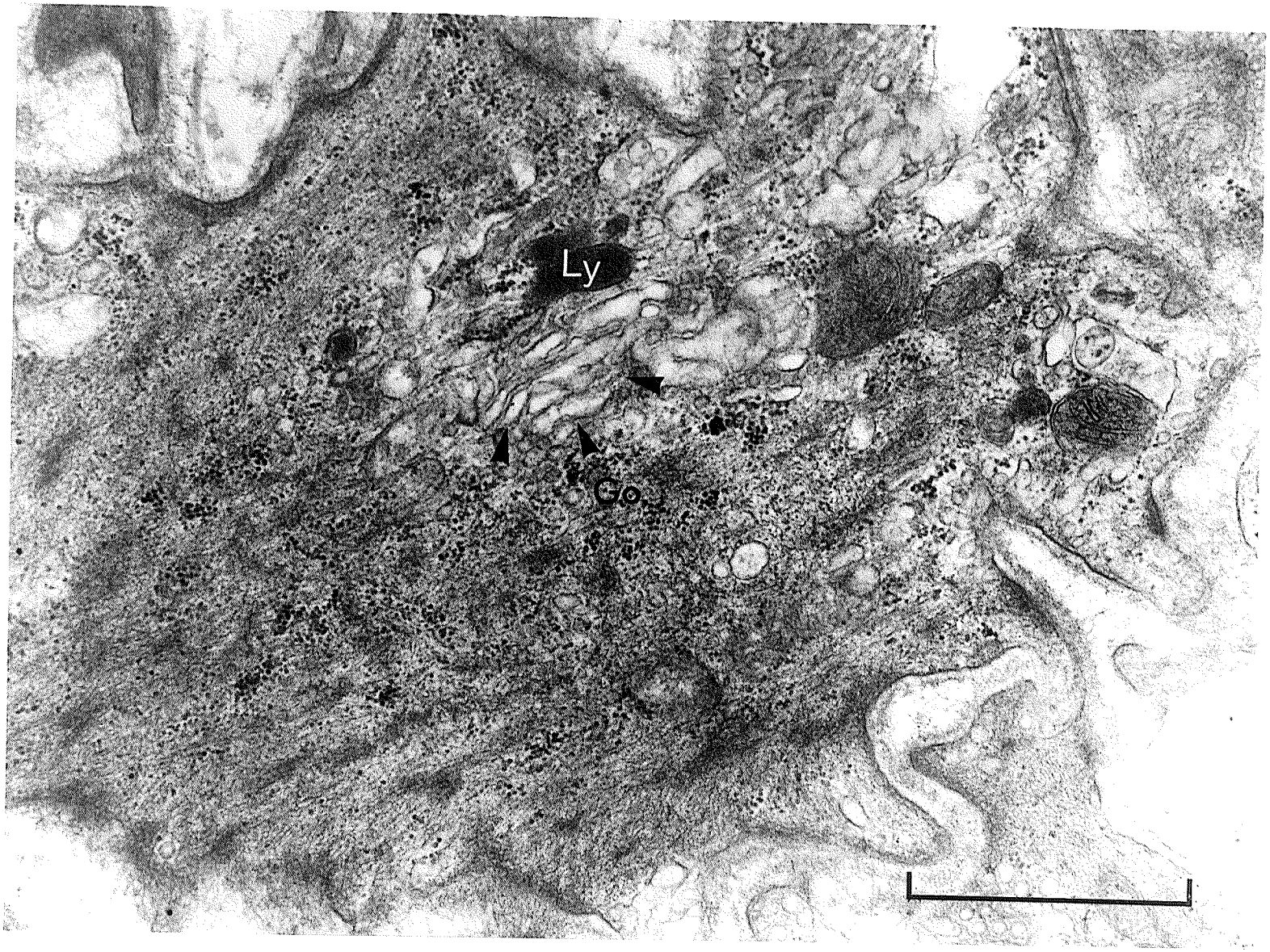

Ly
Go

FIGURE 19.

An example of the possible spiral formation of collagen (Co) around smooth muscle cells. Observe that collagen fibers cut in cross-section are seen to alternate with the elastic filaments (El) which run parallel to the longitudinal axis of the cell. Similar findings have been reported by Gabella. An intermediate junction (ZA) illustrates the symmetrical density of electron dense material on the cytoplasmic side of the two cells involved in the junction. (X 19,830).

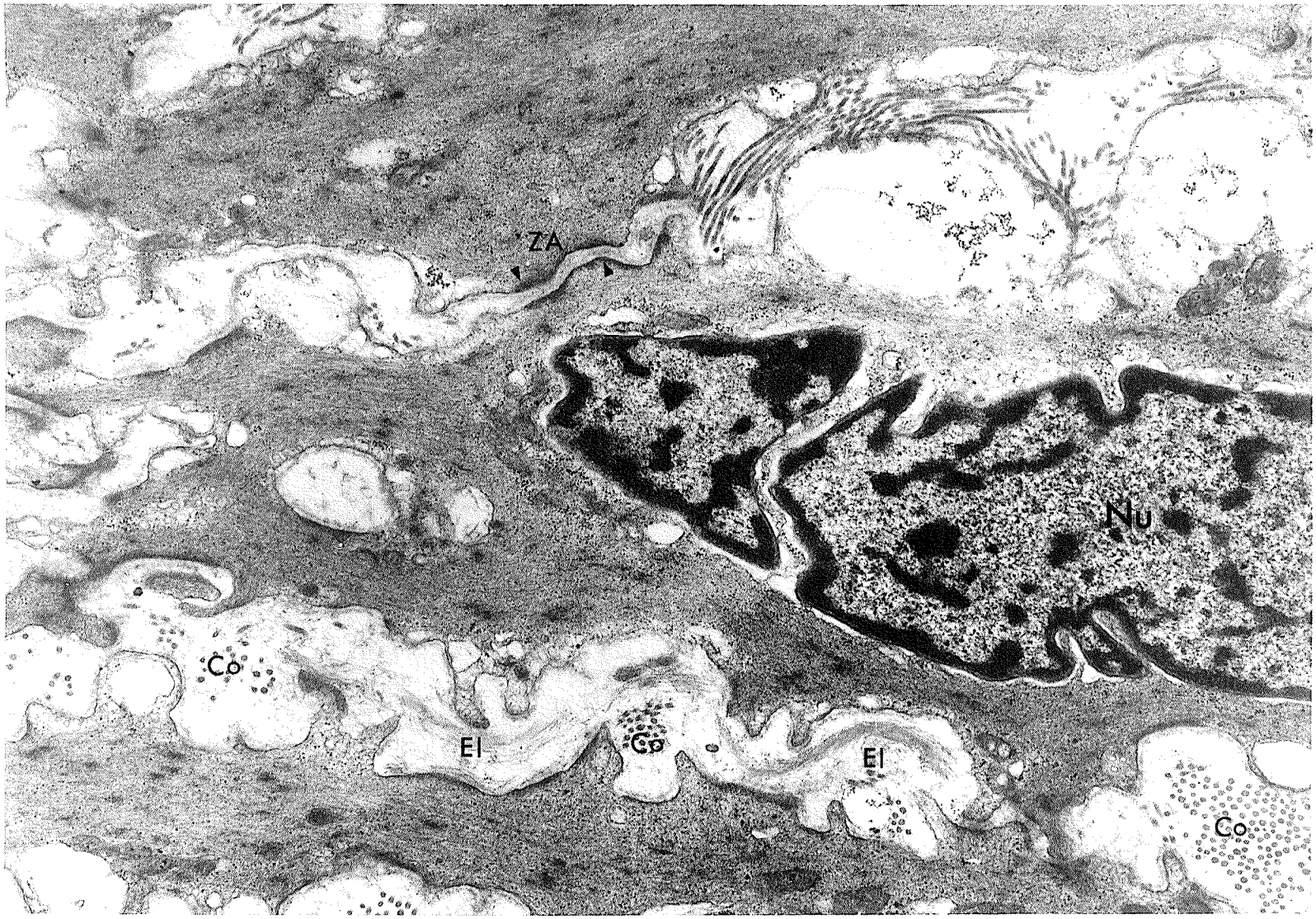

ZA
Nu
Co
El
Cp
El
Co

FIGURE 20.

A micrograph depicting two intermediate types of zonula adherens (ZA) type junctions between three cells. Electron dense material is observed on the cytoplasmic side of both cells involved in a junction. In addition, the characteristic single strand of basal lamina situated in the center of the cleft between the two cells at the site of the junction can be observed. At the top of the micrograph, elastic fibers (El) are seen. (X 23,070).

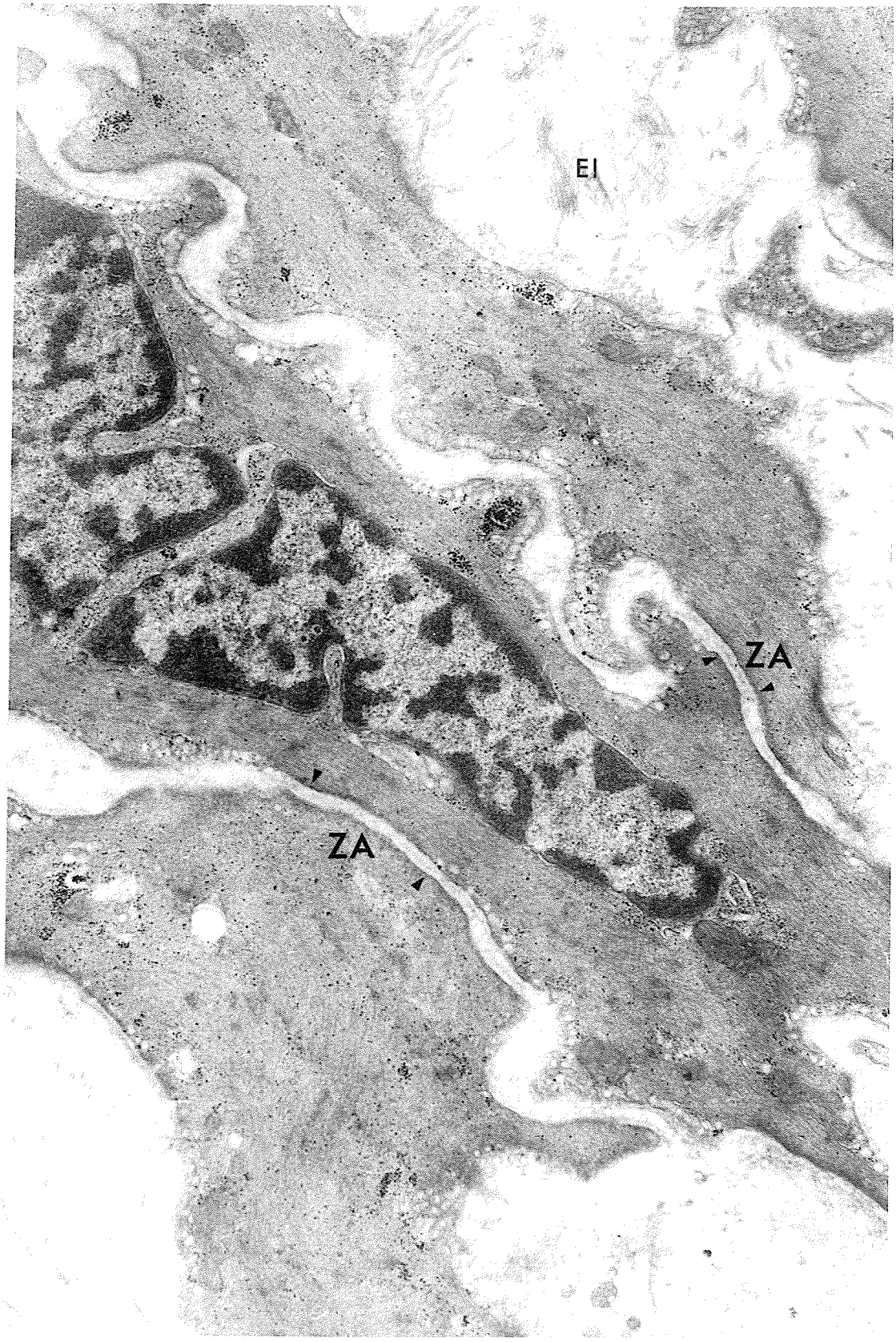

EI
ZA
ZA

FIGURE 21

A micrograph showing the interdigitation between the cell ends of smooth muscle cell forming end-to-end and end-to-side junctions. The long process of cytoplasm of the fibroblast (Fb) at the right is typical of those fibroblasts seen in smooth muscle. Large amounts of collagen (Co) cut at right angles to the long axis of the muscle cell and a group of elastic fibers (E) are also observed in the extracellular space. (X 15,920).

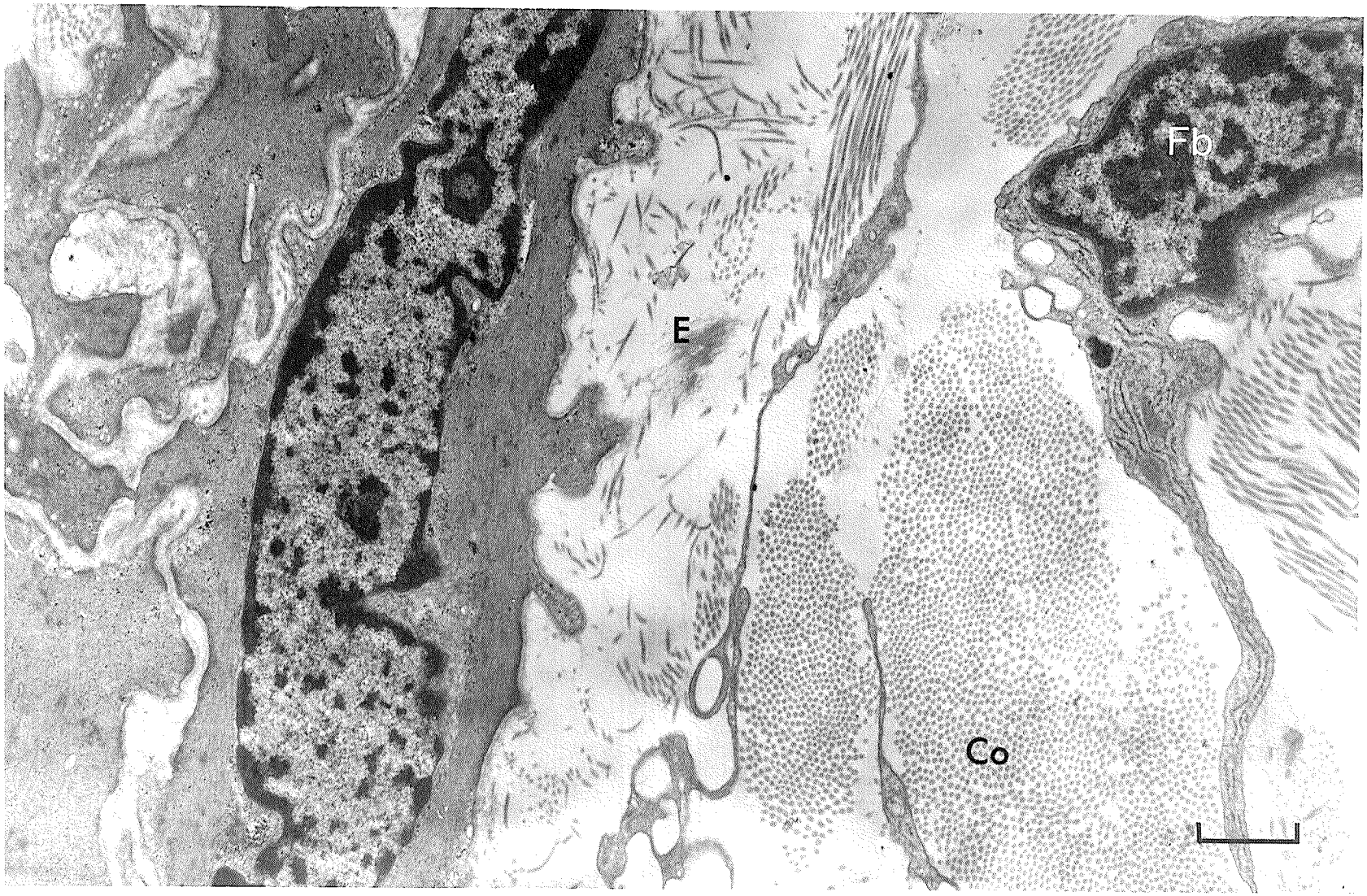

Fo
E
Co

FIGURE 22

Projections or outpouchings of the cell membrane are often seen in tracheal smooth muscle. Pinocytotic vesicles (PV) are seen to line the projections. However dense bands of the membrane are rarely seen on the membrane limiting the projections. A mitochondrion (Mit) located in this peripheral part of the cell can be observed. Several dense bodies in the cytoplasm are seen (db). (x 13,200).

INSET: It can be seen that the sarcoplasmic reticulum associated with the projections often appears dilated.

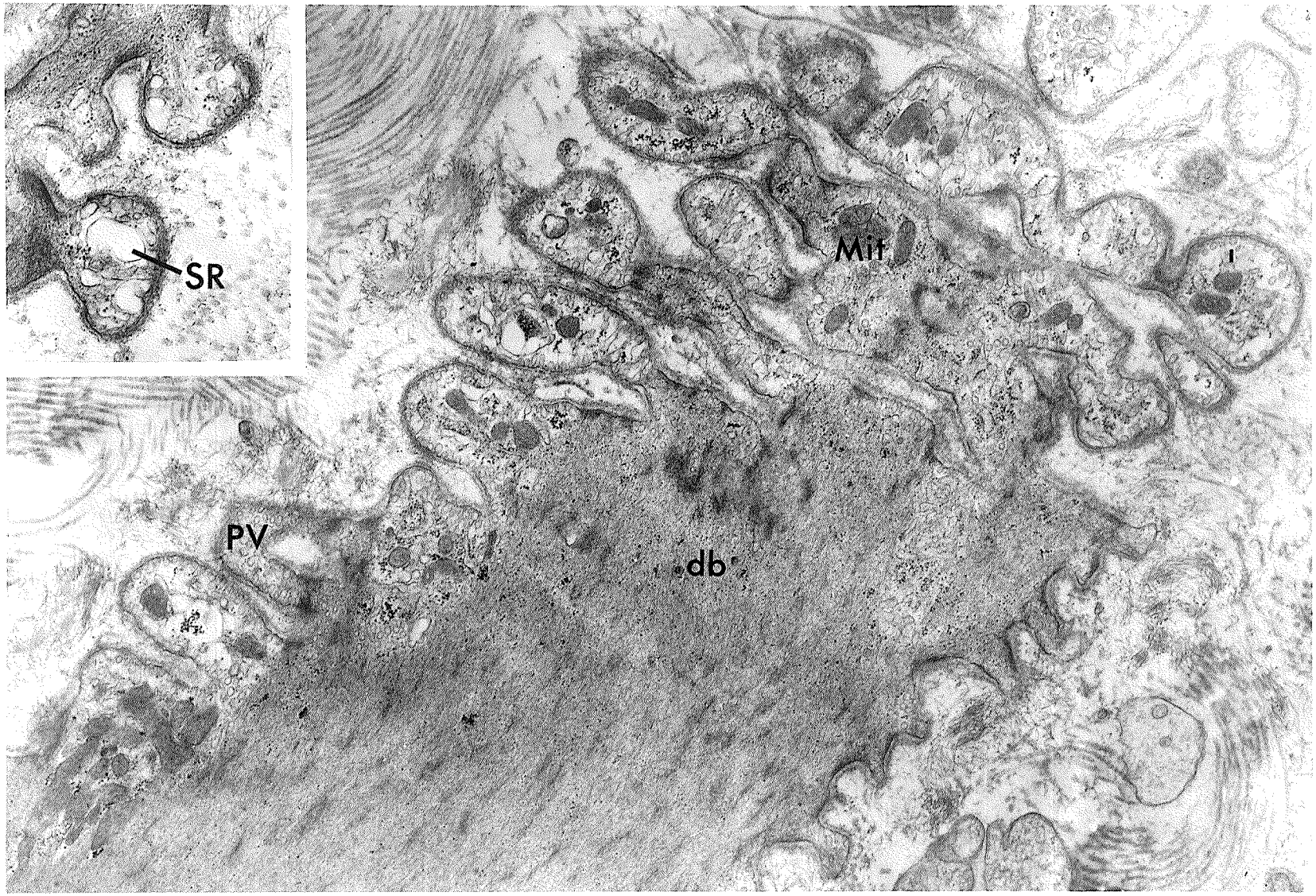

SR
Mit
I
PV
db

FIGURE 23.

In this micrograph several dense bodies (db) can be seen in the cytoplasm of the smooth muscle cells. Some of these dense bodies are often elongated in shape. Mitochondria (Mit) are often seen in the peripheral portions of the cell as seen in the cell in the upper right hand portion of the picture. (X 24,880).

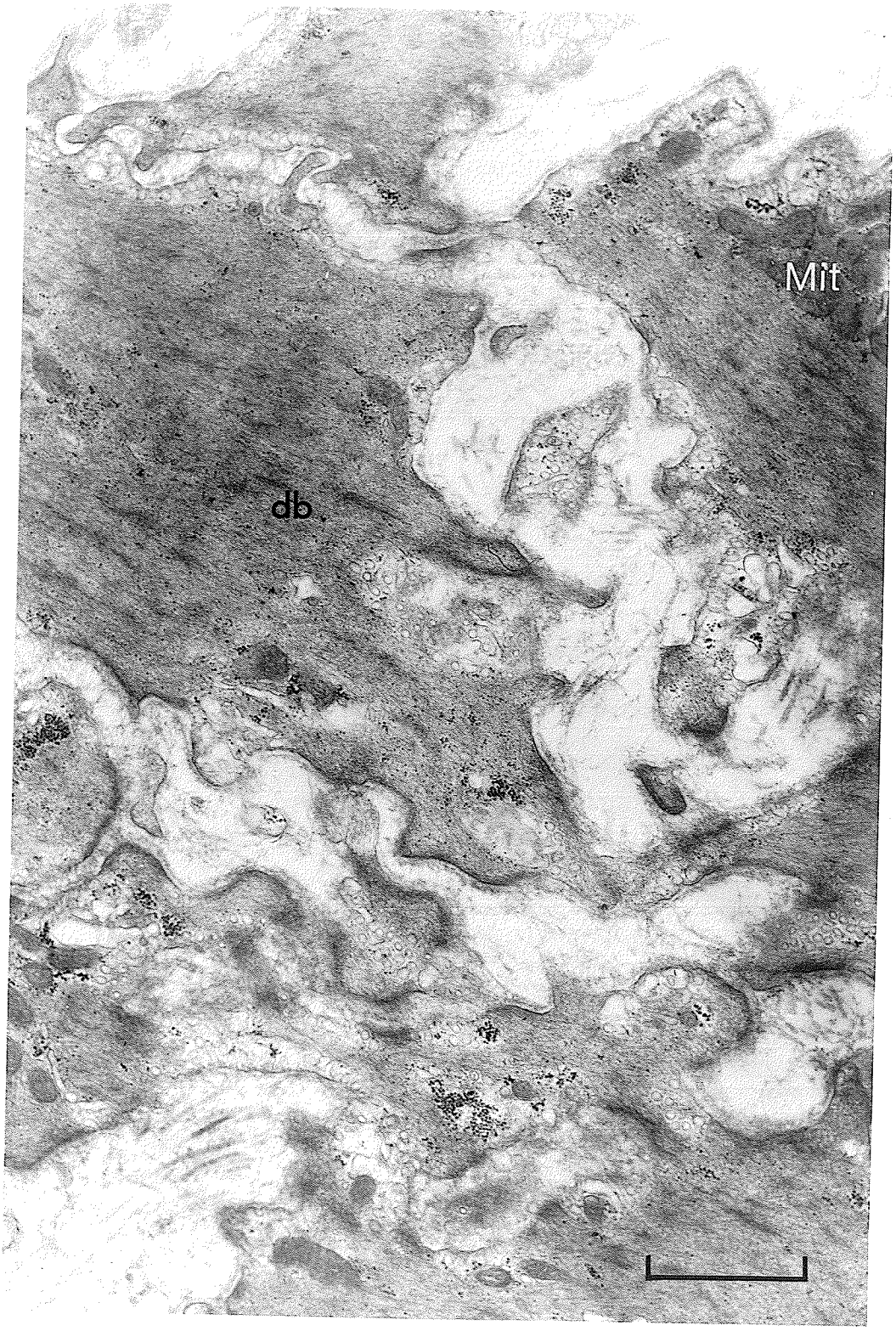
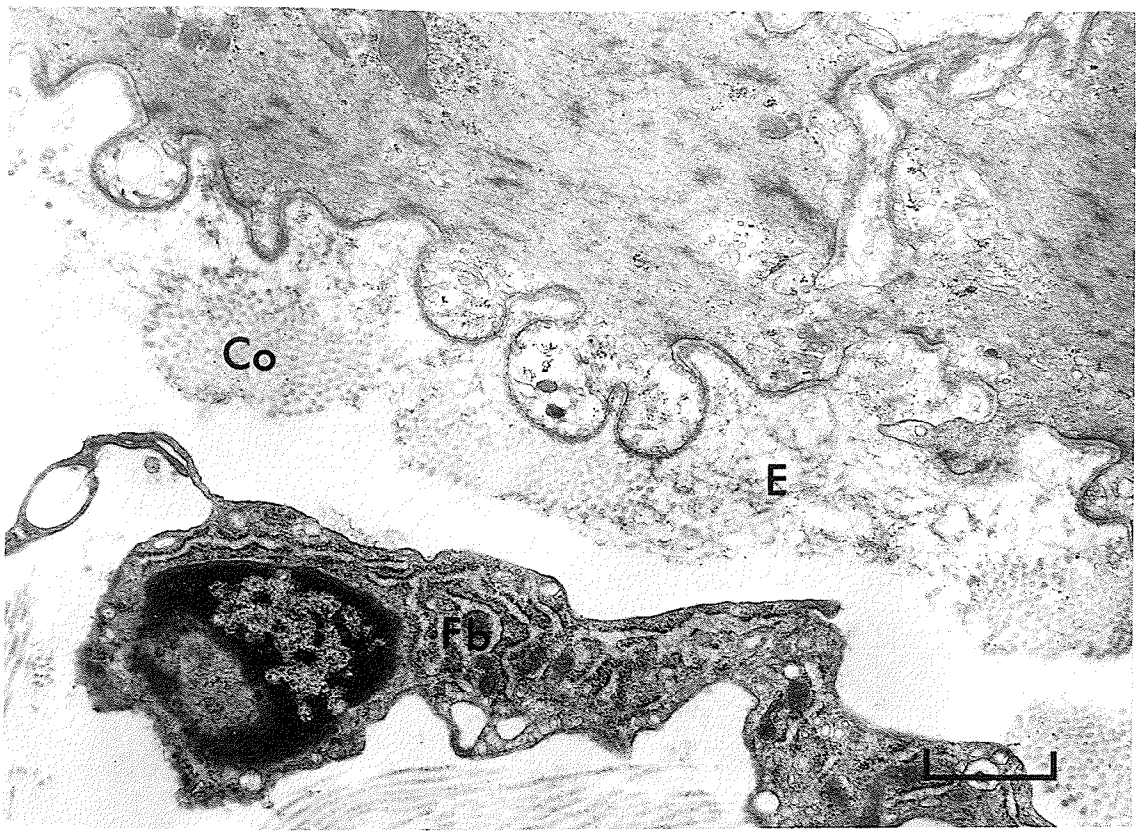

Mit
db

FIGURE 24A.

A micrograph depicting a fibroblast (Fb) in close relation to smooth muscle cells. Numerous cisternae of rough endoplasmic reticulum can be seen within cytoplasm of the fibroblast. Mitochondria and free ribisomes can also be observed. Collagen fibers (Co) running at approximately right angles to the longitudinal axis of the smooth muscle cells can be seen. Elastic fibers (E) are found in close association to the collagen fibers and basal lamina of the muscle. (X 17,320).

FIGURE 24B.

A low power micrograph demonstrating the presence of a capillary containing a red blood cell (RBC) situated in the connective tissue between two smooth muscle bundles (SM). A nearby fibroblast (Fb) can be seen along with nerve varicosities located in the upper right portion of the micrograph. (X 18,440).


Co
E
Fb

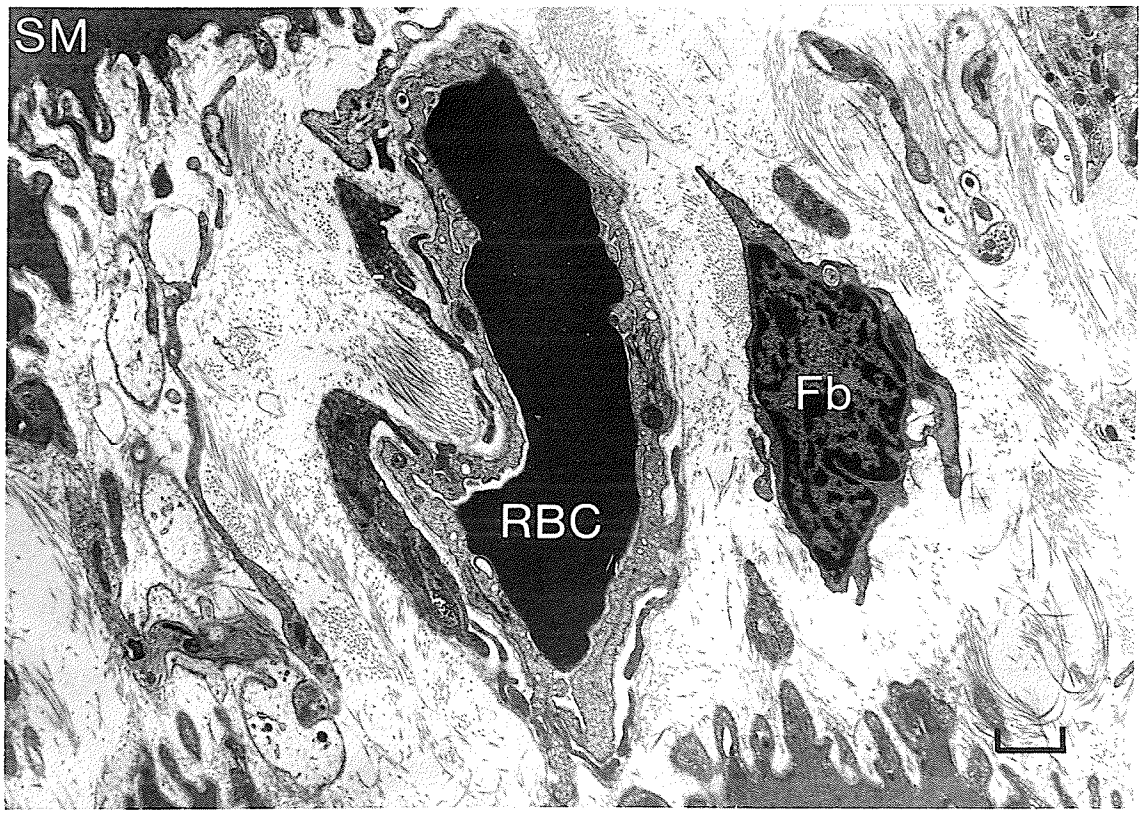
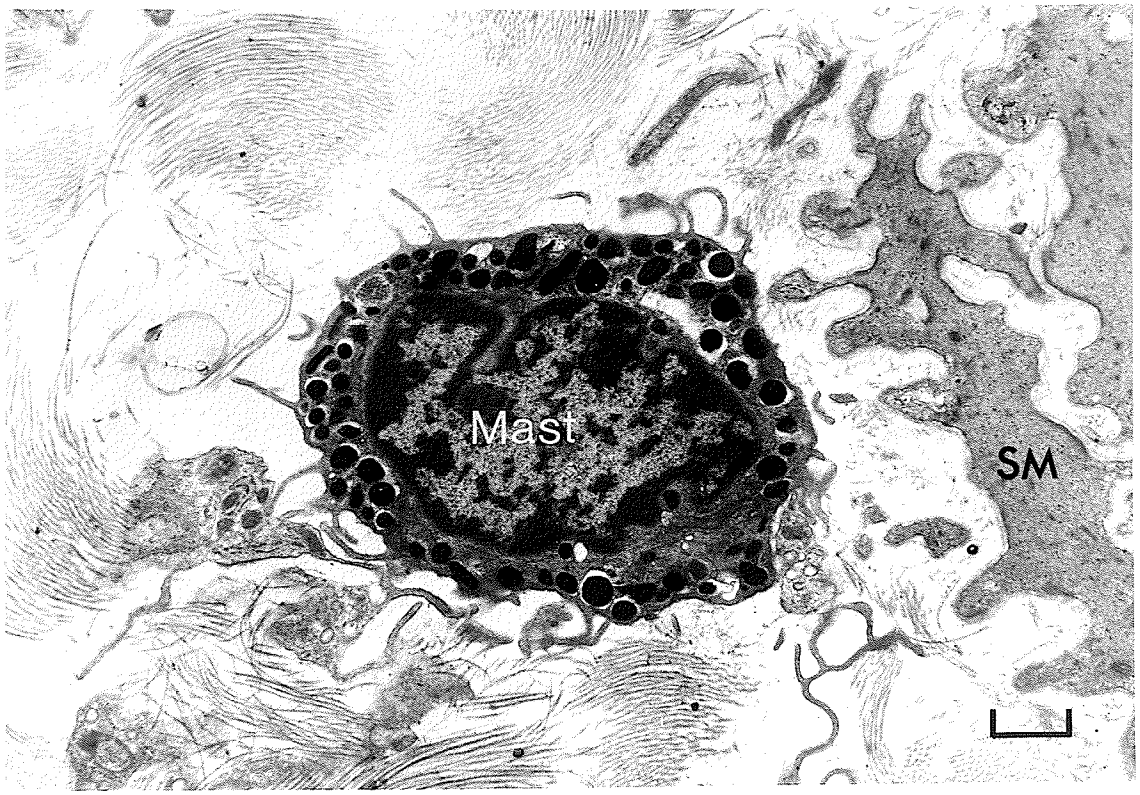

SM
Fb
RBC

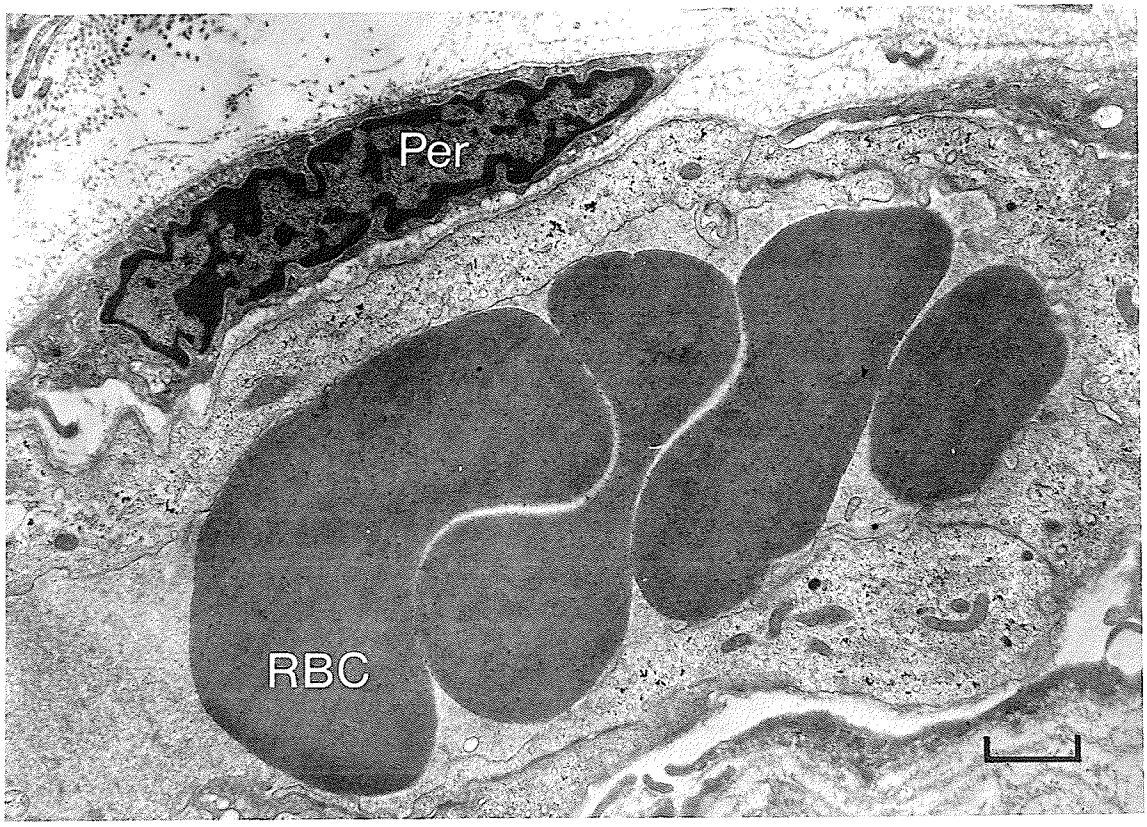
FIGURE 25A. Upper figure

Typical mast cell (MAST) seen in relation to neighbouring smooth muscle cells (SM). Small microvilli can be seen to project from the mast cell. Most secretory granules seen are extremely dense, although a few less dense, immature granules can be observed. Portions of nearby fibroblasts can be seen in close relation to the mast cell.

FIGURE 25B. Lower figure

An example of the type of blood vessels seen in tracheal smooth muscle. A number of red blood cells (RBC) can be seen within the lumen of the vessel. Note the presence of a pericyte (Per) situated outside the endothelial lining of the blood vessel. It has been suggested that pericytes are contractile and can play a role in the regulation of the size of the lumen.


Mast
SM


Per
RBC

B. Quantitative Morphology

Figure 26 is a photograph of a montage assembled from micrographs taken from a portion of longitudinally sectioned muscle fibers. In all of the prints used for quantification purposes only montages of cross sectioned fibers were used and over 200 cells were measured. Approximately 7000 individual measurements were made.

As mentioned in the section Material and Methods, the entire cell circumference, individual lengths of the membrane involved in dense bands, caveolae, and intermediate junctions were measured and recorded. The average length of membrane occupied by these structures is listed in Table 2. It was observed that certain dense bands were either in part, or entirely involved in the formation of an intermediate junction. In most cases the junctional area did not involve the entire length of the dense band. Table 2 also indicates the mean ratio of junction size to the length of the dense band involved.

1. Dense Bands

From qualitative observations of longitudinal and cross-sectioned muscle fibers, the amount of membrane involved in dense band material appeared to increase towards the ends of the muscle fibers. This observation was previously reported by Gabella, (1977). This finding suggested that when observing cross-sections of muscle fibers, cell profiles which exhibited large portions of the membrane involved with dense band material with respect to the cell circumference would be cells that had been sectioned near their ends. Similarly, cells with small portions of their membrane involved with dense bands in proportion to their circumference, would represent cells sectioned

TABLE 2

Mean Value for Individual Dense Band Lengths

0.413 um. ± 0.005 S.E.

± 0.268 S.D.

Mean Value for Intermediate Junction Lengths

0.259 um. ± 0.006 S.E.

0.134 S.D.

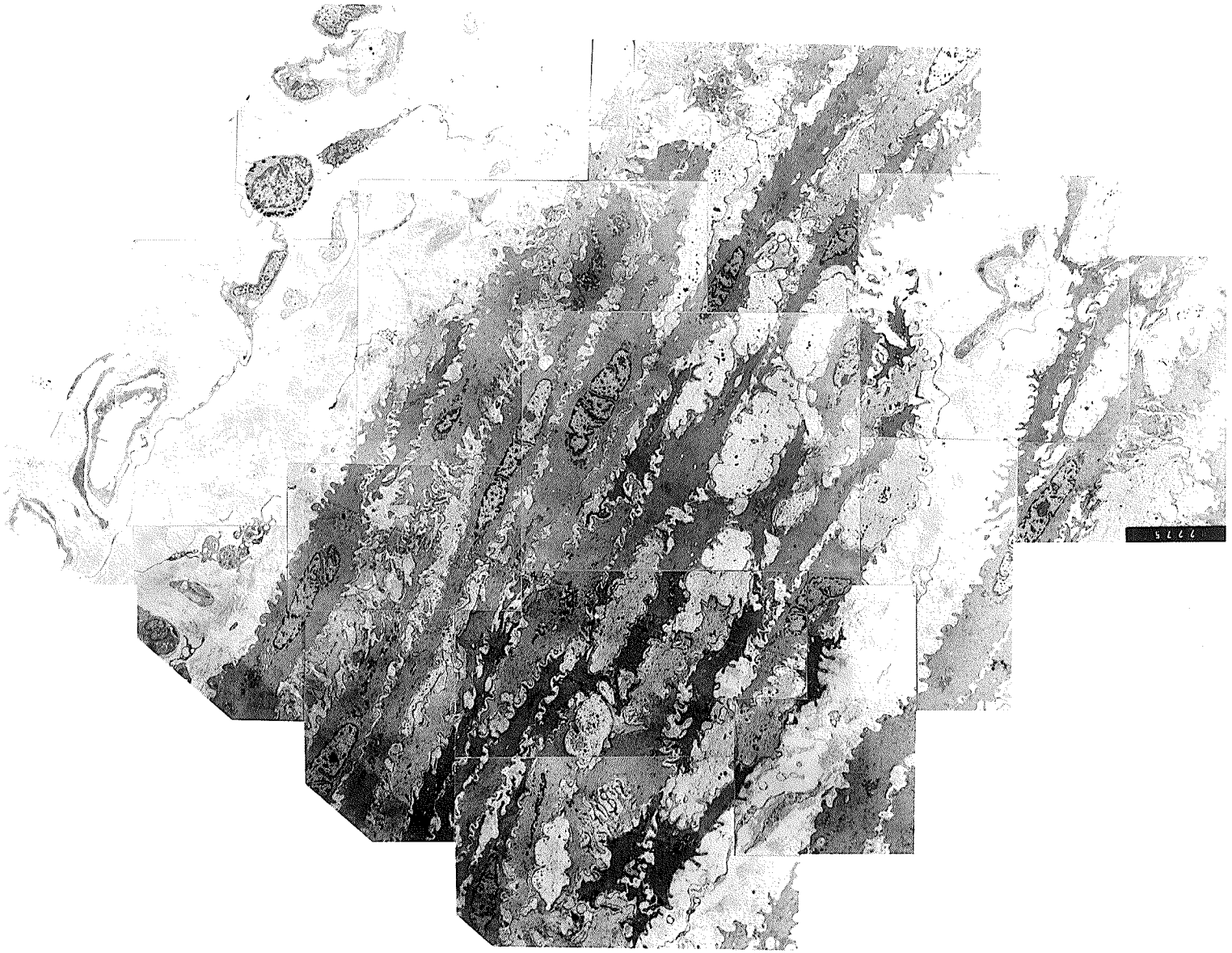
Mean Value of Junction to Dense Band Ratio

0.685 ± 0.014 S.E.

0.308 S.D.

FIGURE 26.

This plate represents a photograph of a montage constructed from a series of micrographs taken from a portion of longitudinally sectioned muscle. The muscle was in a contracted state during fixation. Masts cells, capillaries, and fibroblasts can readily be seen lying in the extracellular space alongside the muscle bundles. (X 1,920).



near their centers or at the level of the nucleus. In other words cells with high total dense band to circumference ratios would indicate cells sectioned near their ends, and cells with low total dense band to circumference ratios would represent cells sectioned near their centers which would generally show a nucleus. Therefore, average dense band to circumference ratios for each cell were made by dividing the sum of the values of the lengths of all the dense bands areas measured in a given cell by the circumference of that cell. These ratios were then plotted as a frequency histogram ranging from 0.0001to 0.4500 at intervals of 0.025. It should be remembered that the cells used for measurement purposes were taken from randomly chosen squares of grids from four animals. The histogram indicates the number of cells which belonged to a particular interval. It also indicates that the majority of cells measured fell within the .1250 to .4000 dense band to circumference ratio. None of the cells used for quantitation fell outside the .5000 to 1.0000 range. However, it was observed that cells near their ends started to subdivide and branch into small finger like projections which contained an extremely dense cytoplasm. The cross sections of these small projections appeared to be surrounded by membranes totally involved with dense bands. It is therefore probable that if the random sample of cells measured had included cells which were sectioned near their ends, and perhaps the small finger-like projections of the cell (designating these small profiles as an individual cross section of a cell), the histogram would have been extended to include ratios in excess of 0.5000 to 1.0000. It should be noted that because of the extreme density of the cytoplasm in

these small projections at the ends of the cells, it was somewhat difficult to clearly define the outline of the incrustation of electron dense material which make up the dense band material at the level of the membrane. As an estimate, it can be said that the majority of cells measured were sectioned approximately between the center (at the level of the nucleus) and the finer specializations at the ends of the cell. To recapitulate, the dense band/circumference ratio for every cell was determined. This resulted in a wide range of ratios. These ratios were then grouped into different intervals. Each interval corresponded to a particular range. Because of the observed increasing regions of dense bands towards the ends of the cells, higher ratios or intervals would indicate cells sectioned nearer their ends. It was reasoned that these intervals when placed in increasing order would in effect represent a longitudinal profile of the cell, starting from the midpoint towards its end. In essence, these randomly chosen cells were assigned a position along the length of a cell, as a result of their dense band to circumference ratio. It should be noted here, that this grouping of cells was used for all further analysis of trends or changes in distribution of the caveolae and intermediate jucntions.

2. Caveolae

For each of the cells which belonged to a particular class interval in the histogram, according to its dense-band-to-circumference ratio, sums of the total lengths of membrane involved in caveolae were calculated. These sums were then divided by the cell circumference in order to obtain a total caveolae to cell circumference ratio,

as was done with the dense band regions of the cell. The mean and standard error of the caveolae-to-circumference ratios for cells belonging to a particular interval were calculated. These mean values were then plotted according to the interval to which they belonged as shown in figure 27B. Fib. 27B indicates that as the amount of dense areas of the membrane increase, or simply as one goes towards either end of the cell, the regions of themembrane involved with caveolae decrease. This arrangement of dense areas and caveolae over the cell surface may be of mechanical importance to the cell and for this reason a rigorous study of their arrangement was undertaken.

3. Intermediate Junctions

A study of the pattern of intermediate cell-to-cell junctions was also performed. Figure 27C and 27D were plotted from cells belonging to class intervals as outlined in figure 27A from ratios of total dense band to circumference measurements. In figure 27C sums of the total lengths of membrane involved with intermediate junctions per cell were divided by the cell circumference, resulting in a junction-to-cell circumference ratio. The mean and standard error of the ratios belonging to a particular interval were calculated and plotted. The slope of the graph indicates at a statistically significant level that a larger portion of the cell membrane is involved in intermediate junctions towards the ends of the cell.

Figure 27D is a graph derived from sums of total junctional lengths divided by total dense band lengths per cell. The mean and standard error of the ratios obtained from cells belonging to each interval were plotted. This graph indicates a lower junction-

to-dense band ratio towards the ends of the cell. Figure 26 would tend to suggest the opposite, however it should be remembered that figure 27C represents the junctional length to circumference ratio. Therefore, although more of the total membrane of the cell towards the ends is involved in junctions, a lesser porportion of the dense band areas of the membrane are involved in cell to cell coupling. Nevertheless, when considering the total junctional length to cell circumference, these results suggest that a greater degree of mechanical coupling may exist as one proceeds from the level of the nucleus or cell center towards either end. It is important to mention at this point that several cells observed near their ends did not necessarily form junctions of the type under discusison, in fact many of the small cell projections displayed no junctions at all in spite of an almost entire or complete membrane circumference involved with dense band material. In several cases these cell projections were some distance away from other cells and their finger-like projections and therefore did not form junctions. In addition it was observed that the basement membrane around these projections appeared especially thick, and was closely associated with elastin and elastic filaments. This organization could serve a junctional function. Gabella (1977) has reported a similar observation.

FIGURE 27 A.

This is a histogram of ratios derived from total dense band lengths divided by the cell circumference. The ratios were grouped according to the class interval listed. The cells belonging to each interval as determined by their dense band to circumference ratio will continue to be grouped together and used for the calculation of caveolae and intermediate junction ratios in figures 27B-D.

INTERVAL RANGES

.0000 thru .0250 = 1	.0250 thru .0500 = 2
.0500 thru .0750 = 3	.0750 thru .1000 = 4
.1000 thru .1250 = 5	.1250 thru .1500 = 6
.1500 thru .1750 = 7	.1750 thru .2000 = 8
.2000 thru .2250 = 9	.2250 thru .2500 = 10
.2500 thru .2750 = 11	.2750 thru .3000 = 12
.3000 thru .3250 = 13	.3250 thru .3500 = 14
.3500 thru .3750 = 15	.3750 thru .4000 = 16
.4000 thru .4250 = 17	.4250 thru .4500 = 18
.4500 thru .4750 = 19	.4750 thru .5000 = 20

The number at the top of each bar of the histogram indicates the number of cells belonging to that particular interval.

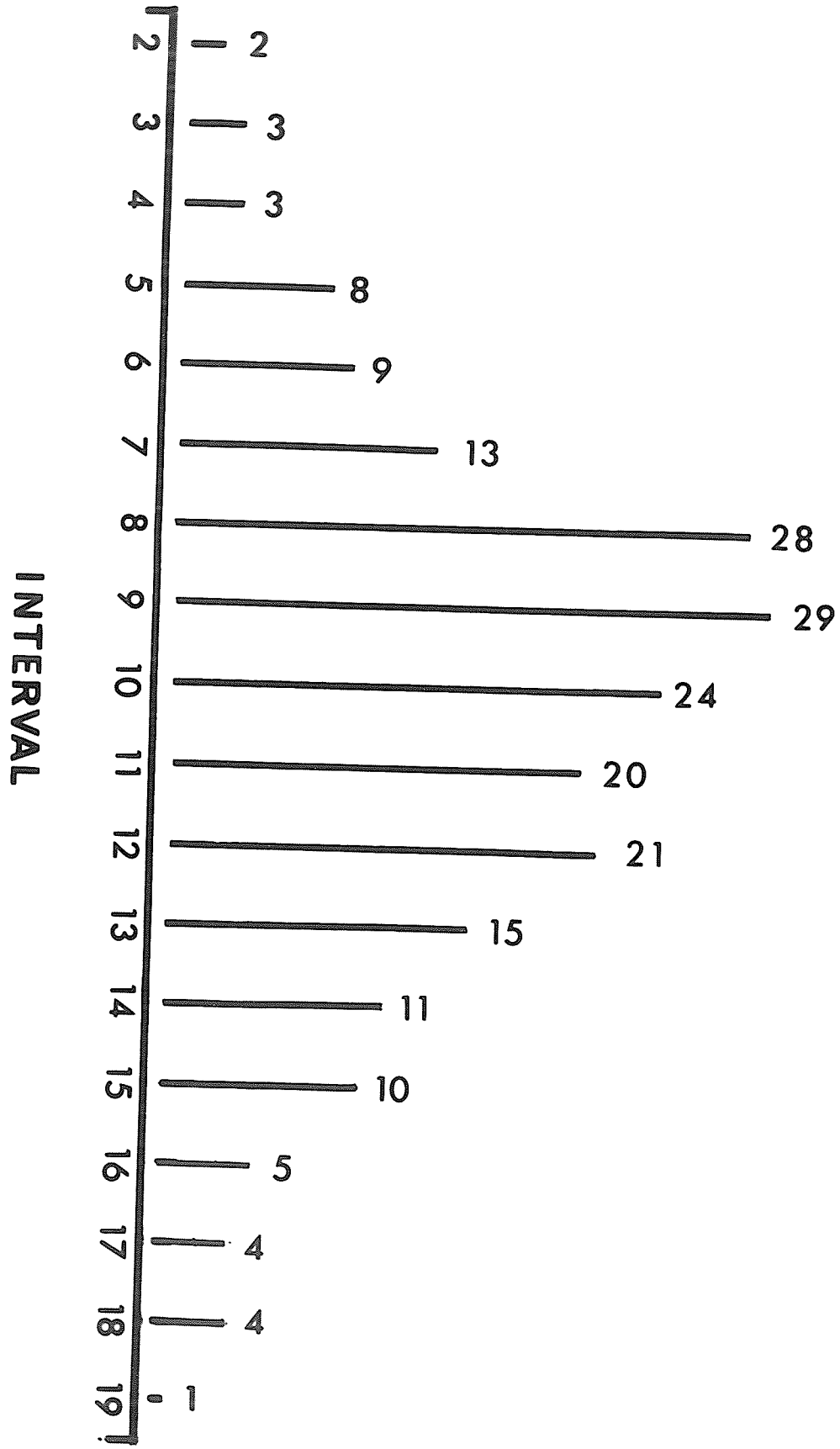

INTERVAL
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
2
3
3
8
9
13
28
29
24
20
21
15
11
10
5
4
4
1

FIGURE 27B.

This graph is a plot of the total caveolae to circumference ratio (Caveolae/Circumference Ratio) for those cells belonging to a particular interval. The mean and standard error values are indicated.

$$\text{Caveolae/Circumference Ratio} = \frac{C_1 + C_2 + C_3 \cdots}{\text{Circumference}}$$ (See Schematic 2)

The linear regression equation fitted to the data is given by:

Cav/Cir. = 0.384 - 0.010 (DB/Cir.)

The fit was highly significant ($P < 0.01$) and the slope value (-0.010) was also significantly different from zero ($P < 0.05$).

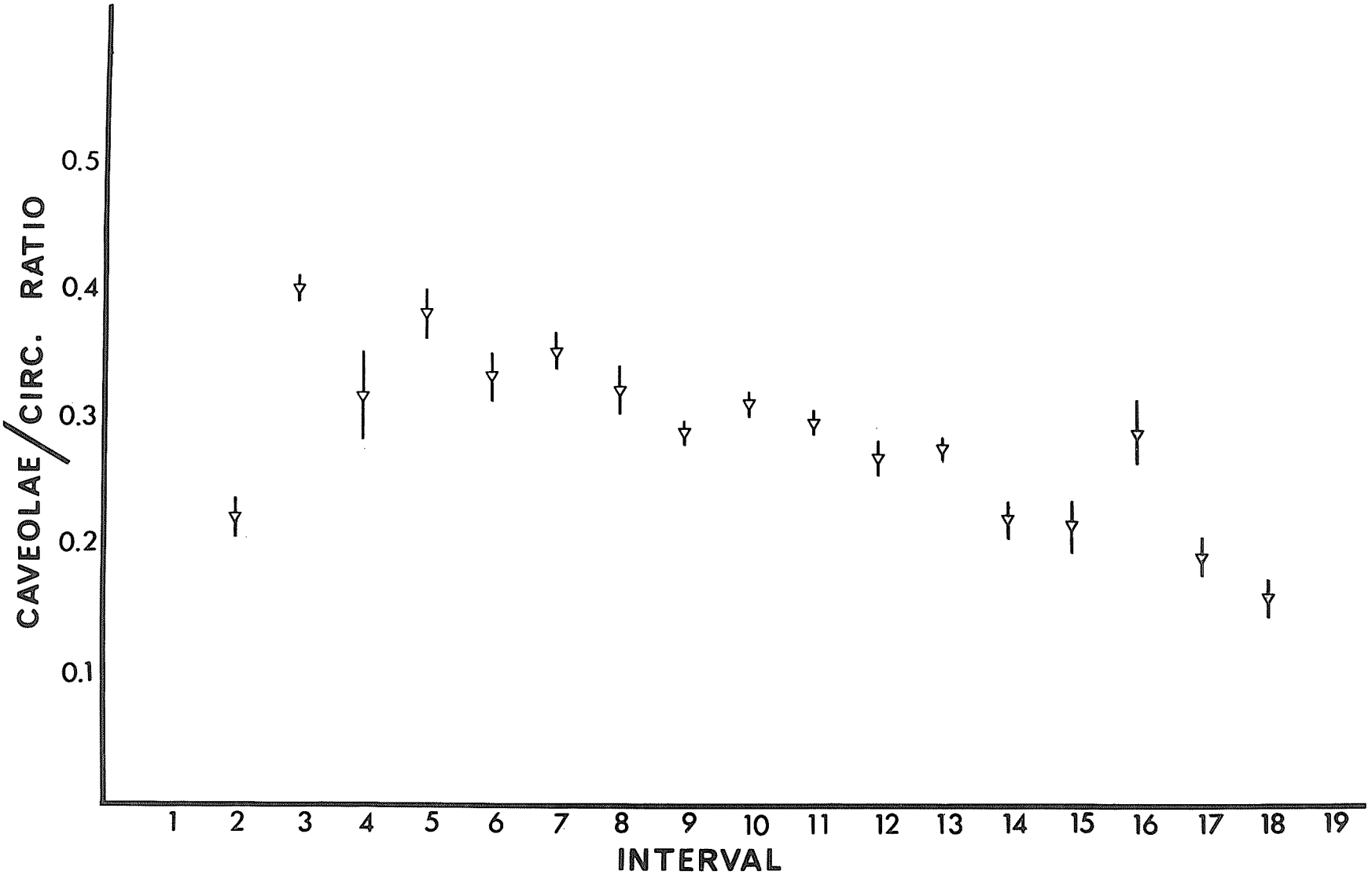

CAVEOLAE/CIRC. RATIO
0.5
0.4
0.3
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
INTERVAL

FIGURE 27C.

This is a graph of the mean and standard error of the ratios derived from the total membrane lengths involved in intermediate junctions divided by the cell circumference, for all cells belonging to a particular interval.

$$\text{Intermediate Junction/Circumference Ratio} = \frac{J_1 + J_2 + J_3 \ldots}{\text{Circumference}}$$

The linear regression equation fitted to the data is given by:

Int. Junct./Cir. = 0.006 + 0.002 (DB./Cir.)

The fit was highly significant ($P < 0.01$) and the slope value (0.002) was also significantly different from zero ($P < 0.06$).

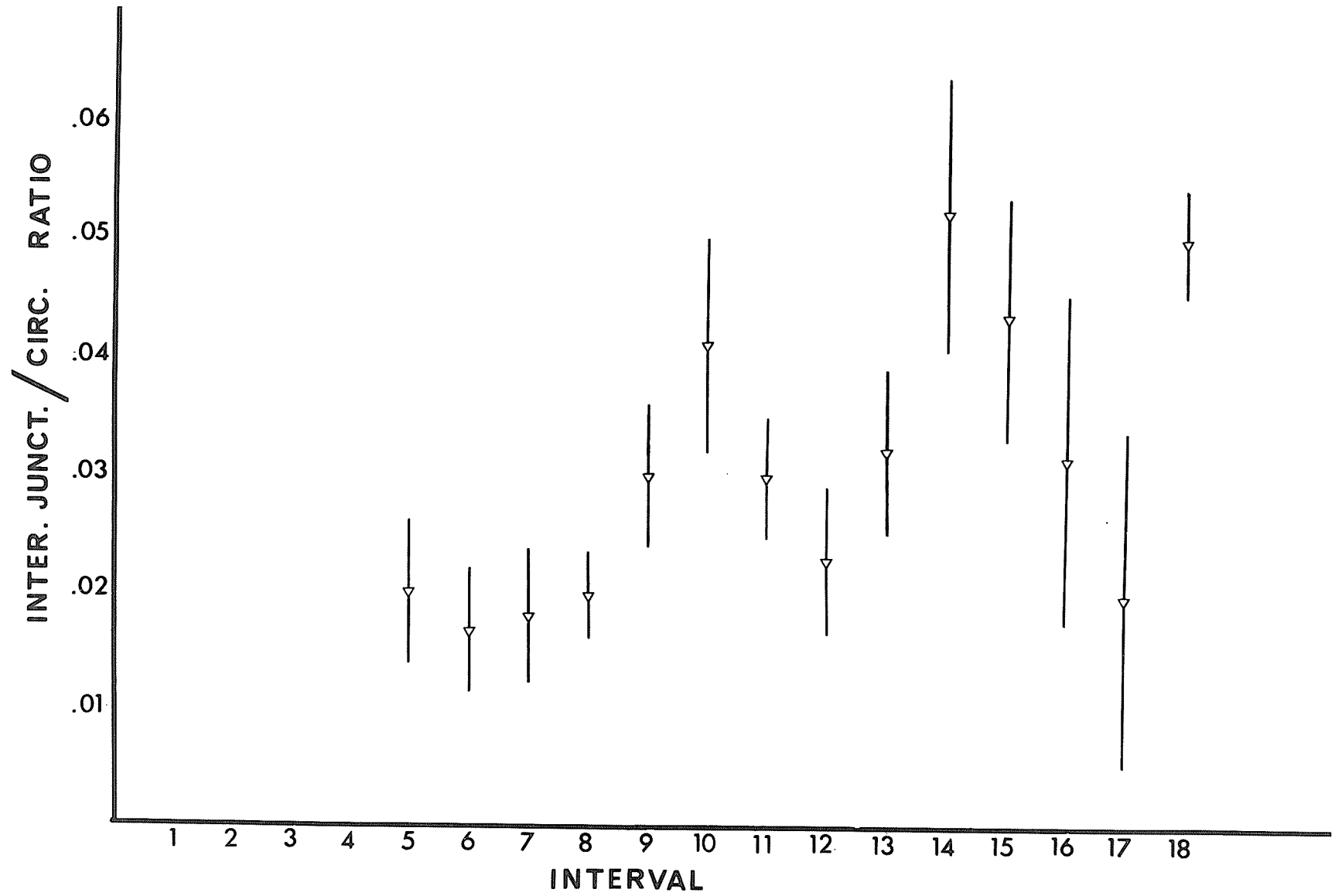

INTER. JUNCT. / CIRC. RATIO
.06
.05
.04
.03
.02
.01
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
INTERVAL

FIGURE 27D.

This is a graph of the mean and standard error of the ratios determined from the total membrane length involved in intermediate junctions divided by the total membrane lengths involved in dense bands per cell, for all cells belonging to a particular interval.

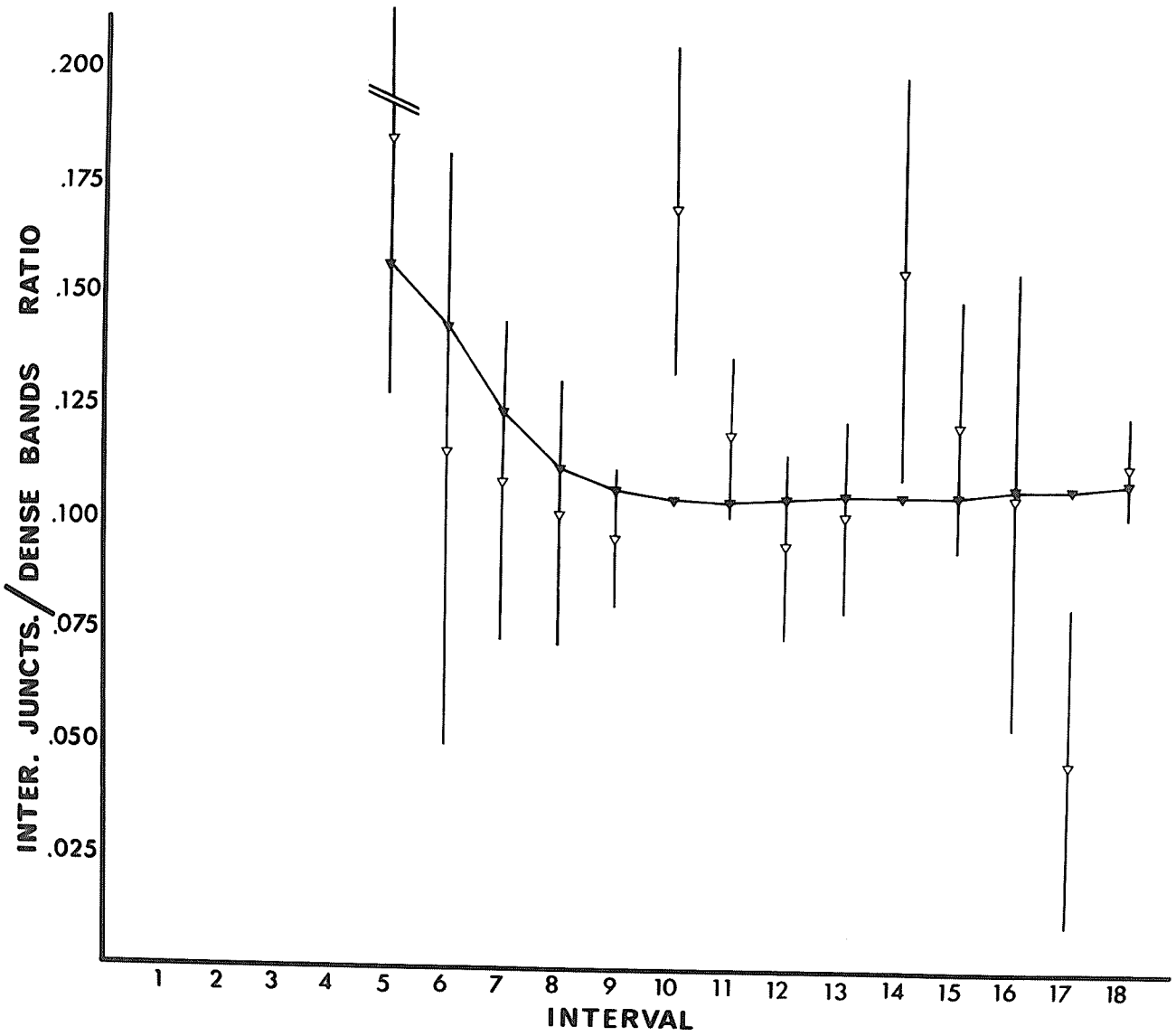
$$\text{Intermediate Junction/Dense Band Ratio} = \frac{J_1 + J_2 + J_3 \ldots}{DB_1 + DB_2 + DB_3 \ldots}$$

The Int/DB ratio was treated as a dependent variable (Y) and for purposes of optimal curve fitting, transformed to its square root. The class intervals were treated as the independent variable and transformed to their reciprocals.

The data can be described by the polynomial function:

$$Y = 0.34 - 4.07X + 41.5X^2 - 110.3X^3 + 133.7X^4 - 3714.6X^5$$

The solid line in the graph is theline obtained for expected values of Y using the equation given above. The function states that increasing values of X result in decreasing values of Y. Goodness of fit of the polynomial equation to the data points was tested using an analysis of variance technique. The fit was significantly good ($P < 0.05$).


.200
.175
.150
.125
.100
.075
.050
.025
INTER. JUNCTS./DENSE BANDS RATIO
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
INTERVAL

C. Innervation - Qualitative Observations

As a general qualitative observation, the degree of innervation seen in tracheal smooth muscle appeared to be rather sparse, as the number of nerve profiles were relatively few in comparison to the large number of muscle fibers seen. Nerve profiles in cross-sections as well as longitudinal were observed. In all sections observed, with the exception of a few cases, all nerves seen in tracheal smooth muslce preparations were unmyelinated. The few myelinated nerves that were seen, were believed to be afferent fibers, probably arising from sensory receptors from the epithelium of the airways (Jones et al., 1980).

The muscle fibers gave the appearance of existing in groups or bundles, separated by collagen and other connective tissue constituents within the extracellular space. It is within this connective tissue septa, or region of the extracellular space, that these unmyelinated nerve profiles are seen. It is important to note that no nerve profiles were seen within the interior of a smooth muscle bundle.

Occasionally, an entire nerve or nerve branch was observed several microns away from the smooth muscle bundles, as seen in figures 28 and 29. These nerve branches contained several Schawnn cells and their axons. The individual Schawnn cells themselves were surrounded by connective tissue comprised of collagen, constituting the endoneurium. In most unmyelinated nerves and some small myelinated nerve fibers seen elsewhere in the body, the Schawnn cells and the enclosed nerve fibers are grouped into bundles or fascicles by another connective tissue sheath, known as the perineurium. Therefore,

in general, large nerve fibers are enclosed by connective tissue knwon as the epineurium and contain many bundles or fascicles of nerve fibers each limited by perineurium. However, this typical arrangement was rarely seen in our tracheal smooth muscle preparations.

As the large nerves extend further into the connective tissue septa, they divide and separate as seen in figure 30. At this point some of the axons often show the presence of synaptic vesicles. As the nerves proceed deeper between the muscle bundles, they continue to separate and spread, achieving closer contact with the smooth muscle cells (Fig. 31).

A closer view of separate nerve profiles can be seen in figures 32 (a & b) 33 (a & b). The nerve endings seen generally conform to the description of most unmyelinated nerves involved elsewhere. A single Schawnn cell is found to ensheath four to six axons. At the point of the fold of the Schawann cell to each axon, a mesaxon is formed. In many cases, the Schawnn cell cytoplasm appears electron-lucent. This may have been due to fixation procedures (Fig. 32a). In several of the axons, synaptic vesicles could be seen within. In addition, mitochondria are often observed inside the axons as well as within the Schawnn cell cytoplasm. Glycogen granules were also often seen in abundance in many of the axons (Fig. 32b).

Agranular vesicles 30 - 40 nm. in diameter found within the axons were termed as cholinergic and designated in the micrographs as "Ach". Figure 33a typifies the morphological appearance of these vesicles seen in several axons. In many axons, microtubules of roughly 5 - 10 nm. in diameter, as well as several neurofilaments, were frequently observed.

Nerve endings that were found in close proximity to smooth muscle fibers were also found to lie near other cells in the connective tissue septa such as mast cells and fibroblasts (Fig. 43). Figure 35 demonstrates several nerve endings lying together alongside smooth muscle fibers. In several cases, the presence of large dense core vesicles, approximately 80 to 120 nm. in diameter was noted within the axons. These vesicles were believed to be representative of adrenergic innervation. In addition, it was observed that some axons contained both cholinergic agranular vesicles as well as the large dense core vesicles, (Fig. 35). This indicated that a single neuron could release more than one type of transmitter.

Figures 36 through 39 illustrate the typical pattern of nerve terminals seen i.e. several terminals lying in the cleft between two smooth muscle bundles. In general, it was observed that of the axons that contained synaptic vesicles, the large majority of the vesicles were of the small agranular cholinergic type, whereas the large dense core vesicles were somewhat rare. Figures 34, 35, 39, 40, and 41, demonstrate a characteristic observed in terminals found lying in close proximity to the smooth muscle fibers. It was often found that the axons of these terminals were only partially ensheathed by their associated Schawnn cell, leaving portions of the axons exposed to the extracellular space. This aspect is seen quite clearly in figure 41, where agranular vesicles can be found in an axon partially devoid of its Schawnn cell sheath. It is important to note that closest nerve to muscle association was still approximately 240 nm. in distance and in many cases 1 - 2 microns away. No neuro-muscular junctions in the classical sense with close nerve terminal

to muscle cell contacts could be identified.

FIGURE 28.

A micrograph of a group of unmylinated nerve fibers found lying some distance from the smooth muscle bundles. Several Schawnn cells and their axons can be seen cut in cross-section. (X 11,290). INSET: The inset is an enlargement showing the large dense core vesicles (DCV) found inside one of the axons. (X 20,420).

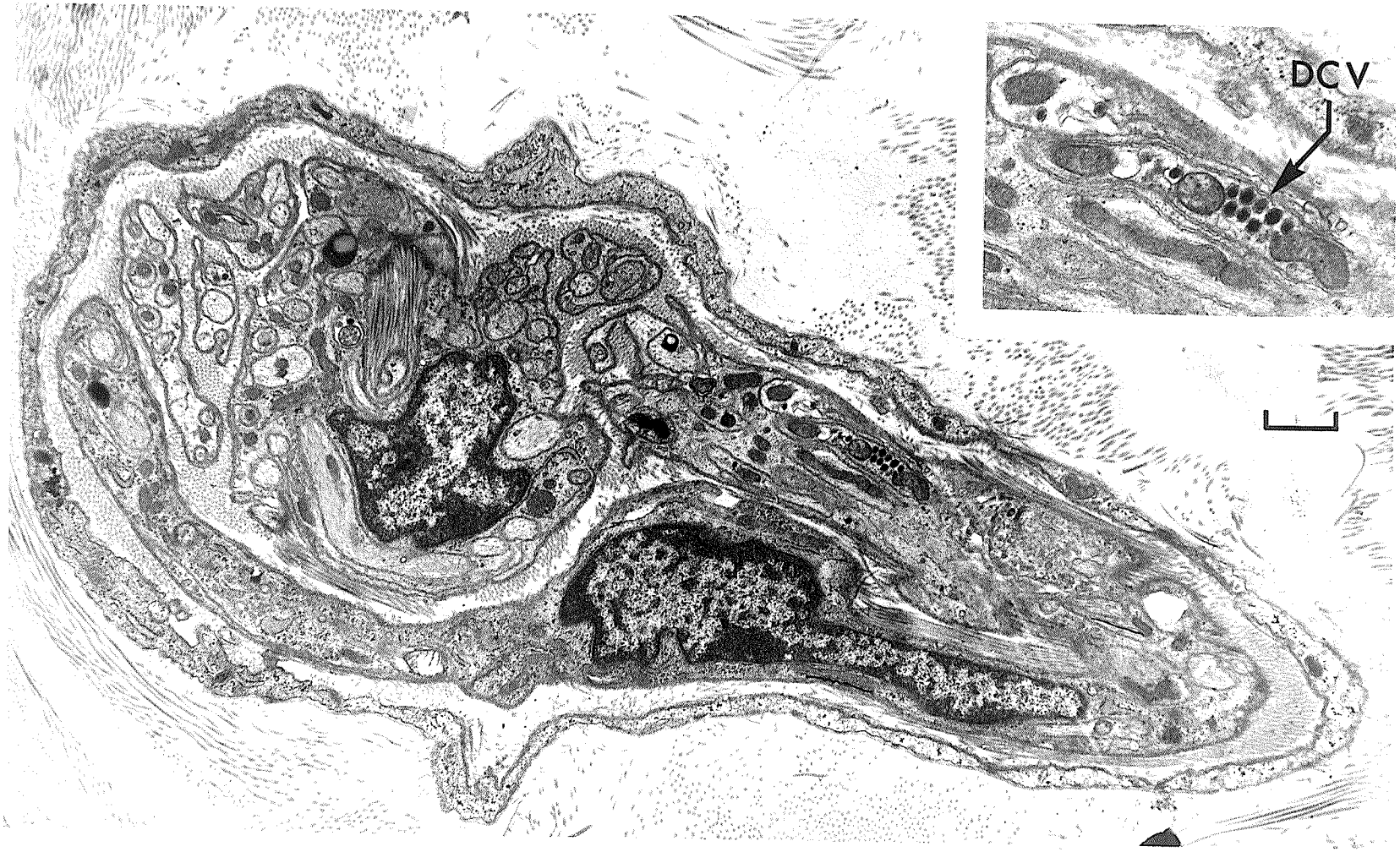

DCV

FIGURE 29.

This micrograph is a higher power magnification of figure 28. The nuclei of two Schawnn cells and their axons are seen. The connective tissue separating the Schwann cells in the form of collagen, is usually referred to as the endoneurium and can be clearly seen, although the arrangement is somewhat atypical. (X 18,410).

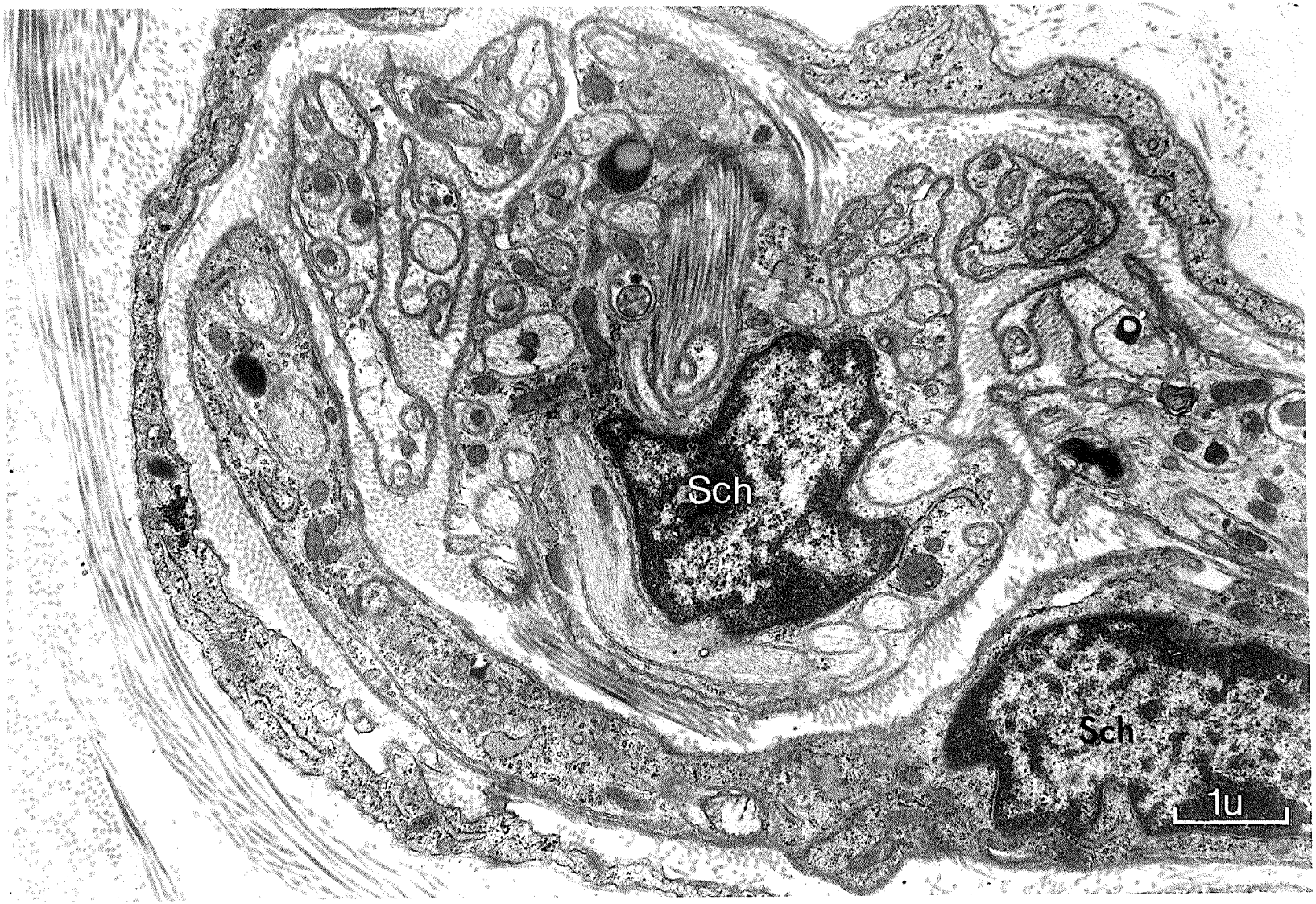

Sch
Sch
1u

FIGURE 30

A micrograph demonstrating several nerve profiles, which are often seen in bundles before they divide and separate when they reach the muscle fibers (SM). A fibroblast (Fb) can also be seen between the nerve profiles. (X 8,380).

Fb
MS
SM

FIGURE 31.

A low power micrograph illustrating the spacial relationship between nerve terminals (NT) and the smooth muscle cells (SM) as well as extracellular components such as fibroblasts (Fb) and blood vessels (BV). The blood vessel or capillary seen in this micrograph is difficult to discern because much of the lumen appears to have been lost,perhaps during fixation. The nucleus of the Schawnn cell (Sch) of one of the terminals can be seen. The muscle cells appear very contracted in this micrograph, with both light and dark cells being present. Two adjacent dark cells near the top of the micrograph demonstrate a pronounced zonula adherens or intermediate junction. The other cells also show a close relationship ot each other as many of the numerous fold in the sarcolemma between adjacent cells are complementary, perhaps indicative of good cell to cell mechanical coupling. (X 7,660).

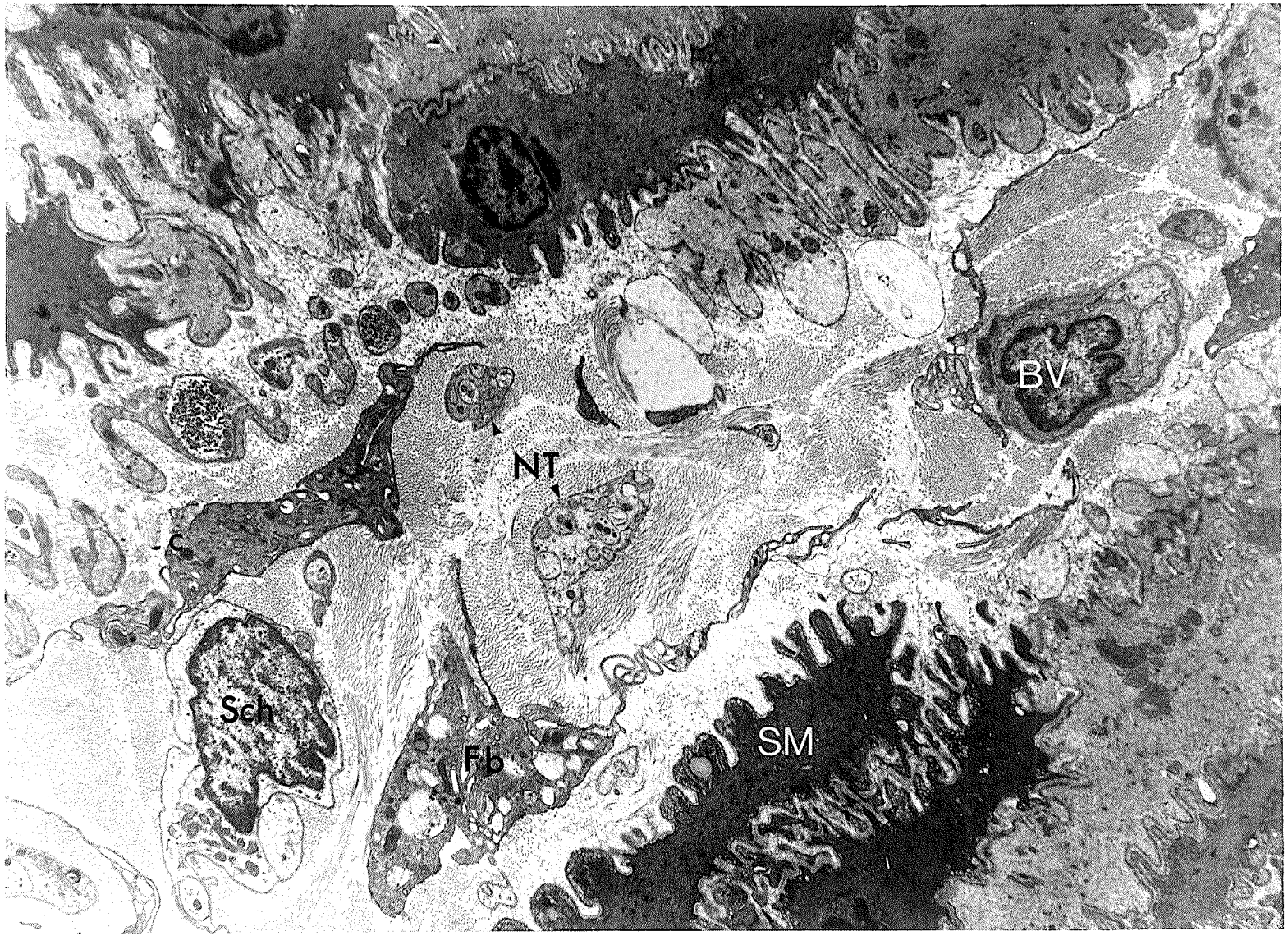
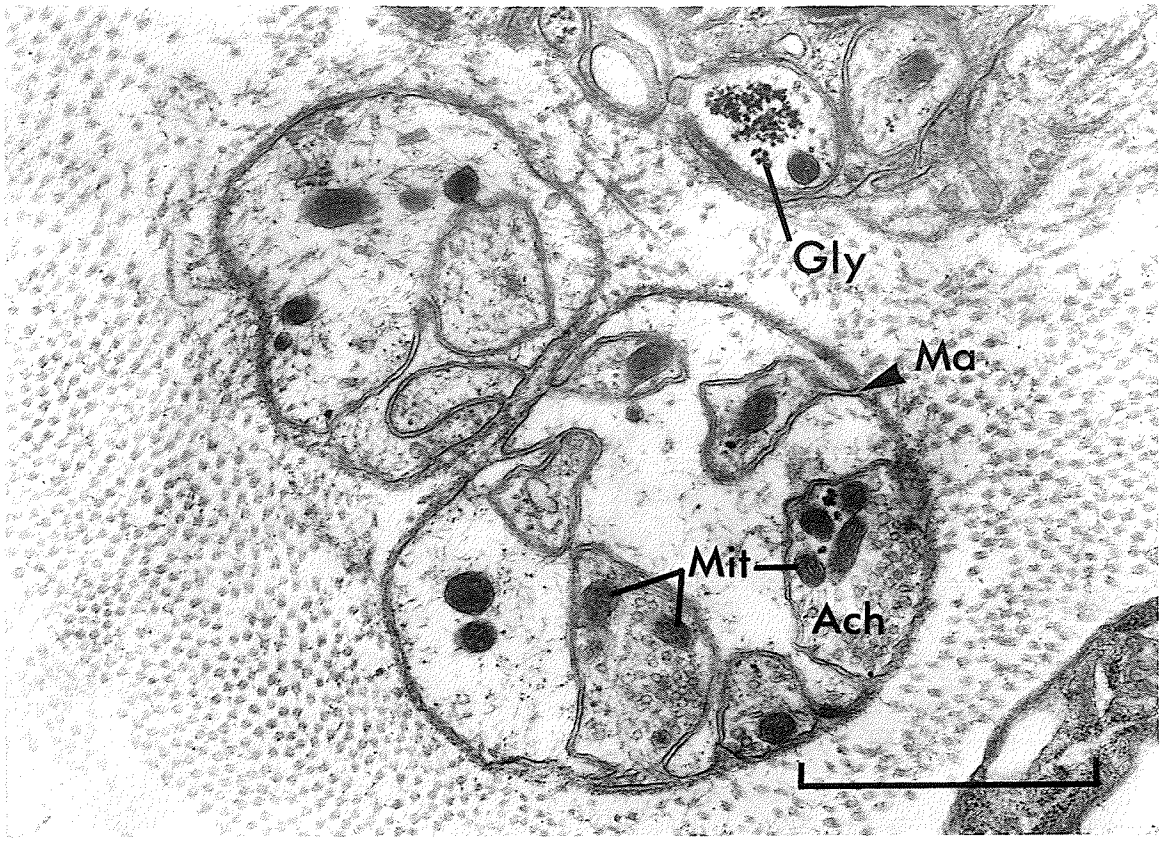

BV
NT
C
Sch
Fb
SM

FIGURE 32A. (Upper)

A high power micrograph of a typical nerve profile. The Schwann cell process is seen to surround the axons, forming a mesaxon (Ma), i.e. an invagination of the Schawnn cell plasma membrane. Cholinergic vesicles (Ach), alongwith mitochondria (Mit) and glycogen (Gly) can be found inside the axonal terminals. (X 38,300).

FIGURE 32B. (Lower)

A micrograph depicting a Schwann cell (Sch) alongwith its nucleus and several axons (Ax). Glycogen (Gly) can also be observed within one of the axons. (X 21,950).


Gly
Ma
Mit
Ach

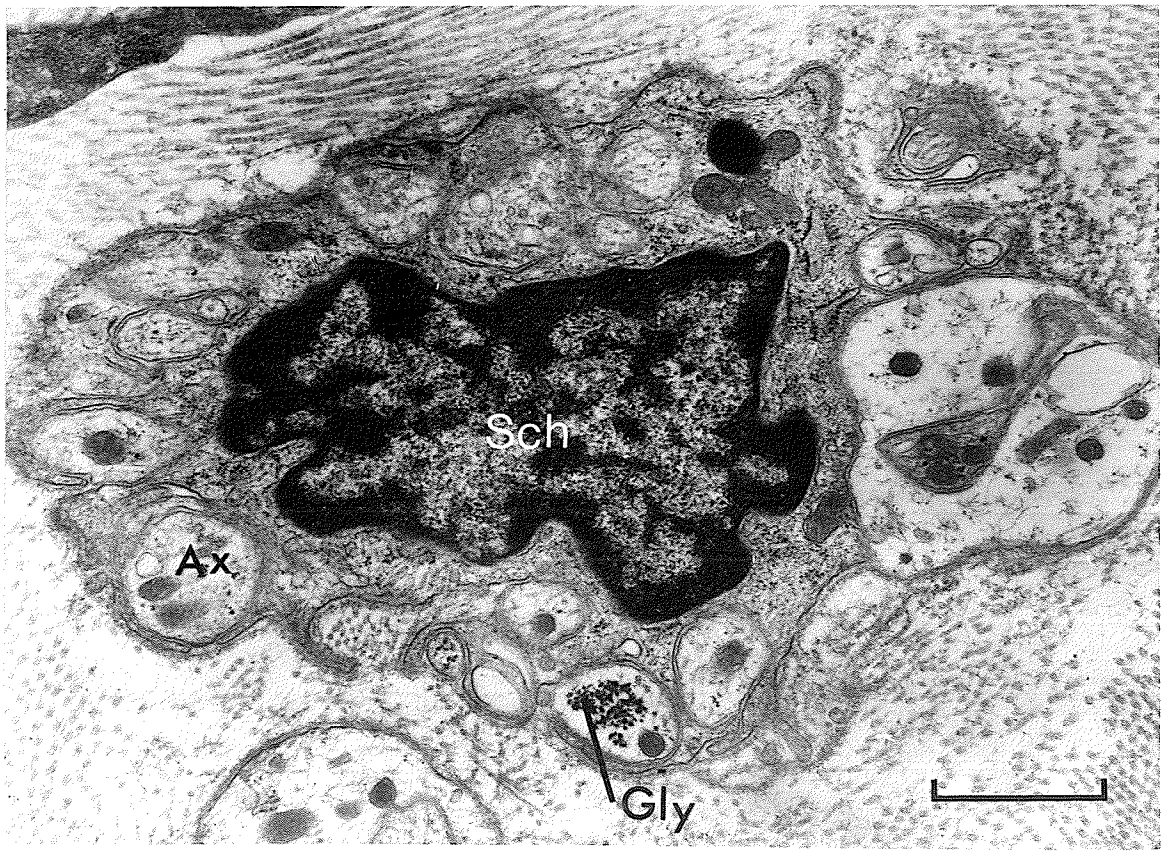

Sch
Ax
Gly

FIGURE 33A. (Upper)

A high power micrograph of a nerve terminal containing mitochondria (Mit) and several cholinergic vesicles. Note that the Schwann cell has not completely surrounded the axon at the top. (X 43,260).

FIGURE 33B. (Lower)

This micrograph demonstrates a nerve terminal, lying beside a smooth muscle fiber (SM), clearly depicting the Schwann cell (Sch) and its nucleus. Some of the axons in this terminal are seen to contain neurotubules (NF) of approximately 10 nm. in diameter. The closest distance between the nerve axon and the muscle cell appears to be roughly 750 nm. (X 27,530).

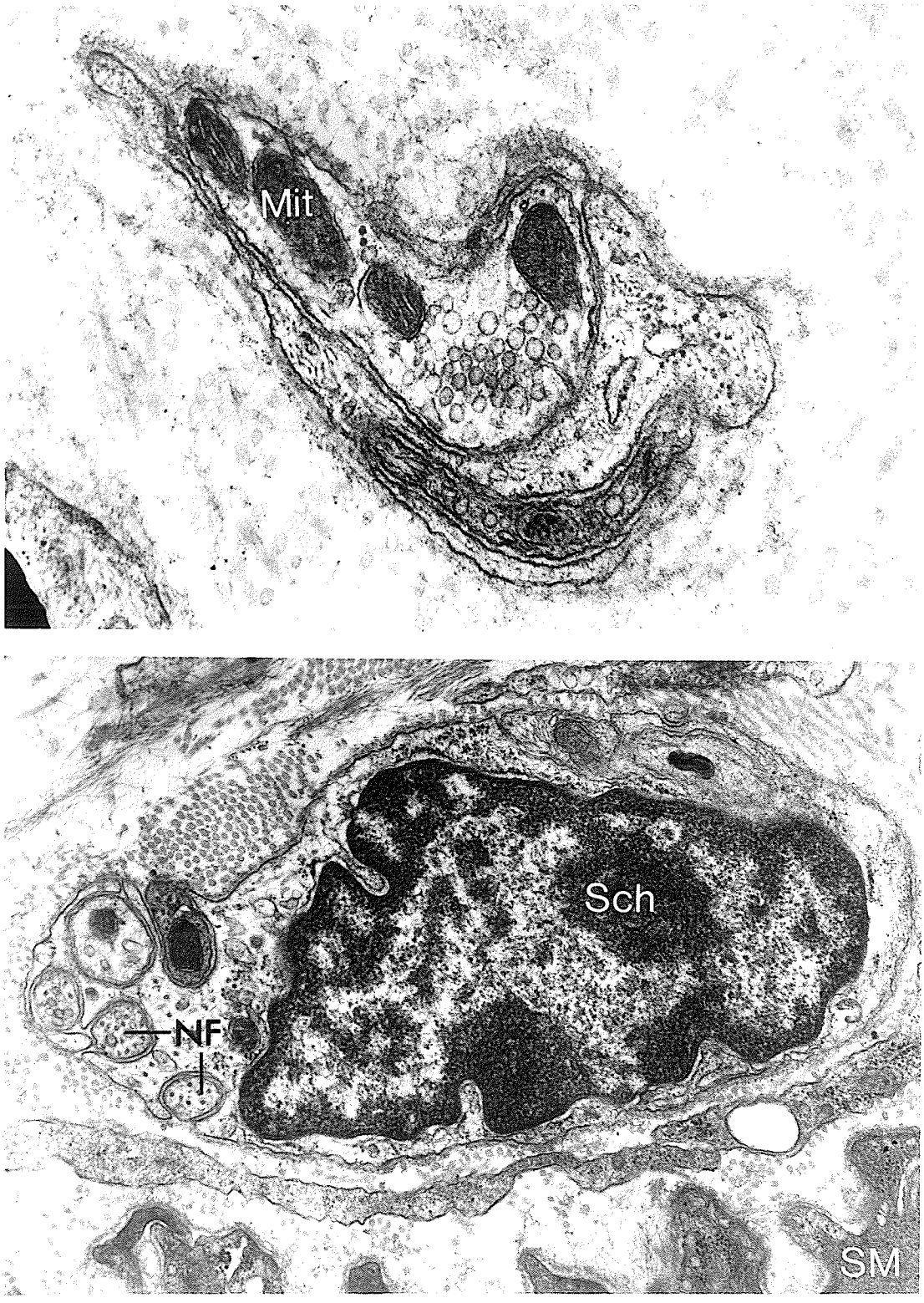

Mit
Sch
NF
SM

FIGURE 34

This micrograph demonstrates the relationship often seen of the extracellular components to the smooth muscle cells. It shows the close association of a fibroblast (Fb) and a mast cell (Mast) alongside a nerve process. The closest neuromuscular distance seen in this micrograph is approximately 240 nm. The basis for the close association of the fibroblast and mast cell seen here can only be speculated upon at the present time. The close proximity to each other tends to suggest a certain degree of intercellular communication. Note that one of the axons (Ax) encompassed by the Schwann cell (Sch) contains both small agranular vesicles alongwith a few larger granular vesicles. Parts of a smooth muscle cell (SM) can also be seen. (X 15,900).

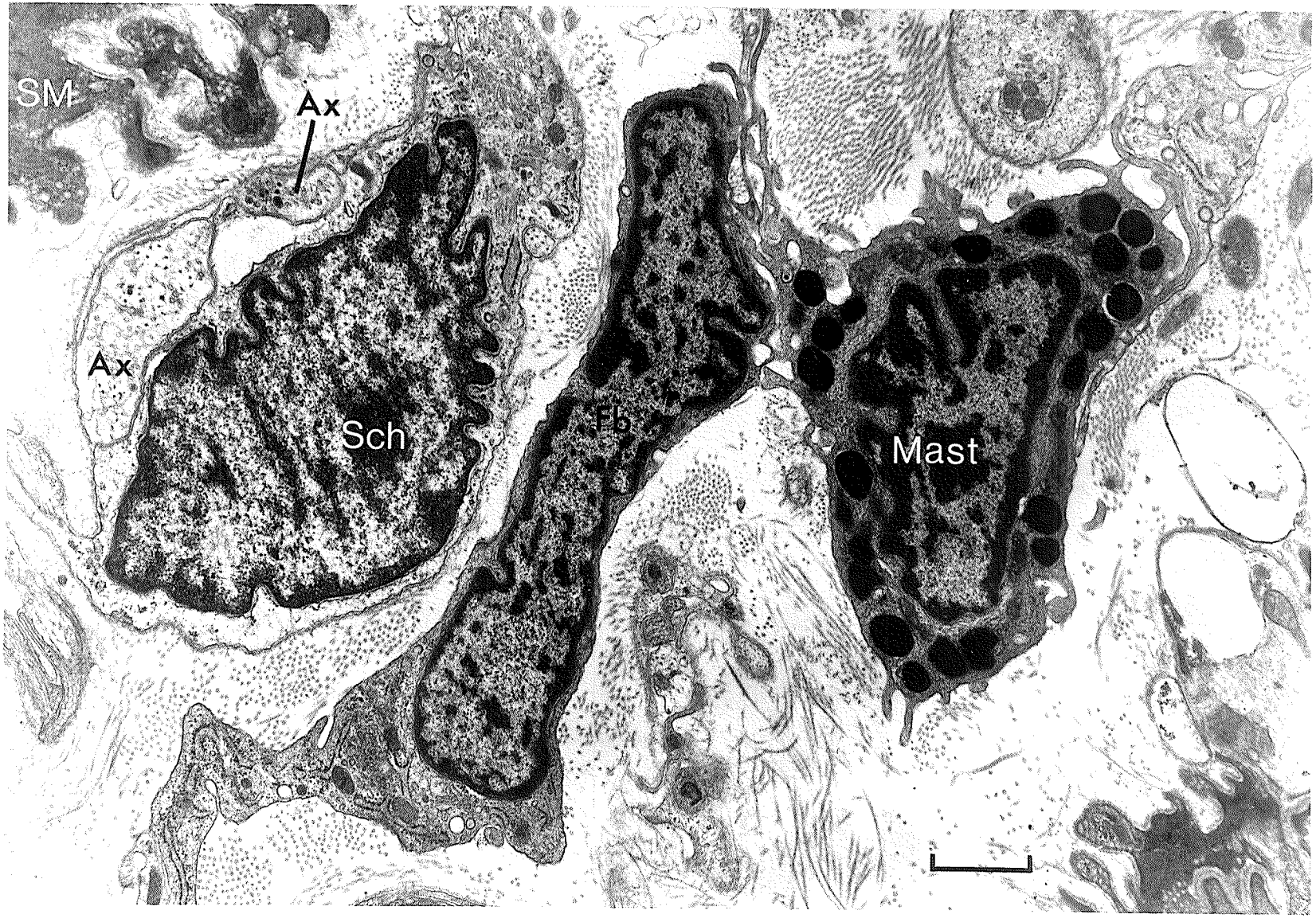

SM
Ax
Ax
Sch
Fb
Mast

FIGURE 35.

This micrograph demonstrates a series of nerve terminal encounters often seen lying alongside the muscle fibers (SM). Both cholinergic (Ach) and adrenergic (DCV) vesicles can be seen inside the axons. Note that some of the axonal terminals contain both agranular as well as the dense core vesicles. At the bottom of the micrograph, a portion of a fibroblast can be seen (Fb). (X 23,860)

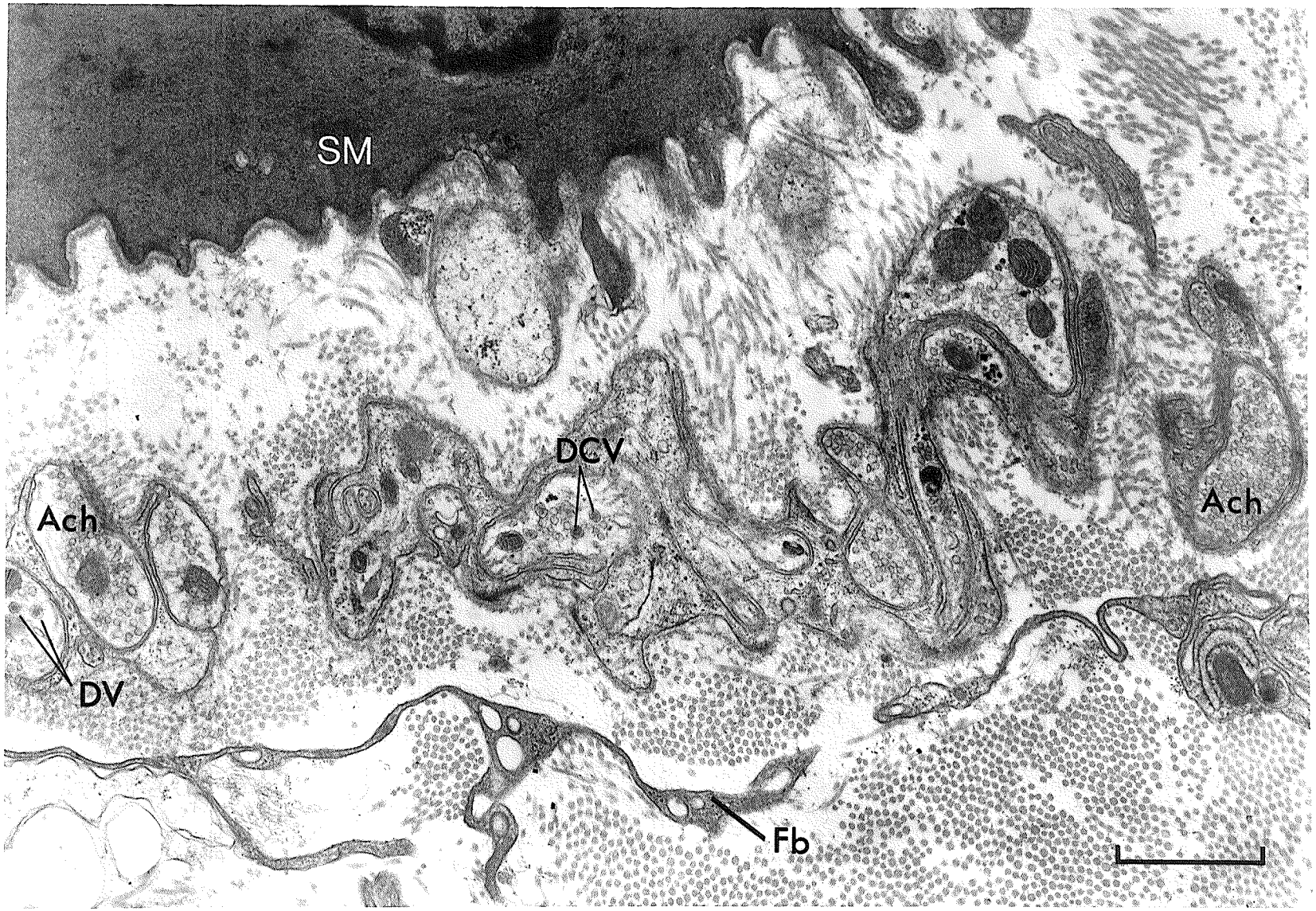

SM
DCV
Ach
Ach
DV
Fb

FIGURE 36.

In this micrograph, several nerve terminals can be seen in the extracellular space between two smooth muscle (SM) bundles. Dense core vesicles (DCV) and cholinergic vesicles (Ach) can be identified within the terminals. Fibroblasts can also be observed within the extracellular space. (X 10,940).

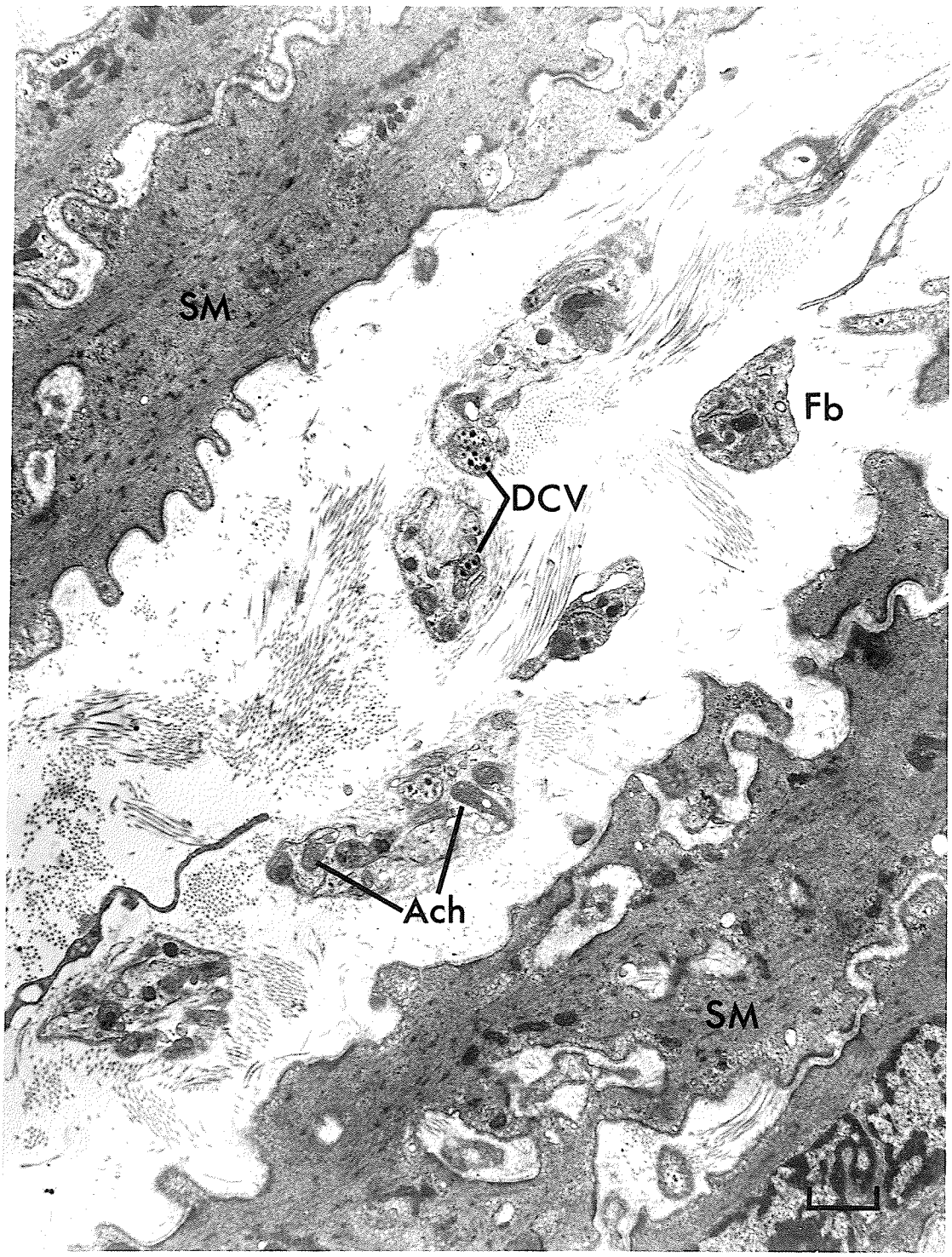

SM
Fb
DCV
Ach
SM

FIGURE 37.

Two nerve terminals are observed, located between two longitudinally sectioned muscle fiber bundles. Cholinergic (Ach) endings can be seen within one of the axons. (X 20,030).

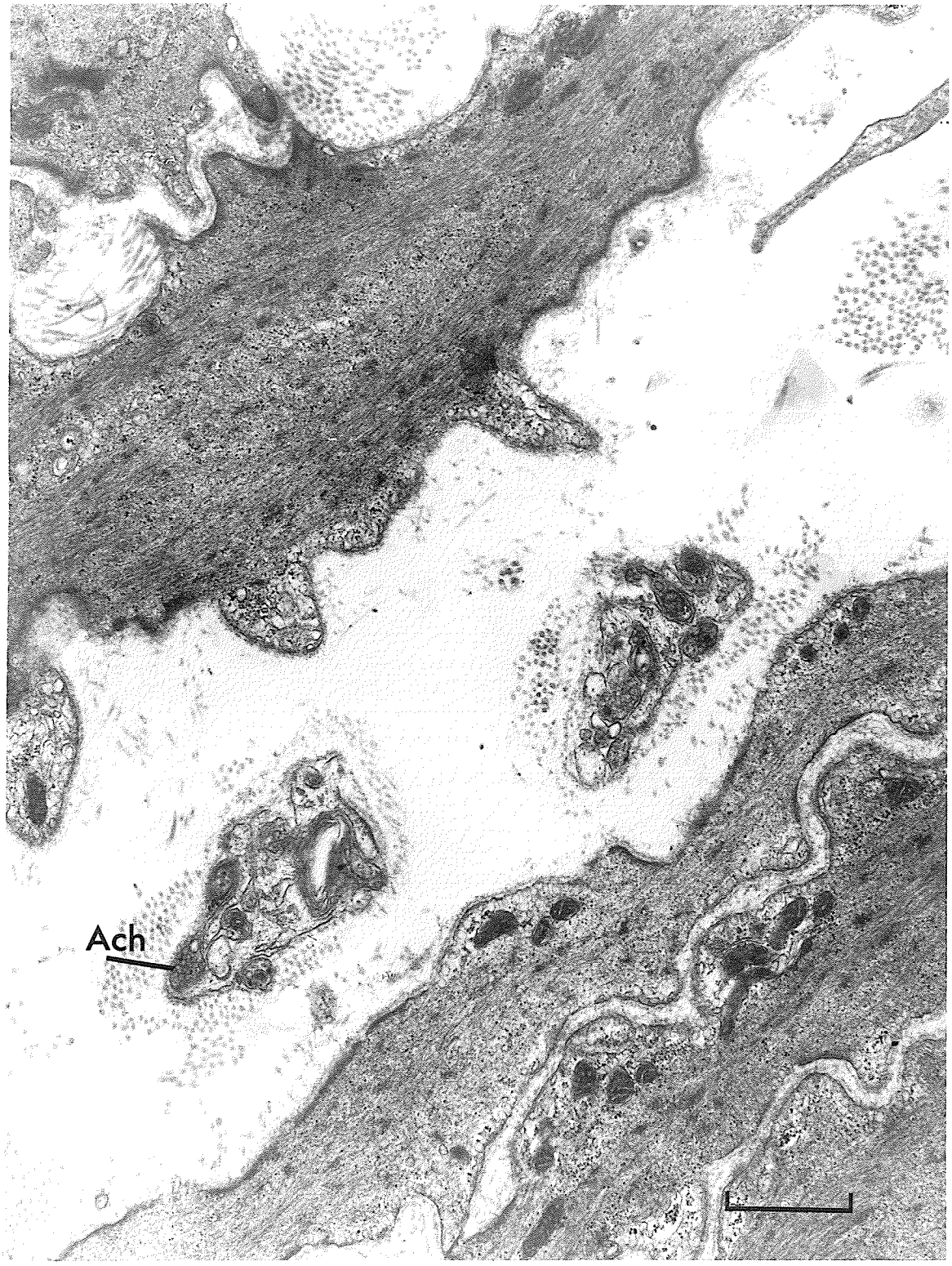

Ach

FIGURE 38.

A slightly higher power micrograph of figure 37. Note the presence of large numbers of cholinergic (Ach) endings. (X 13,830).

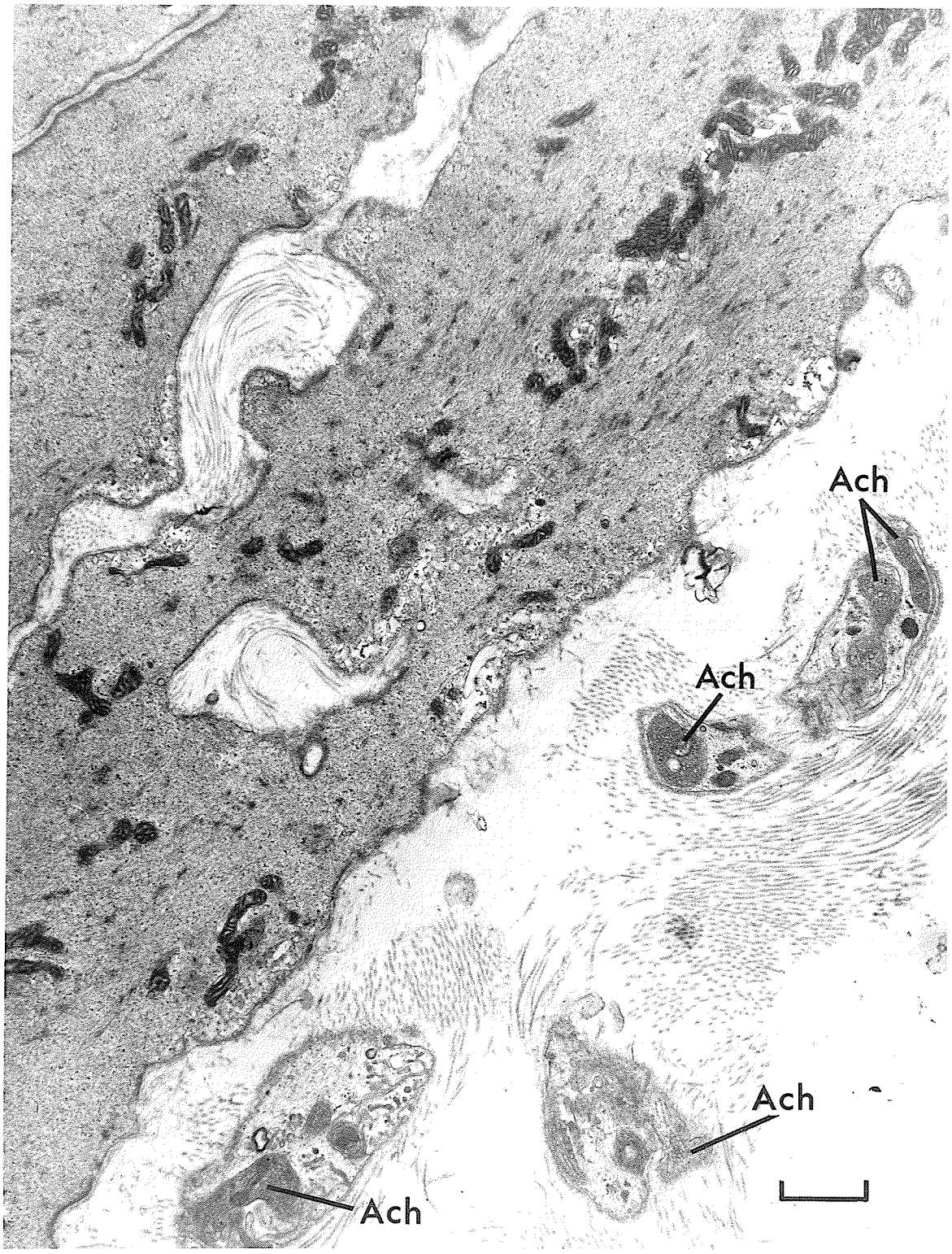

Ach
Ach
Ach
Ach

FIGURE 39.

A high power micrograph of a nerve terminal seen in figure 37 showing several dense core vesicles (DCV). These vesicles exhibit a halo around the dense core, surrounded by a membrane. Numerous cholinergic (Ach) vesicles can also be observed. The muscle cells at the top of the micrograph depict the presence of a gap junction (GJ). At the bottom of the picture, a portion of a fibroblast (Fb) can also be observed. (X 28,980).

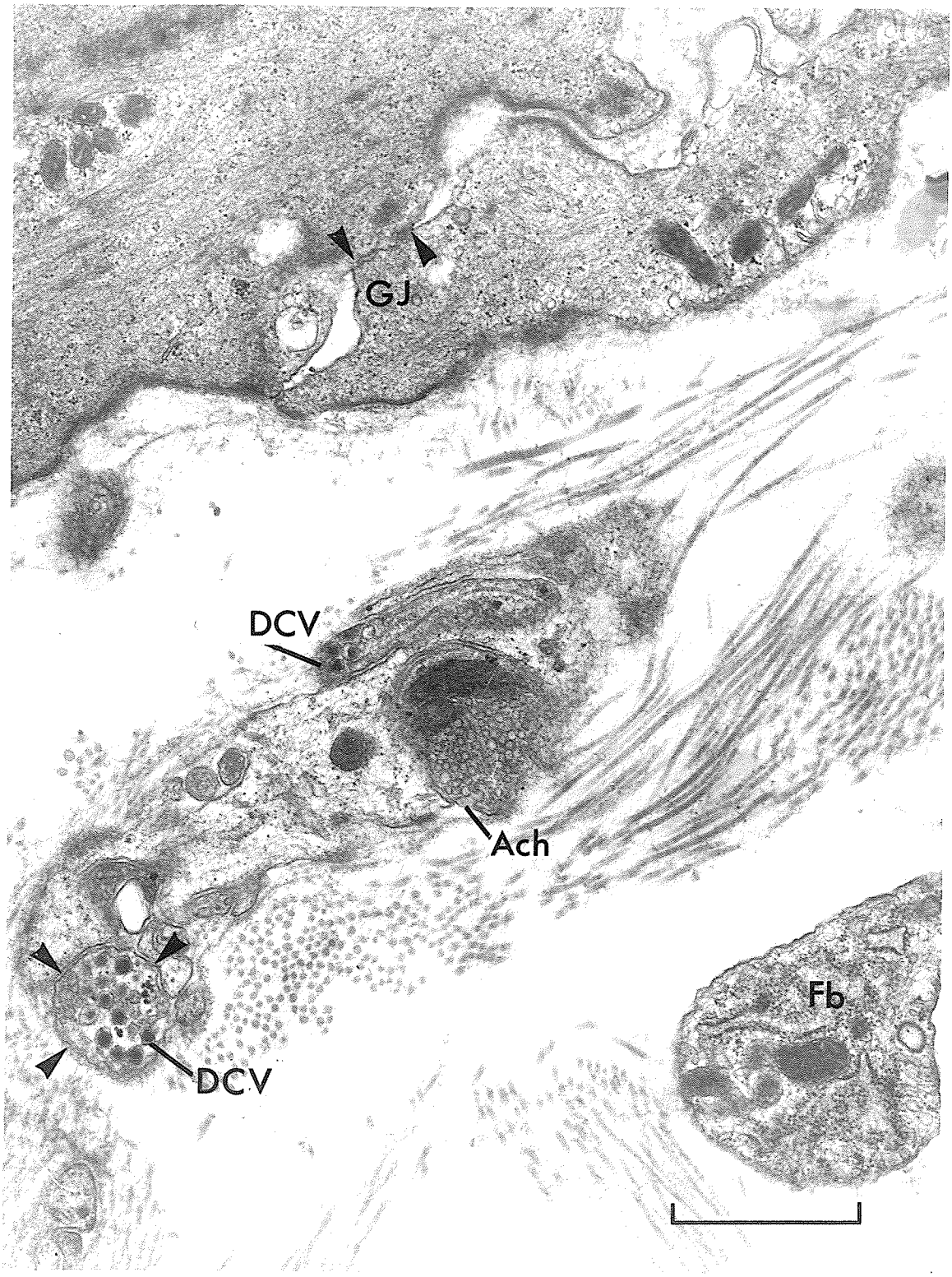

GJ
DCV
Ach
DCV
Fb

FIGURE 40.

A micrograph depicting a nerve terminal (NT) in fairly close opposition to a smooth muscle cell. Two mast cells which are almost entirely devoid of vesicles can be seen at the lower portion of the micrograph. Some of the granules are filled with dark, homogeneous material, some with a light gray material and some are empty. These, have been associated with different states of activity of cellular secretion. (X 18,240).

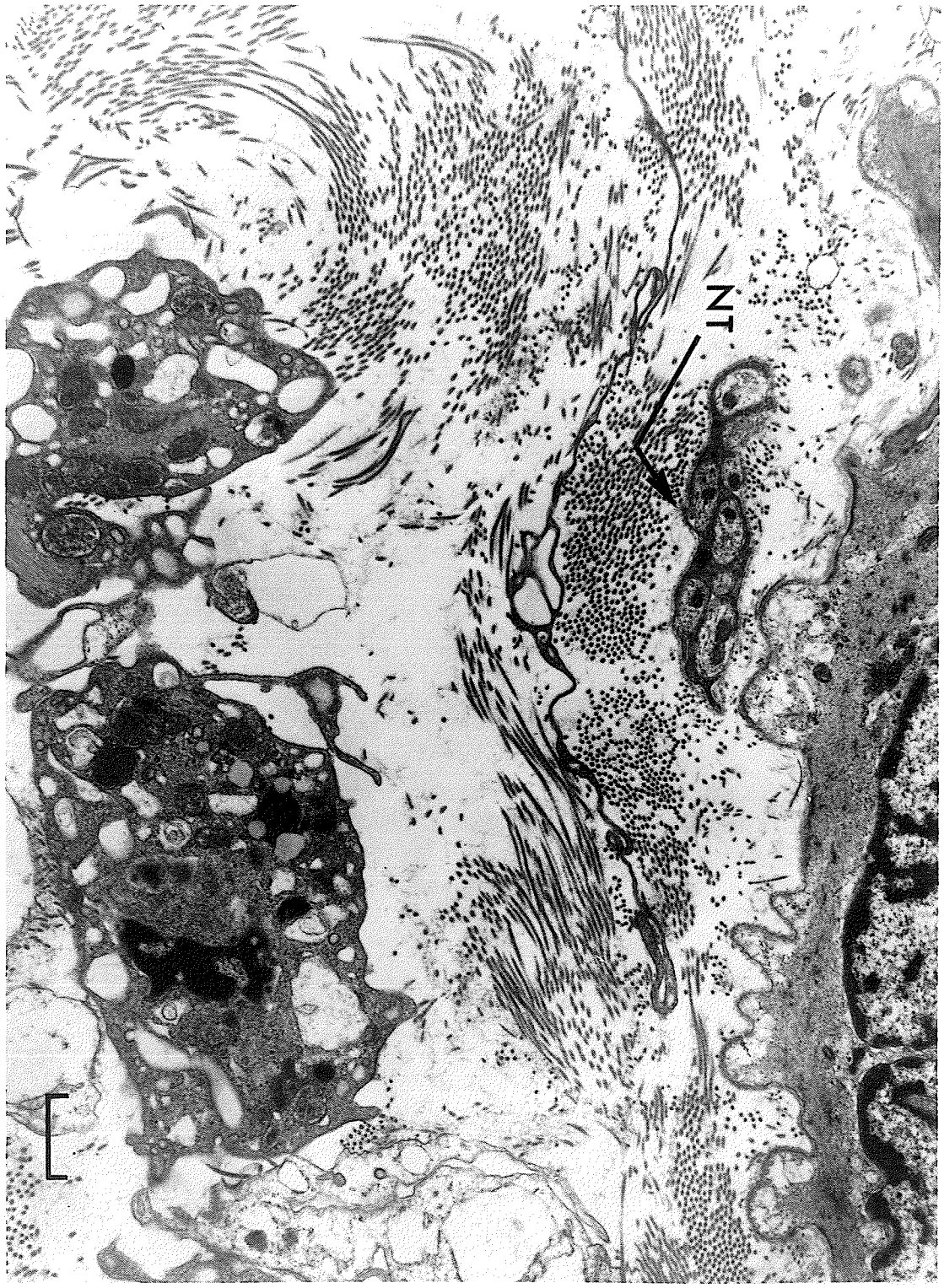
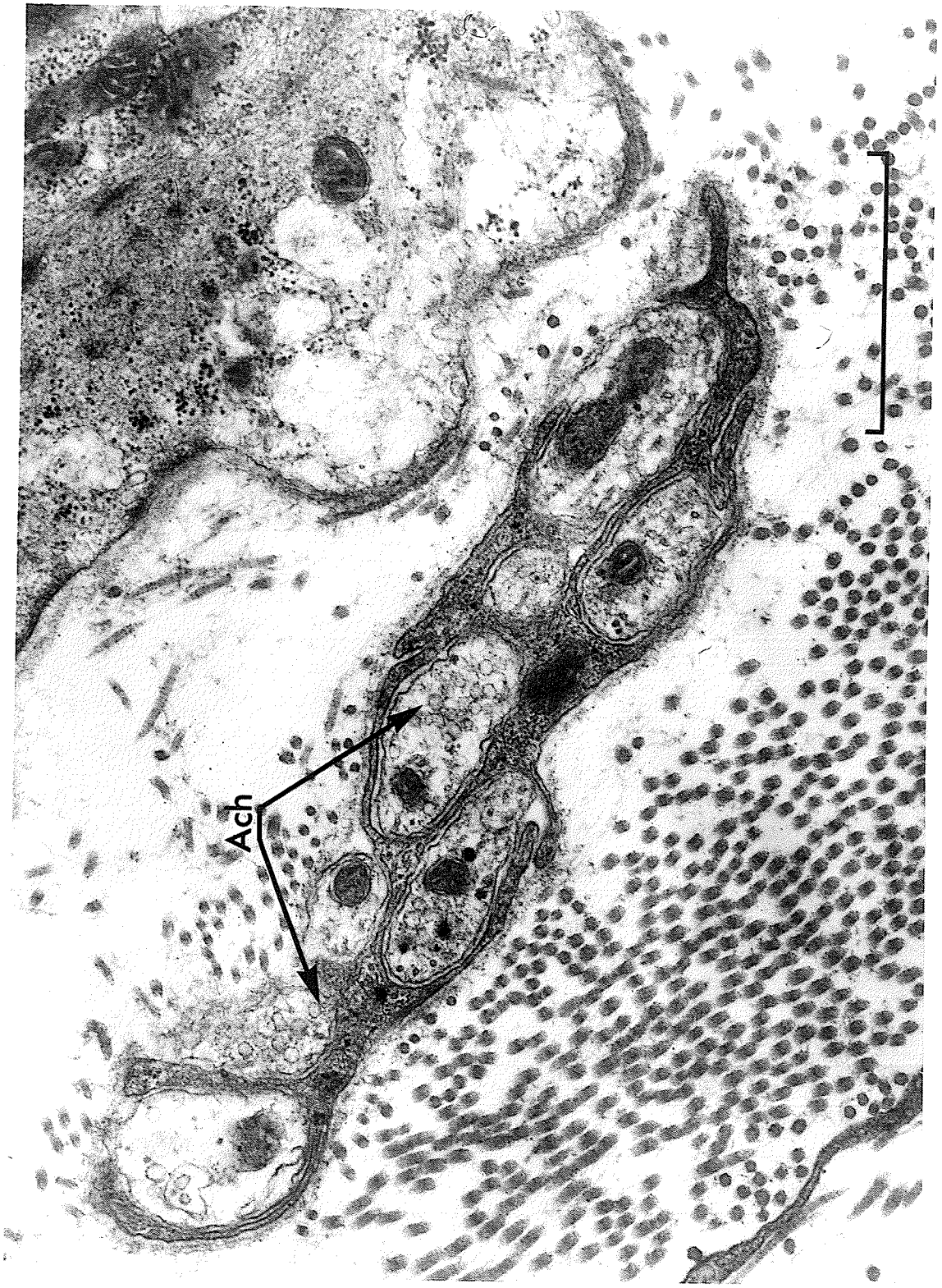

NT

FIGURE 41.

A high power micrograph of the nerve terminal seen in figure 41. The presence of cholinergic (Ach) vesicles can be observed inside the axons. Note that the Schwann cell does not always form a continuous enclosure around the axons (arrows). (X 45,090).


Ach

D. Quantitative Innervation

A quantitative study of the nerve profiles found lying in the connective tissue surrounding the smooth muscle cells was performed using cross sections of tracheal smooth muscle tissue taken from four animals. The grid squares used for counting purposes were selected on the basis outlined in the section on Materials and Methods. The results are listed in Table 3. Table 3 lists counts for each animal as well as total counts as a sum of all four animals.

Approximately 11 axons were present per 100 smooth muscle cells, with approximately 50 per cent of the axons containing only small agranular or cholinergic vesicles. All axons that contained even one clearly identifiable agranular vesicle (30 - 40 nm. in size) were termed cholinergic. However, most cholinergic axons seen contained at least 2 - 5 small agranular vesicles and axons which contained only one vesicle were somewhat rare. On the other hand, large dense core vesicles, believed to be representative of the adrenergic nerves, were much less frequent. In several of the axons containing these vesicles, only one or two vesicles were seen within a particular axon. As a general statement, the axons seen containing the large dense core vesicles appeared with approximately one-tenth the frequency of that of those containing cholinergic vesicles.

Large clear or opaque vesicles (120 - 160 nm. in diameter) were also observed, although with very low frequency. These vesicles were found occasionally in the axons without the presence of other vesicles but most regularly in axons containing cholinergic vesicles. The nature of these vesicles could not be discerned at present, and were classified together due to morphological appearance.

Small granular vesicles, similar in appearance to the large dense core vesicles, but similar in size ot that of the small agranular or cholinergic vesicles were seen in 2 or 3 axons. The identity of these structures was rather questionable, and may perhaps have been partially sectioned large dense core vesicles.

It should be remembered at all times that the axons observed either containing synaptic vesicles or those found empty were taken to represent an encounter of a particular axon at a particular position within the muscle tissue. Therefore the values given for the total number of axons for example, indicates the total number of encounters of axons seen and not an absolute figure of the number of axons actually present. It is conceivable that a particular axon or nerve process (i.e. Schawnn cell and its associated axons) may be observed more than once within a given section.

TABLE 3

Animal Group	I	II	III	IV	Total
Total # of cells	952	949	1031	1084	4016
Total # of axons	196	72	49	120	437
Pure Cholinergic	99	19	30	54	202
Pure LDV	3	3	1	1	8
Pure LCV	3	-	3	-	6
Chol. + LDV	4	8	1	8	21
Chol. + LCV	3	-	-	3	6
Chol. + LDV + LCV	-	2	-	-	2
Small Granular Ves. (SGV)	2	-	2	-	4
SGV + Chol.	1	4	-	-	5
Chol. + LDV + SGV	2	1	-	-	3
Chol. + LCV + SGV	-	1	-	-	1
15 nm. Vesicles	5	-	-	-	5

Mean # of axons 109.5 / 1004 cells
S.D. 64.96 / S.D. 65.47
SE. 32.45 / S.E. 3274

Pure Chol. Axons Mean 50.5/ 1004 cells
S.D. 35.48
S.E. 17.74

Abbreviations: Cholinergic vesicles (Chol.). Large Dense core vesicles (LDV). Large clear vesicles (LCV). Small Granular vesicles (SGV).

DISCUSSION

in the past canine tracheal smooth muscle has been described as a multi-unit smooth muscle on the basis of electrophysiological and ultrastructural findings (Bozler, 1948; Kroeger and Stephens, 1975). The activity of multi-unit smooth muscle is believed to be controlled by the diffusion of transmitters from the nerve terminals. A high density of nerve terminals and close nerve-muscle association has also been shown to exist in multi-unit smooth muscles (Bennett and Merrillees, 1966; Gabella 1974). However, since tracheal smooth muscle demonstrates properties akin to both single unit and multi-unit muscles, its classification into a specific muscle type may depend upon the extent of its autonomic innervation, as suggested by Burnstock (1970). It has therefore become important to establish an accurate quantitative assessment of the type and degree of innervation in this muscle. This information, in addition to aiding in the classification of the muscle, would help in understanding the degree of control offered by the nervous system and also to perhaps suggest the requirements for cell-to cell electrical coupling.

The study by Kannan and Daniel (1980) revealed a muscle cell to nerve axon ratio of 29 : 1 with roughly one-third of the axons containing cholinergic vesicles. The findings in the present investigation reveal a muscle cell to nerve axon ratio of approximately 10 : 1, with at least 50% of the axons containing cholinergic vesicles. The difference in the percentage of the axons containing vesicles may be accounted for by the method used in evaluating these axons. Kannan and Daniel (1980) classified axons as being cholinergic or adrenergic only if 3 or more vesicles were present in any given

axon. Although it may be slightly more reliable to observe 3 or more vesicles within an axon for classification purposes, it is also possible that this may lead to an abnormally low and perhaps inaccurate count. In the majority of cases in the present study, the presence of one or two vesicles readily enabled their identification, especially with respect to the large dense core vesicles. Therefore, in the present study, any axon that contained a structure that had the morphological characteristics of a particular type of vesicle was catagorized and counted accordingly. The large dense core vesicles, indicative of adrenergic innervation were present in roughly 8% of the total number of axons counted. This value confirmed the predominance of the cholinergic or parasympathetic system in this muscle (Cabezas et al., 1971). The closeness of the adrenergic and cholinergic terminals or varicosities to each other suggested the possibility of presynaptic modulation of neurotransmitter release. In fact, Vermeire and Vanhoutte (1977) had reported selective attenuation of cholinergic responses to electrical field stimulation by β - agonists in canine trachealis. The present structural findings are therefore supportive of such interaction between nerves. Additionally, in a number of cases both cholinergic and adrenergic vesicles were found within the same axon. The significance of such an arrangement of vesicles is not known. However, it has been proposed by Burn and Rand (1959) that stimulation of sympathetic fibers result first in the release of Ach, which in turn cause the release of norepinephrine to act on the effector organs. It is apparent that a single neuron may produce more than one type of transmitter.

Examination of the micrographs could not conclusively demonstrate the presence of purinergic innervation. Nevertheless, the presence of large clear vesicles (LCV) was noted in a few of the axons counted. Unfortunately, these large clear vesicles were not always spherical in shape, which made it difficult to positively identify them. Large opaque vesicles have been suggested as storage sites of ATP (Burnstock, 1979; Richardson and Ferguson, 1979), but it could not be determined accurately whether the vesicles seen in the present study corresponded to these large opaque vesicles. It is presently not known whether or not the purinergic system exists in the canine trachealis. Suzuki et al., (1976) could not demonstrate the presence of purinergic nerves in canine tracheal smooth muscle. Purinergic innervation has nonetheless been identified in airway of the guinea pig (Coleman and Levy, 1974; Coburn and Tomita, 1973; Hakansson et al., 1976), human (Richardson and Beland, 1976) and in the baboon trachealis (Middendorf and Russel, 1978). Further electrophysiological studies will have to be carried out to help determine their presence or absence in canine trachealis.

It should be mentioned that small granular vesicles were occasionally seen within a few axons. Although their presence was noted, they have not been termed adrenergic because of the difficulty in their identification. They appeared in extremely low frequency. In a number of instances, these vesicles may have been large dense core vesicles which were only partially sectioned. Kannan and Daniel (1980) have however termed only these granular vesicles as adrenergic in their study.

It is important to note that this study did not exclude axons that were found near blood vessels, however, if these axons were excluded, they would not have significantly altered the muscle cell to nerve axon ratio. At present there is concern by investigators in the field as to what extent the nerves lying alongside blood vessels may influence the contractile response of isolated muscle strips in vitro during electrical field stimulation. In general, when examining micrographs of canine trachealis it was observed that the blood vessels found lying in the connective tissue alongside the muscle fibers, were not blood vessels in the classical sense, but were capillaries. These capillaries were found to consist of only one layer of endothelial cells, and did not exhibit the presence of smooth muscle cells which could be used for regulation of lumen diameter; they would thereby be unresponsive to motor nerves. On one occasion, a pericyte was seen in association with a blood vessel. Pericytes are believed to be immature or developing smooth muscle cells. which upon maturity may provide the basis for the regulation of lumen size of blood vessels. However, as mentioned above, a pericyte was observed on only one accasion, therefore the effect of these cells on the regulation of lumen diameter may be minimal. Thus it is quite conceivable that the nerve axons which were found in relatively close proximity to the capillaries, may have little or perhaps no effect on these vessels. As a result, neurotransmitters released from these terminals may have their primary effect on the airway smooth muscle. In the light of this suggestion, it is possible that undue concern has been placed on whether these nerves are playing a primary role in vasomotion.

From a qulititative standpoint, nerve terminals were often seen lying 1 - 2 microns away from the muscle fibers, in the clefts between the muscle bundles. Terminals were not found in close association with individual muscle fibers within a particular bundle. Similar observations were quoted by Kannan and Daniel (1980). It would therefore appear that transmitters released from terminals would have to travel considerable distances before reaching the muscle fibers. Burnstock (1979) has previously stated that transmitter released from varicosities greater than 1000nm. in distance from muscle fibers would be ineffective. However, since canine tracheal smooth muscle does not exhibit the classical neuromuscular junctions or relationships seen in other autonomically innervated tissues, it is possible that such limitations in neurotransmitter distance may not be applicable. It was also observed that several nerve terminal profiles found some distance from the muscle fibers had axons which were partially devoid of their Schwann cell sheathes. Several of the "unsheathed" axons were found to contain synaptic vesicles. This finding coupled with the observation that no close nerve-muscle contacts were seen between nerve terminals and muscle fibers implicated these axons as being probable sites of transmitter release. The large distances between terminals and muscle fibers also suggests the possibility that transmitters released from an axon terminal may excite more than one muscle cell. This suggestion is basically speculative at this point.

When attempting to quantitize the neural elements of the muscle, nerve terminals, ie. Schwann cells and their associated axons, were considered as nerve terminal encounters. This was done in order to account for the possibility that any single nerve process may enter

and re-enter the plane of section at several points. This may have unfortunately lead to an inaccurate and perhaps high value for the number of nerve endings per muscle cell, a value that is crucial in our understanding of the nerve-muscle relationship. The extent of innervation involved in the muscle is important in determining whether canine tracheal smooth muscle belongs to the single unit or multiunit classification of muscle. From analysis of the distribution of vesicles in the micrographs, it was conceivable that a single unmyelinated nerve axon may have many points of transmitter release and may therefore be said to functionally innervate a large number of cells. If this sugestion is proved to be correct, perhaps through the use of serial sections, it would indicate that although the nerve to muscle cell ratio could be low, there is in fact a high "functional" nerve to muscle cell ratio. Such a finding would tend to place tracheal smooth muscle into the multiunit category of muscle fibers. The suggestion that a particular varicosity or axon terminal could excite more than one cell (as mentioned previously), would also support this mechanism. This explanation of the nerve muscle relationship works quite well when considering muscle fibers which lie on the outside layers of the muscle bundles. Unfortunately, it does not account for the many fibers in the interior of the bundles since nerve terminals were not found to penetrate within the bundles. However, an electrophysiological study performed by Kroeger and Stephens (1975) had demonstrated a space constant of 1.6mm., which is sightly less than twice the length of an individual fiber as reported by Suzuki et al. (1976). Therefore it appears that a certain degree of electrical coupling does exist between cells which would help depolarize cells located within the interior of the bundles when the outer cells were

excited through release of transmitters from nerve terminals. In a study using taenia coli of the guinea-pig, Gabella (1976) observed that bundles of smooth muscle cells were found to lose their individuality within tens of microns of their length, splitting into or merging with other bundles. It may be possible that a similar situation exists in canine trachealis. If this proves to be true, the splitting and merging of bundles would expose fibers located in the interior of bundles to the connective tissue septa, which would contain nerve terminals. Therefore, this may allow interiorly located fibers to be directly exposed to transmitters at a particular point along their length, and in doing so would perhaps make up for any deficiencies in cell to cell electrical coupling. Perhaps in general, muscle fibers located on the exterior of the muscle bundles would be thought of as being multiunit whereas fibers located within the interior could be regarded as being single unit.

Conclusively, canine trachealis is perhaps best described as an intermediate type of smooth muscle since it has properties pertaining to both multiunit and single unit smooth muscles. This study suggests that canine trachealis appears to be multiunit in nature as it is predominantly controlled by nervous input, however its requirements for cell to cell electrical coupling make it akin to single unit preparations.

With regards to muscle fiber morphology, the smooth muscle cell of the trachealis possesses structural features similar to most smooth muscles. Its centrally located nucleus was flanked on either of its poles by numerous organelles such as the Golgi apparatus, smooth and rough endoplasmic reticulum, lysosomes, glycogen, and mitochondria. These regions of the cell were devoid of myofilaments and therefore do not contribute to the development of contractile force. There were

also myofilaments which ran alongside the nucleus and its poles. These filaments were seen to run parallel to the long axis of the cell. The mitochondria were located in two main regions of the cell. Large numbers were seen along the central aspect of the cell extending from the poles of the nucleus and were surrounded by myofilaments. Several mitochondria were often found near the cell surface in close association with the caveolae and the sarcoplasmic reticulum (SR). It has been suggested that mitochondria play a role in excitation-contraction coupling through their involvement in calcium metabolism (Popescu, et al., 1974). These organelles are believed to represent a "buffer" compartment for free Ca^{+2} rather thana source for the release of activator calcium (Raeymaekers et al., 1977). However, Stephens and Wrogeman (1970) have shown that mitochondrial protein in smooth muscle is only approximately 10% of that of skeletal muscle, and therefore the number of mitochondria present in smooth muscle may be too few to play a significant role in calcium metabolism.

The sarcoplsmic reticulum was observed directly below and occassionally in contact with the caveolae. This undoubtly subserves its role in the regulation of intracellular free Ca^{+2} levels during the contraction-relaxation cycle as in skeletal muscle (Popescu, 1977). In the middle regions of the cell, near the level of the nucleus, the SR was most abundant, however it was less apparent near the distal portions of the cell and was essentially absent in the smallest distal profiles. This suggests a lesser degree of excitation-contraction coupling through the SR at these end regions of the cell. As mentioned above, the majority of cytoplasmic organelles are present at either poles of the nucleus. It would therefore appear that this central region of the cell

is the site of major homeostatic mechanisms of the cells such as protein synthesis, carbohydrate metabolism and energy production. Towards the ends of the cell, the predominance of myofilaments suggests that this region of the subserves the functional or mechanical role of the cell. The result of the quantitative morphology section support this concept.

The quantitative morphology section of the present study involved the study of dense band regions of the cell membrane and intermediate junctions, both of which are of primary importance in the development of tension and transmission of force between muscle cells. The regions of the membrane involved in caveolae were also studied in an attempt to detect changes in patterns of caveolae at defferent regions along the cell length.

As explained in the Review of Literature and Results sections, Gabella (1977) had stated that dense band regions of the cell membrane increase from the midpoint to the ends of the cell. This was also confirmed by our observations of longitudinal and cross-sectioned fibers. Therefore cross sections of cells which were used for measurements were grouped according to their dense band to circumference ratios. The aspect of dense band areas increasing as one goes towards the ends of the cell suggest an increase in the sites for filament attachment. The importance of this finding lies in the suggestion that a major portion of force development may take place at these regions of the cell.

The measurements of the regions of the membrane involved with caveolae indicated that as the dense band to circumference ratios of the cells increased, the caveolae regions decreased. In other words, the maximum number of caveolae decreased significantly towards either end of the cell. Had the cells which were chosen randomly for measurement

purposes included the smallest of the distal portions of the cell, it would have been found that no caveolae were present, and that the membrane would have been comprised entirely of dense bands. These features were also observed from qualititative assessment of the micrographs.

Intermediate junctions formed by the opposition of two dense band regions from adjacent cells were determined to be sites of mechanical coupling between cells (Henderson et al., 1971) and were therfore also studied from a quantitative standpoint. The results indicated the area of membrane involved in intermediate junctions increased from the mid-c point to either ends of the cells. This is a not unexpected finding since the amount of dense band material in the membrane also increased towards the ends of the cell. However, the junctional area of dense band ratio decreased somewhat inspite of the finding of an overall increase in junctions. Although this may appear to be contradictory, it is simply a statement of the fact that though both junctional regions and dense band regions increased toward the ends of the cells, the dense band regions increased at a greater rate, resulting in a slightly lower junction to dense band ratio. It may be helpful to suggest at this point that perhaps all dense band regions, in addition to their obvious importance in association with myofilaments, may be potential sites for intermediate junction formation. The decrease in junction to dense band ratio towards the ends of the cell may indicate that certain conditions must be met before junctions will form. It should also be noted that these intermediate junctions may be transient in nature. There may also exist a constant turnover or formation and breakdown ot these junctions by the cell, depending upon specific conditions present at a particular

time. Perhaps the membrane at the central portions of the cell may be more suitable for junction formation than regions towards the ends. It was observed that the basement membrane near the cell ends was especially thick. This basement membrane was also observed to be intimately involved with elastin and elastic fibers which in turn were involved with the collagen fiber network. The increase density of the basement membrane may perhaps in some way affect the ability of the cells to form junction, hwereas the slightly reduced density of the basement membrane in the middle of the cell may have also contributed to the higher junction to dense band ratio observed there. Numerous other factors may also be related. These findings suggest perhaps that much of the force transmitted between cells near their ends involves the connective tissue network which comprises in part the parallel and series elastic component of the muscle. In contrast, regions towards the center of the cell may relie more on intermediate junctions for force transmission. The collagen network, as mentioned in the Results section appeared to form spirals around the individual muscle fibers, and were often at right angles to elastic fibers which were found associated with the basement membrane. The significance of this arrangement is obscure at the present time.

The findings of this study have suggested a number of morphological features which may help explain force development and transmission in canine tracheal smooth muscle. This study has also brought to attention the difficulty involved in attempting to quantify relatively unspecialized structures such as smooth muscle fibers, or perhaps any other structure. It may be appropriate to mention here that the more random in nature or structure a cell appears, the more sensitive the approach that will have

to be employed in order to quantify subtle features of its morphology.

Furthur studies on tacheal smooth muscle will have to include serial sections of the muscle over large distances. Perhaps the use of these sections coupled with three dimensional reconstruction of individual cells and groups of cells may enable a better understanding of the spatial relationship between muscle cells, pattern and size changes in cell to cell junctions, as well as structural changes within the cells. It is hoped that this study will prove to be helpful in achieving a more complete understanding of the innervation and mechanical aspects of canine tracheal smooth muscle and other smooth muscles.

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