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# An ultrastructural study of intramuscular melanocytes in PET/Wmr mice

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AN ULTRASTRUCTURAL STUDY OF INTRAMUSCULAR MELANOCYTES  
IN PET/Wmr MICE

BY

CHARLES AUGUST GOMER III

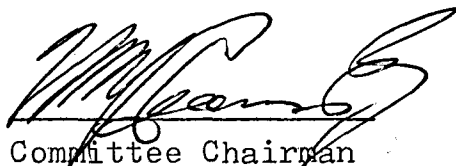
A.B., UNIVERSITY OF NORTH CAROLINA, 1971


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OF RICHMOND IN PARTIAL FULFILLMENT OF THE REQUIREMENTS  
FOR THE DEGREE OF MASTER OF SCIENCE IN BIOLOGY


AUGUST, 1978

AN ULTRASTRUCTURAL STUDY OF INTRAMUSCULAR MELANOCYTES  
IN PET/Wmr MICE

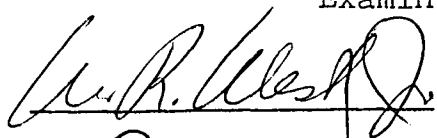
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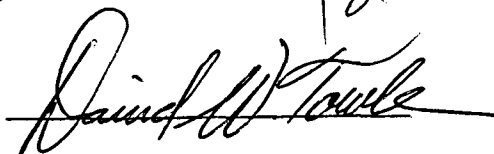
  
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## TABLE OF CONTENTS

I. Abstract .....	ii
II. Acknowledgements .....	iii
III. Introduction .....	1
IV. Materials and Methods .....	5
V. Observations .....	8
VI. Discussion .....	15
VII. Literature Cited .....	20
VIII. Figures .....	23
IX. Vita .....	35

## ABSTRACT

An examination of the lateral head of the gastrocnemius of the PET/Wmr mouse was made with an electron microscope. Cells of the perimysium and endomysium were described and the relationship of these cells to muscle cells was characterized. The relationship between melanocytes and muscle cells found in this strain was the major focus. Melanocytes were seen to be present in the perimysium and endomysium with the greatest concentration in the endomysium. Melanocytes of the perimysium appeared segregated from the adjacent tissue by fibroblasts, which surrounded the melanocytes with their cytoplasmic processes, as well as by collagen fibers. Melanocytes of the endomysium were seen to have no direct communication with the muscle cells though there were conical protrusions of the sarcolemma at areas of juxtaposition between the perikaryon of the melanocytes and the muscle cells.

## ACKNOWLEDGEMENTS

I am indebted to Dr. Willie M. Reams, Jr. for his invaluable advice, guidance, and technical assistance throughout the course of this thesis. His sincere desire to serve has been a mainstay to this end.

Furthermore, I wish to acknowledge the contributions of Dr. Francis B. Leftwich and Dr. William S. Woolcott, members of my committee, given in criticism in the writing of this thesis.

I should like to thank Ms. Shirley Craig, Department of Anatomy, Medical College of Virginia, for her technical assistance and suggestions. I also want to express a deep appreciation to Mr. Harry E. Mitchell, Jr., formerly of the University of Virginia, for his practical assistance in mastering the finer points of electron microscopy.

Lastly, I want to thank my wife for her stalwart support and understanding.

## INTRODUCTION

The pigment terminology used here conforms to that of Fitzpatrick, et al. (1966) - melanoblast: an undifferentiated dendritic cell of neural crest origin which may be stimulated to become a melanocyte; melanocyte: a differentiated dendritic cell of neural crest origin having the capacity to produce melanosomes; melanosome: a specific organelle produced by a melanocyte capable of undergoing the biosynthetic process of melanization.

Mammalian pigmentation has generally been described as the direct association of melanocytes with the derivatives of ectoderm; the skin being the primary example. However, melanocytes do occur in extra-epidermal tissues and their intramuscular colonization has been documented (Reams, 1966; Mayer and Reams, 1962; Reams and Hollyfield, 1962).

The neural crest origin of epidermal melanocytes in mammals is known from the work of Rawles (1940, 1947). Rawles was able to demonstrate and map the medio-lateral spread of melanoblasts from their neural crest origin to all major regions of the body by the end of 12 days of embryonic development.

Melanoblasts migrate from the neural crest to two principal communities: the ectoderm and the

mesoderm ventral to the neural crest. The melanoblasts of the mesodermal migration pattern have been found to locate themselves in the connective tissues of the non-integumentary portions of the body (Reams, 1966; Nichols and Reams, 1960). The melanoblasts of ectodermal migration become skin components and as such contribute to the production of melanin pigmentation of the skin and its derivatives (Weston, 1963). Ectodermal melanoblasts in the advancing margin of invasion proliferate and leave a residual complement of melanoblasts which in turn proliferate in the developing skin. The migration and proliferation continue until the skin has been completely colonized.

Mayer and Reams (1962) extended the studies of Rawles (1940, 1947) with a detailed analysis of the migratory patterns of melanoblasts into the legs of embryonic mice of the PET strain. The dorsal ectoderm of the PET embryo leg received melanoblasts by day 12 and the ventral ectoderm by day 13. Melanoblasts of the ventral leg skin had emigrated into the adjacent mesoderm and populated the leg musculature by day 14. Following colonization, the melanoblasts in the differentiating muscle ultimately appeared as melanocytes in the endomysium and perimysium of the muscle, and in the superficial connective tissue of the muscle and the leg in



general. Pigment cell colonization is apparent in the gastrocnemius, plantaris, soleus, and peroneals of the leg once discrete muscle groups have been formed but is rarely observed in the remaining muscles. Skin grafting studies of the colonization of the leg musculature by the melanocytes have shown that the initial melanoblast migration to the leg musculature and ensuing proliferation are sufficient to supply all the melanocytes found within the leg of the adult (Reams and Baird, 1960).

The pigmented posterior muscles, such as the gastrocnemius, have within them profusely branched, highly dendritic melanocytes (Mayer and Reams, 1962). The processes may extend across several muscle fibers, though at times they are found to be oriented parallel to muscle fibers and extend to some distance from the nuclear region of the melanocyte. In contrast to the diffuse nature of the melanocytes of the distal regions of the leg musculature, the melanocytes of the proximal regions are found to be fusiform and with few dendrites which extend to adjacent muscle fibers (Mayer and Reams, 1962).

Although the melanocyte population of skeletal muscles is well documented, no work has been done on the anatomical relationship of these cells to muscle fibers. To this end an electron microscopic study

was undertaken to examine the gastrocnemius and its melanocytes in the  $PET/Wmr$  mouse.

## MATERIALS AND METHODS

The tissues used in this study were obtained from mice of the PET/Wmr strain (Reams, 1967) maintained at the University of Richmond. Mice of this strain were used because of their availability and the accessibility of background information on their melanocyte development and migration (Reams, 1966; Mayer and Reams, 1962).

For a light microscopic examination, 4-day old and adult mice were killed by decapitation and the left hind legs cut away at the site of articulation of the head of the femur and the acetabulum. The skin and superficial connective tissue surrounding each leg was removed and the entire leg placed in absolute ethanol for dehydration. Three changes of ethanol were carried out at 24-hour intervals. The limbs then were cleared in oil of wintergreen and examined in toto using a stereoscopic microscope or as whole mounts of thick sections using a compound microscope.

In preparation for electron microscopic examination, 4-day old and adult mice were killed and the legs skinned as described above. The

gastrocnemius was dissected away from the legs and examined with a stereoscopic microscope to ascertain the presence and location of melanocytes. After inspection of the muscle, it was separated via an incision bisecting the acute angle at the medial conversion of the fibers. The lateral head was further separated parallel to the plane of the muscle fibers to produce 4 strips of muscle (1.0 x 4.0 mm).

The samples were placed in 1% gluteraldehyde-veronal acetate buffered fixative (pH 7.4) for 2 hrs. at 2 C. At the end of this period, the samples were again sectioned to produce blocks of muscle 0.5 x 1.0 mm. The superficial layer of muscle (0.5 x 0.5 mm) from each block was isolated and placed in fresh gluteraldehyde fixative for 1 hr. at 2 C. Following immersion in the fixative, the samples were rinsed in 3 changes of veronal-acetate buffer at 2 C for 5 min. each. Samples then were placed in a 2% osmium tetroxide, veronal-acetate buffered fixative (pH 7.4) at 2 C for 90 min. (Pälade, 1952). Dehydration of the samples was carried out through a graded series of acetone.

The infiltration and embedding process made use of Epon 812 resin. The embedding medium was made using 5 ml Epon A, 5 ml Epon B, and 0.15 ml DMP-30 accelerator. The samples were passed through

a series of acetone/Epon solutions to undiluted Epon medium. The samples were left in pure Epon medium overnight. The embedding medium was changed the next day and the tissue blocks placed in fresh medium for 2 hrs. Individual samples then were placed centrally on the surface of Epon medium which had been placed previously in Beem capsules. The capsules were then incubated in an oven at 60 C for 48 hrs.

Under a stereoscopic microscope, the blocks of Epon and tissue were then trimmed with a single-edged razor blade which had been rinsed with acetone. The face of the block was shaped to measure 0.25 x 0.25 mm and thin sections were obtained using a Reichert "OmU-2" ultramicrotome with a diamond knife. Thin sections (60-90 m $\mu$ ) were mounted on copper grids which had been cleaned in a sonicator for 10 min.

Thin sections were routinely stained using a saturated aqueous solution of uranyl acetate for 2 min. and post-stained with lead citrate for 3 min. (Reynolds, 1963; Watson, 1958).

Tissue sections were examined on a Philips 201C electron microscope and electron micrographs were made using 3 $\frac{1}{4}$ " x 4" cut film (Kodak #4889 High Contrast Film, Eastman Kodak Corp., Rochester, New York) from which prints were made by photographic enlargement.

## OBSERVATIONS

## Light Microscope

The distal portion of the lateral head of the gastrocnemius of 4-day old mice had a high concentration of melanocytes in the superficial layer anterior to the aponeurosis of the Achilles tendon. Melanocytes in the proximal portion of the muscle were sparse. Examination of whole mounts of the superficial layer of the gastrocnemius revealed that melanocytes resided in the perimysium and endomysium, as well as in the superficial connective tissue. The melanocytes observed were characteristic in their branched appearance with dendritic processes passing across several muscle fibers and at times orienting themselves parallel to the plane of the muscle fibers with their dendrites extending great distances from the cell body (Fig. 1).

Examination of the adult muscle revealed a diffuse population of melanocytes with no region having a local concentration. The melanocytes were again characteristic in their numerous dendritic processes; however, there appeared to be a decrease in the dendrite

length and degree of melanization.

## Electron Microscope

### Muscle Cells

The nuclei were elongated ovoid structures oriented parallel to the plane of the muscle fibers. On occasion, several nuclei were observed at the periphery of a single muscle fiber just within the sarcolemma. The elongated unbranched multinucleated fibers were found to contain the sarcoplasm, consisting of a homogeneous ground substance, and morphologically distinguishable mitochondria, free ribosomes, attached ribosomes, sarcoplasmic reticulum, an occasional lysosome, and glycogen granules. Myofilaments and sarcomeres were conspicuous. At the interface between two adjacent muscle fibers was an endomysium characterized by its homogeneous appearance. Irregular shaped, sarcolemmal protrusions occurred at several muscle fiber to muscle fiber junctions (Fig. 2 and Fig. 3). Some of the protrusions contained vesicles and free ribosomes. Discrete fascicles were surrounded by a complex matrix of areolar connective tissue having constituents of fibroblasts, macrophages, and an infrequent mast cell. The most significant feature of the perimysial connective tissue was the presence of large amounts of collagen fibers.

## Fibroblasts

Fibroblasts were found in great numbers in the perimysium and were represented as conspicuous, long, branching cells (Fig. 4). A fibroblast was characterized by an ovoid nucleus with an occasional indentation which was surrounded by a coarse appearing cytoplasm. Within the cytomembrane were complex folds of granular endoplasmic reticulum consisting of many interconnecting cisternae. A Golgi complex was present with numerous, well developed cisternae, and vesicles at the margins. Mitochondria were in evidence as large circular organelles but were few in number. Cytoplasmic processes were apparent in close proximity to the vast network of collagen fibers which were associated with the fibroblasts (Fig. 5). These fibers were noted to be oriented in all planes around these cells. Lipid droplets were present as poorly defined electron dense areas within the margins of the cells.

## Melanocytes

A typical melanocyte had a large, irregular, ovoid nucleus surrounded by a prominent nuclear membrane. Mitochondria were diffuse throughout the cytoplasm and were characteristically large and ovoid with well developed cristae (Fig. 6). The endoplasmic reticulum, though not as extensive as that of the



fibroblast, appeared well developed and coursed throughout the cytoplasm adjacent to the mitochondria. Both rough and smooth endoplasmic reticula were noted. The cytoplasm had a granular appearance with the presence of electron dense areas suggestive of lipid droplets and vacuoles. A large Golgi complex was present and had well developed vesicles and cisternae which were oriented around the nucleus. Melanosomes were in all stages of development and appeared to be membrane-bound, elongated structures with longitudinally oriented filaments undergoing melanization in young melanosomes. Mature melanosomes were electron opaque (Fig. 7 and Fig. 8). Dendritic processes extended out from the perikaryon with varying concentrations of melanosomes.

#### Fibroblast : Muscle Cell Relationship

Fibroblasts of the perimysium were characterized as having an elaborate endoplasmic reticulum. Collagen fibers were in close association with the cell membrane and extended throughout the connective tissue matrix (Fig. 7). The fibroblast frequently was so oriented that the nucleus of the fibroblast was in close association with the muscle fibers. The majority of collagen fibers were at the outer boundaries of

the perimysium although a number of fibers were in close association with the muscle cells. Cytoplasmic extensions were seen to course along the length of the muscle cells with collagen fibers forming a thick sheath around them.

#### Fibroblast : Melanocyte Relationship

The majority of the melanocytes observed in the perimysium were at the external margin of the connective tissue with only an infrequent pigment cell at the junction of the muscle cell (Fig. 7). Fibroblasts associated with the melanocytes in a manner suggestive of the fibroblasts surrounding the pigment cell with cytoplasmic extensions and actively producing collagen. Dendritic processes of the melanocytes were frequently enclosed by the cytoplasmic processes of the fibroblasts and were surrounded by a high concentration of collagen fibers. Contrasted to the orientation of the fibroblasts along the path of the muscle fibers, the pigment cell orientation to the fibroblast was at random. The fibroblasts were apparently capable of phagocytizing melanosomes from melanocytes as evidenced by the presence of a melanosome within a fibroblast vesicle (Fig. 9).

## Muscle Cell : Melanocyte Relationship

Melanocytes in the perimysium were not closely associated with the muscle cells but rather were in the superficial layer of the perimysium separated from the muscle cells by fibroblasts, collagen fibers, mast cells, and macrophages. The orientation of the melanocytes in the perimysium had no distinctive feature. Cytoplasmic processes from the fibroblasts encircled the melanocytes and their dendrites. Collagen fibers were present in large numbers in the area adjacent to the pigment cell dendrites. Pigment cells which were closer to the muscle fibers had a surrounding matrix consisting of collagen fibers and had an association with fibroblast processes.

Melanocytes of the endomysium had cytomembranes closely apposed to the sarcolemma (Fig. 10). The majority of the dendrites of the pigment cells were parallel to the muscle fibers. No direct communication was seen between the cytomembrane of a pigment cell and the sarcolemma of a muscle fiber (Fig. 11). Muscle fibers had conical protrusions of the sarcolemma which extended towards the melanocyte perikaryon and melanocyte dendrites at areas of a thickness of two or more melanosomes. No such modifications of the

sarcolemma were noted in regions where the pigment cell dendrites were of a thickness of one melanosome or less. At areas of high concentrations of melanosomes the sarcolemmal projections contained vesicles and/or free ribosomes (Fig. 11 and Fig. 12). Fibroblast nuclei and processes were intermittent throughout the endomysium. The processes were oriented in a fashion which caused the melanocyte to be sandwiched between the process and the sarcolemma of the adjacent muscle fiber (Fig. 12). Collagen fibers were seen in an otherwise homogeneous endomysial matrix. No free melanosomes were seen. The presence of desmosomes was not demonstrated.

## DISCUSSION

The neural crest origin of melanocytes has been examined and the wide distribution of pigment cells documented in extra-epidermal tissues (Nichols and Reams, 1960). Extended studies of the migratory patterns of melanoblasts have demonstrated emigration by these cells from the ventral skin into the adjacent mesoderm with the ensuing proliferation of melanoblasts in the differentiating leg musculature (Mayer and Reams, 1962). In the present study examination of the 4-day old mouse gastrocnemius with a light microscope revealed a high concentration of melanocytes in the perimysium and endomysium of the superficial layer of muscle. The pigment cells had characteristically branched appearances with dendritic processes extending across several muscle fibers (Fig. 1). Examination of the adult gastrocnemius revealed a diffuse population of melanocytes again characterized by branching dendrites though both the dendrite length and degree of melanization appeared reduced. This is the result of the postnatal cessation of proliferation of pigment cells but the continued growth of the maturing muscle.

To extend the observations made with the light

microscope, the relationships of the primary cells of the gastrocnemius of the PET/Wmr mouse were examined by the use of an electron microscope. Tissues of the gastrocnemius of 4-day old mice suggested that there was high metabolic activity within the muscle as evidenced by high concentrations of free and attached ribosomes, the complex endoplasmic reticulum associated with fibroblast deposition of collagen fibers in the perimysium and endomysium, and the presence of immature melanosomes in several pigment cells. Mast cells and macrophages were seen but, as they were not related to the intent of this study, not commented upon.

Fibroblasts were in great numbers with the highest concentration in the perimysial tissue. The deposition of collagen by the fibroblasts suggested that these cells also had a high metabolic rate (Fig. 5). The fibroblasts appeared to recognize the presence of melanocytes in the area in that fibroblast processes were oriented around the pigment cells in a manner which suggested an attempt to segregate the pigment cells from the myisial tissue by collagen deposition (Fig. 8). This was more apparent in the perimysium than in the endomysium. The fibroblasts of the endomysium were few and appeared to cause

a closer association between the muscle cells and the melanocytes. This was evidenced by their having cytoplasmic processes sandwich the pigment cells and pigment cell dendrites between the fibroblast and muscle fibers (Fig. 11). It was noted that a melanosome had been phagocytized by a fibroblast in the perimysium (Fig 9). This brings up the question of whether a fibroblast received the melanosome directly from a pigment cell dendrite or picked up a melanosome which had been released into the tissue matrix. But, as no free melanosomes were seen, the former proposition seems more appropriate (Fig. 9).

Melanocytes were found to reside in all areas of the perimysium and endomysium. These cells were represented as having ovoid nuclei, a large Golgi complex, elaborate endoplasmic reticulum, free ribosomes, and melanosomes in all stages of melanization (Fig. 8). The degree of maturation of pigment cells was determined by the number of mature fully-melanized melanosomes present in the dendrites and the perikaryon. Melanocytes were generally found in the superficial layer of the perimysium where they were more or less segregated from the muscle fibers by fibroblasts or collagen fibers which surrounded the pigment cells. The fibroblast cytoplasm was oriented in a manner

which suggested recognition of the melanocyte and an attempt by the fibroblast to isolate the cell. There were no free melanosomes seen in the perimysium.

Melanocytes of the endomysium were more directly apposed to the muscle cells. The dendritic processes of the pigment cells were found to be sandwiched next to the muscle cells, without actually communicating with the sarcolemma (Figs. 11 and 12). The melanocytes coursed throughout the endomysium along the border of the sarcolemma. At areas where there was a high concentration of melanosomes in the perikaryon and proximal portions of dendrites, there were regular-shaped, conical protrusions with vesicles and free ribosomes extending from the sarcolemma to the cytomembrane of the melanocyte (Figs. 11 and 12). The protrusions had a thick base which tapered to a tip next to the pigment cell cytomembrane. Protrusions and vesicles were absent where the concentration of melanosomes was lower (Fig. 10). The areas with the protrusions resemble neuro-muscular junctions. The sarcolemmal modifications at melanocyte associations differed from the observed muscle to muscle junctions in that the protrusions were of regular conical shape in the former and highly irregular in the latter (Fig. 2 and Fig. 3). At no point along the associative path of the melanocytes and the muscle fibers was



there the indication that desmosomes or other tight junctions were present. The significance of the observed associative junctions remains to be elucidated.

In sum, with regard to the relationships of melanocytes to muscle cells, there were no desmosomes nor tight junctions observed. However, the association between a pigment cell and a muscle cell was characterized by conical protrusions of the sarcolemma which contained free ribosomes and vesicles. These modifications of the sarcolemma approached the cytomembrane of the pigment cell perikaryon and thicker areas of its dendrites. Melanosomes were not observed in the muscle proper, either as free melanosomes or as inclusions in the muscle cells.

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Figure 1. Whole mount of superficial muscle layer from lateral head of gastrocnemius from 4-day old PET/Wmr mouse. Note melanocyte orientation. Melanocyte, MC; Muscle Fiber, MF. X 1000.

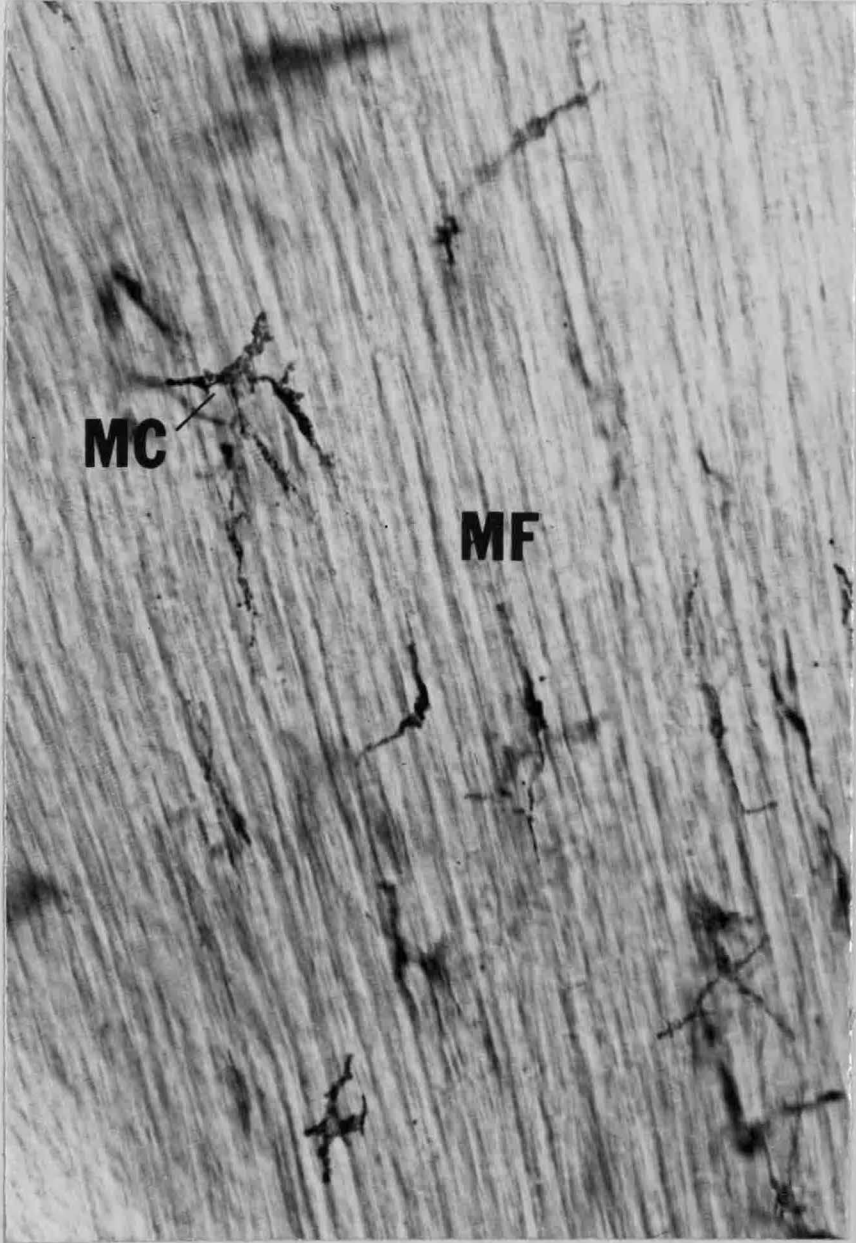


Figure 2. Electron micrograph of junction of two muscle cells of lateral head of gastrocnemius. Note suggestion of recognition by cells in the form of the interdigitating protrusions. Note vesicles. Endomysium, E; Mitochondrion, M; Muscle Fiber, MF; Protrusion, P; Ribosomes, R; Sarcomere, S; Vesicles, V. X 53,000.

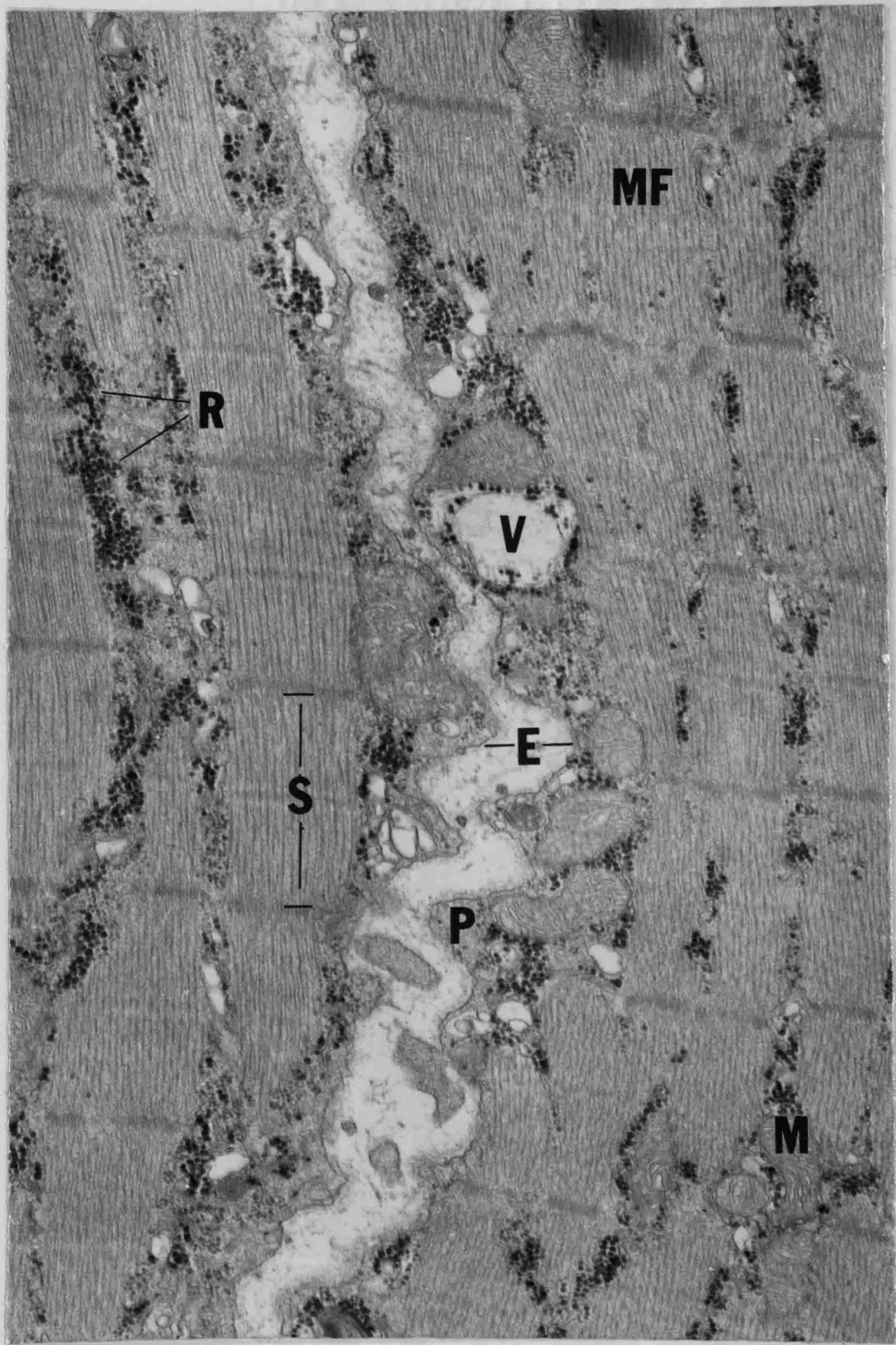
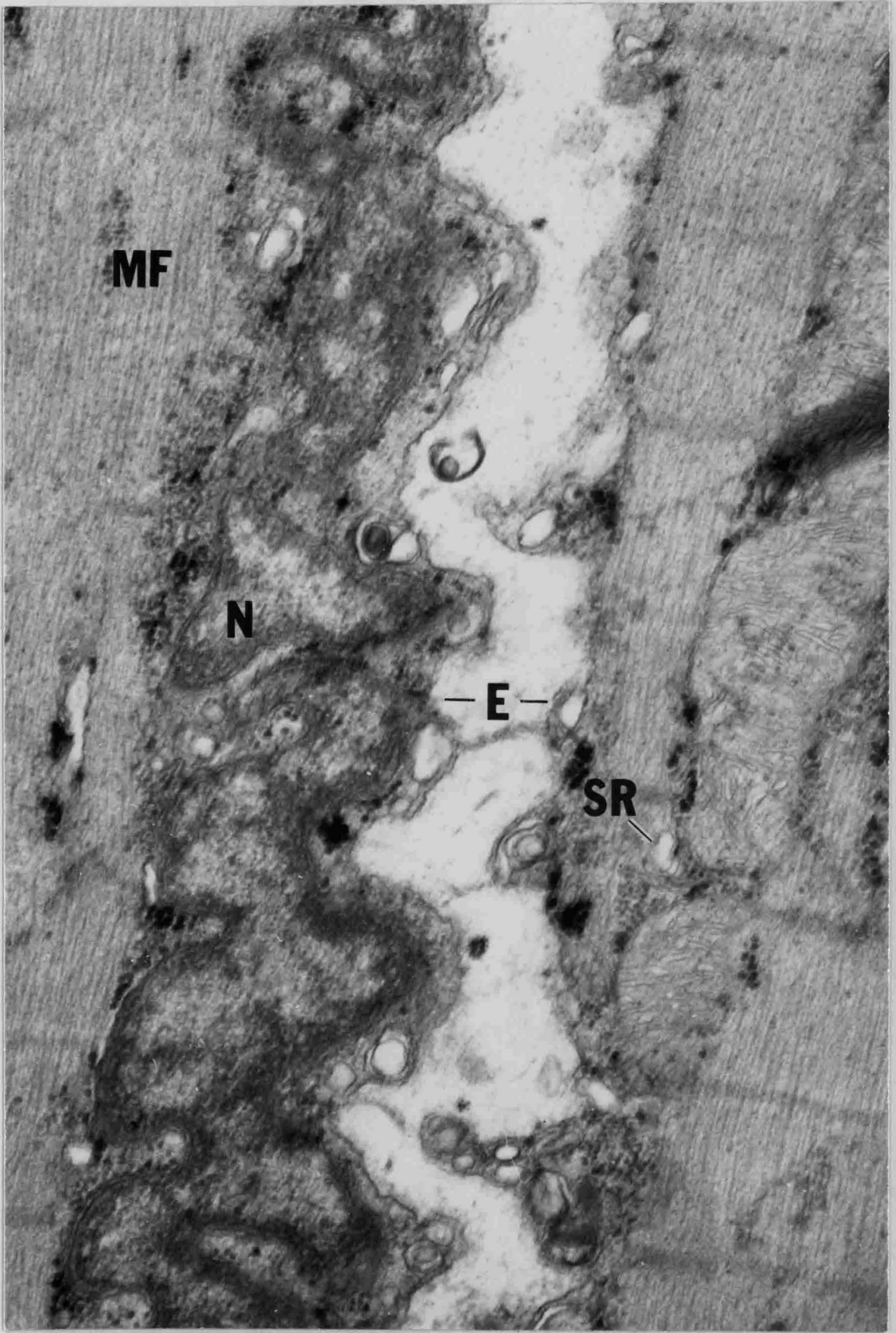




Figure 3. Electron micrograph of junction of two muscle cells of lateral head of gastrocnemius showing nucleus and sarcolemma. Note suggestion of recognition by cells in the form of interdigitating protrusions. Endomysium, E; Muscle Fiber, MF; Nucleus, N; Sarcolpasmic Reticulum, SR. X 73,000.



**MF**

**N**

**— E —**

**SR**

Figure 4. Electron micrograph showing relationships between the perimysium and the muscle cells. Collagen, C; Fibroblast, F; Macrophage, MP; Muscle Fiber, MF; Perimysium, P. X 45,000.

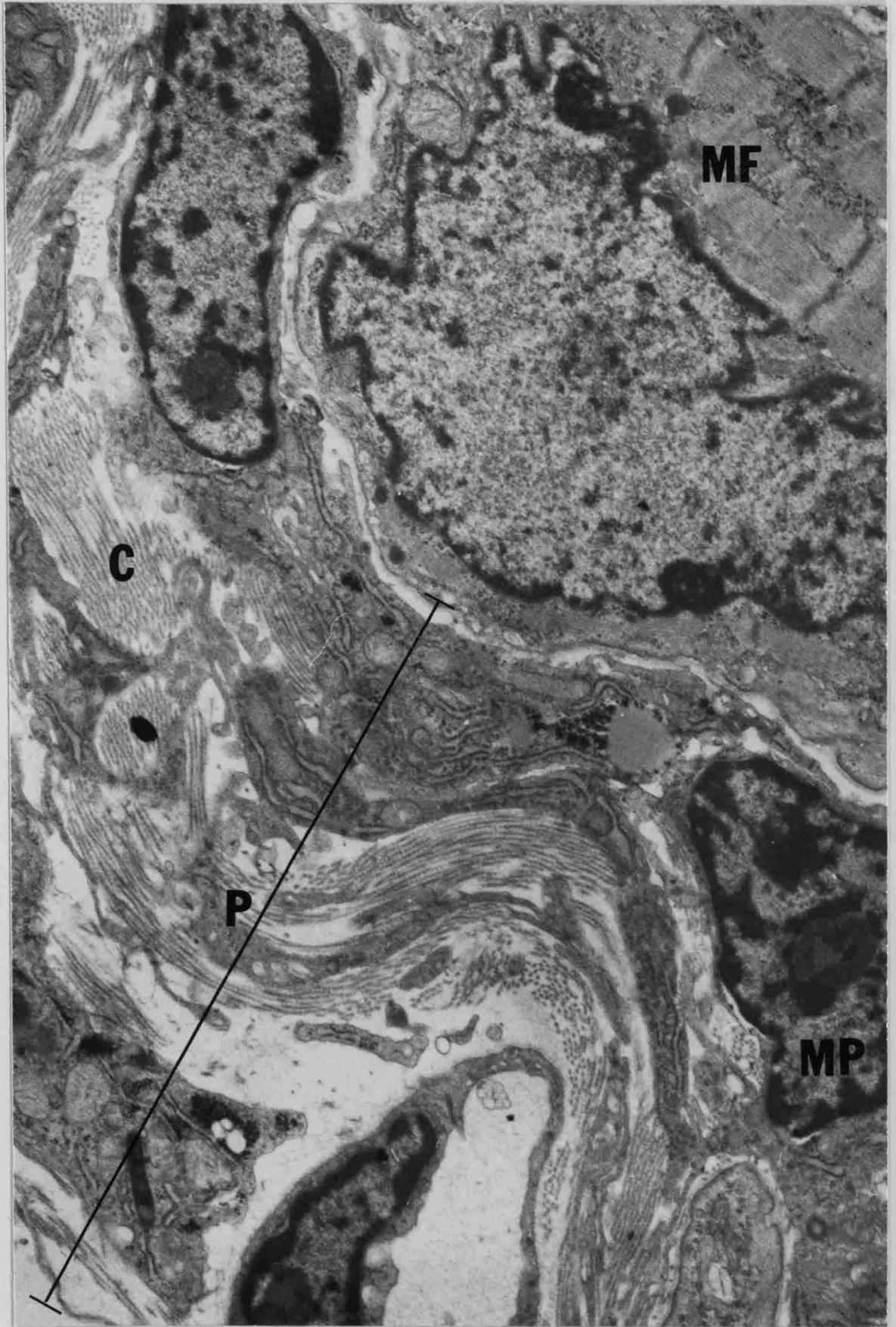


Figure 5. Electron micrograph of perimysium showing nucleus of muscle cell, complex endoplasmic reticulum of fibroblast, collagen fibers, and melanocyte with melanosomes. Collagen, C; Endoplasmic Reticulum, ER; Melanocyte, MC; Melanosome, MS; Muscle Fiber Nucleus, N.  
X 29,000.

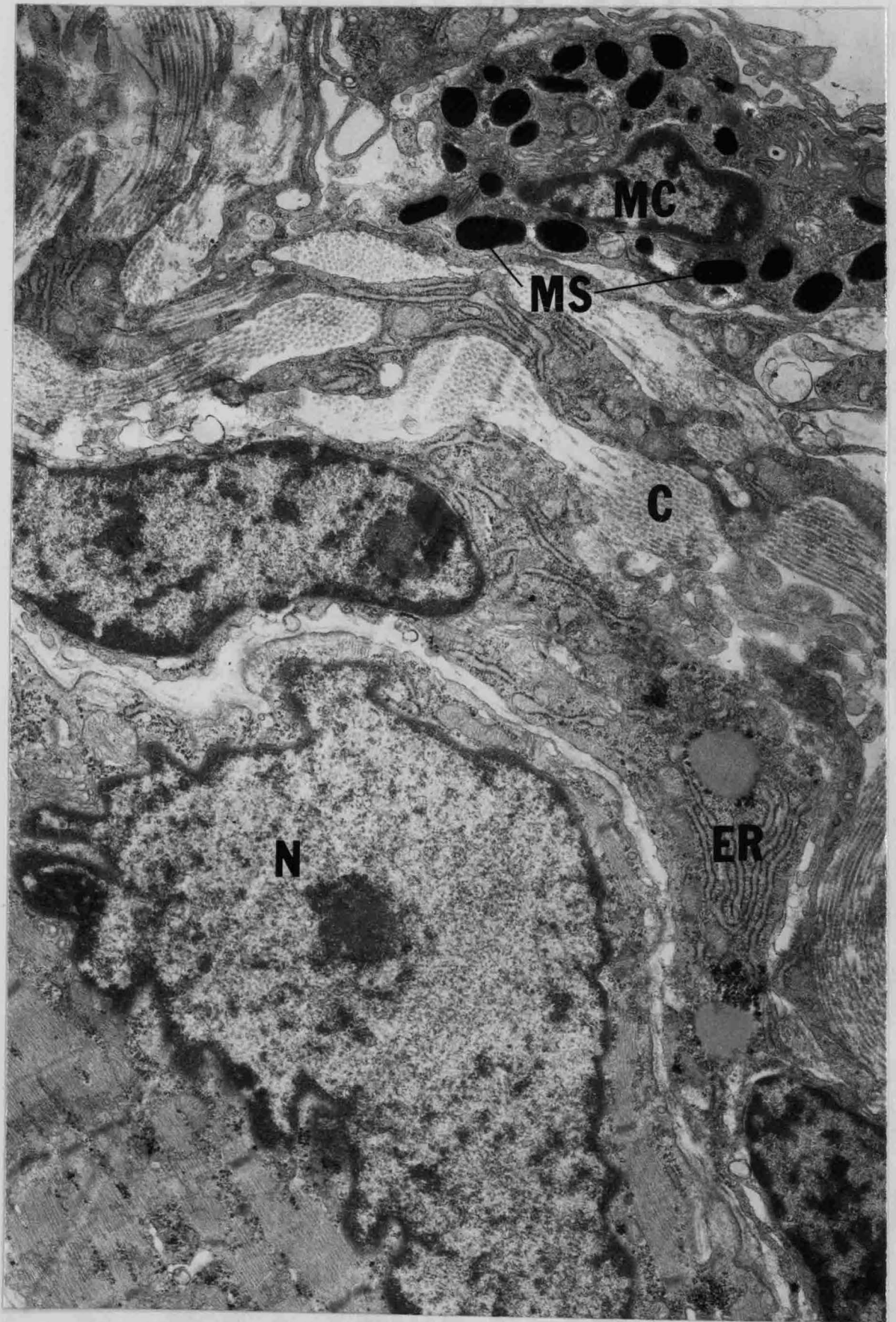


Figure 6. Electron micrograph of melanocyte of gastrocnemius showing perikaryon with numerous melanosomes. Also seen are numerous collagen fibers and fibroblast processes. Collagen, C; Fibroblast Process, FP; Melanocyte, MC; Melanosomes, MS. X 30,000.

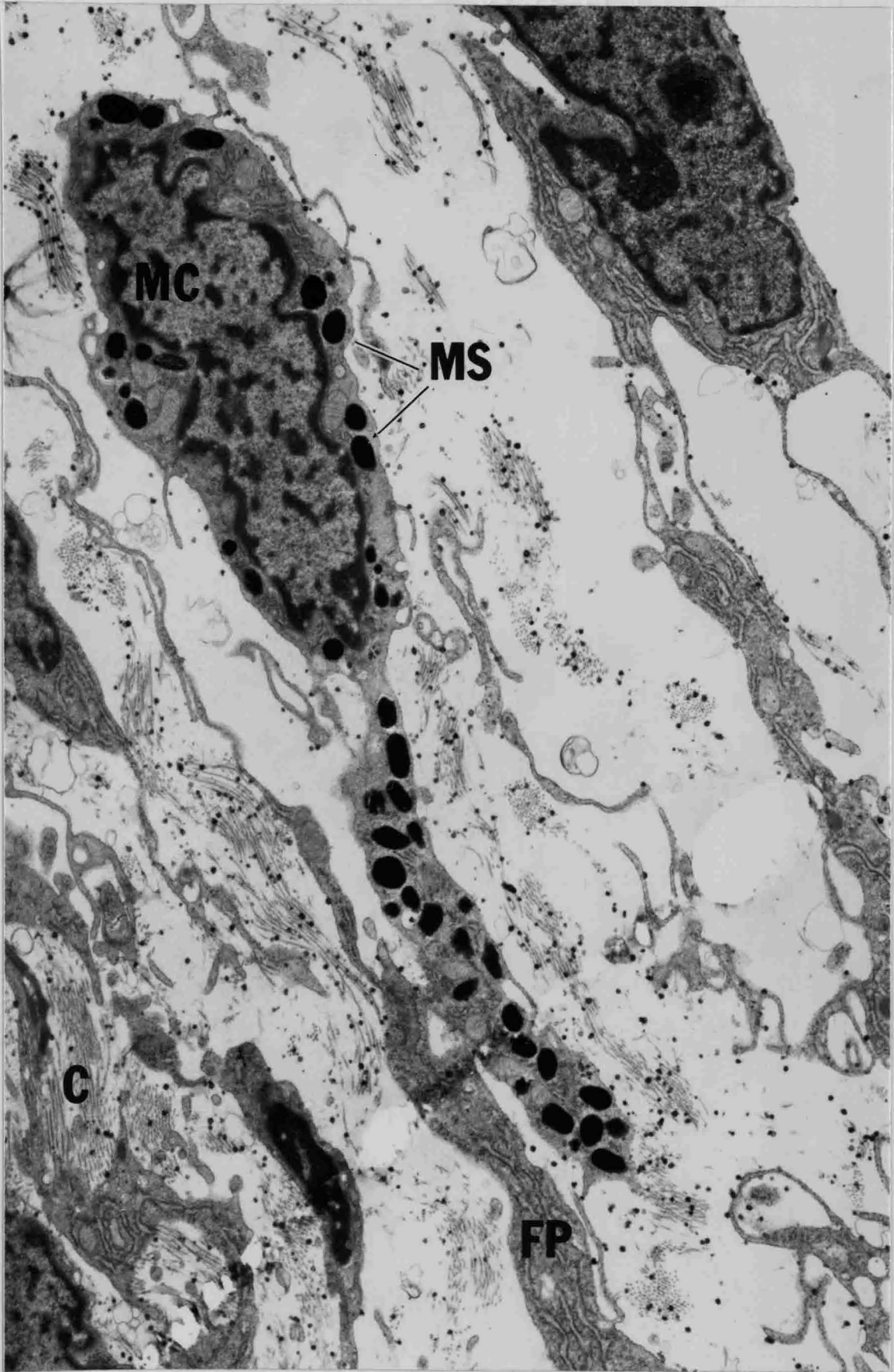




Figure 7. Electron micrograph showing perimysium of gastrocnemius with fibroblast, melanocyte, muscle cell, macrophage, and collagen. Collagen, C; Fibroblast, F; Macrophage, MP; Melanocyte, MC; Muscle Fiber, MF. X 16,000.

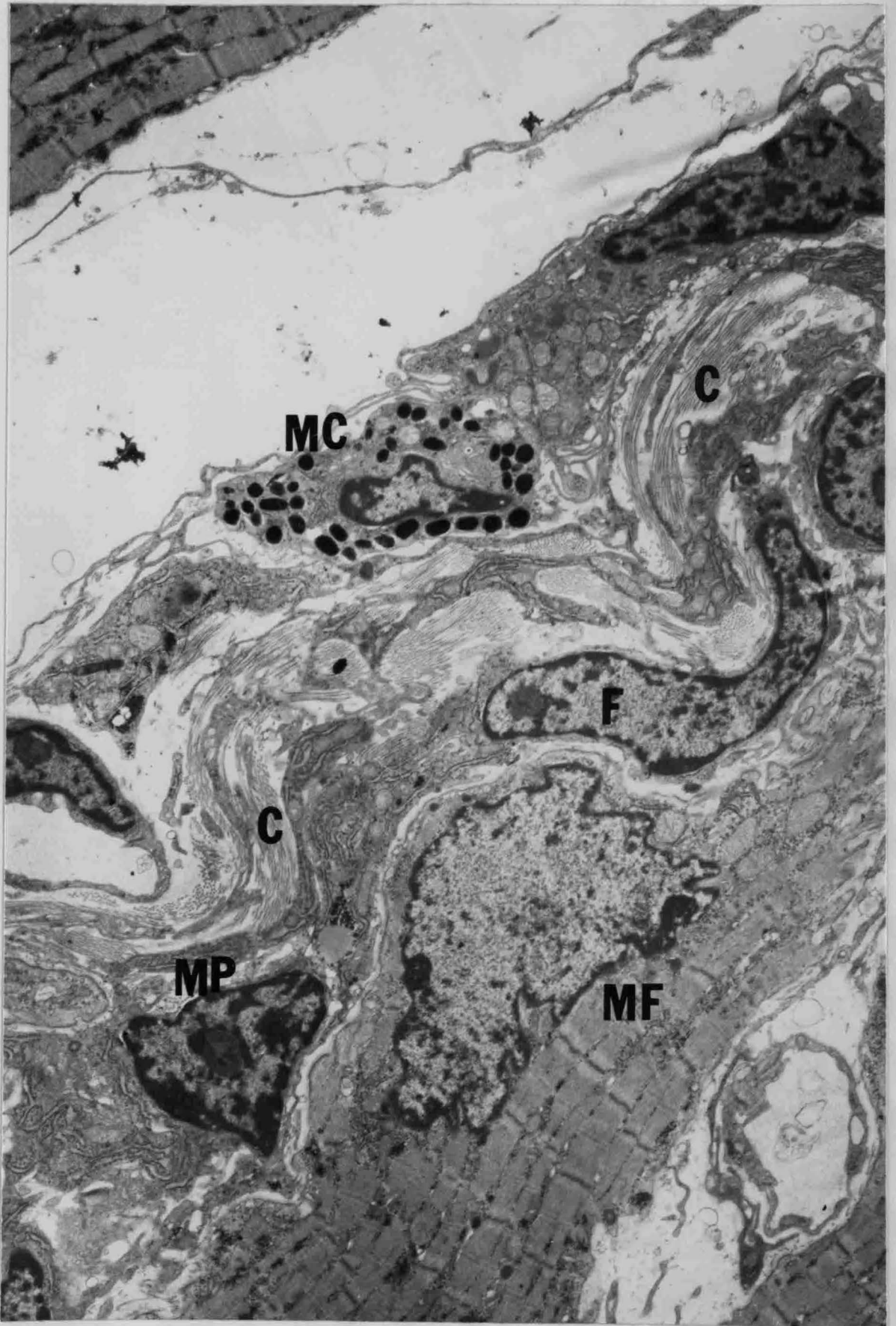


Figure 8. Electron micrograph of melanocyte of perimysium showing various stages of melanosome formation. Collagen, C; Fibroblast, F; Melanocyte, MC; Melanosome, MS. X 53,000.



Figure 9. A. Electron micrograph of fibroblast with phagocytized melanosome. B. Comparison of phagocytized melanosome in fibroblast and melanosomes of melanocyte. Fibroblast, F; Fibroblast Process, FP; Melanocyte, MC; Melanosome, MS; Muscle Fiber, MF. X 35,000.

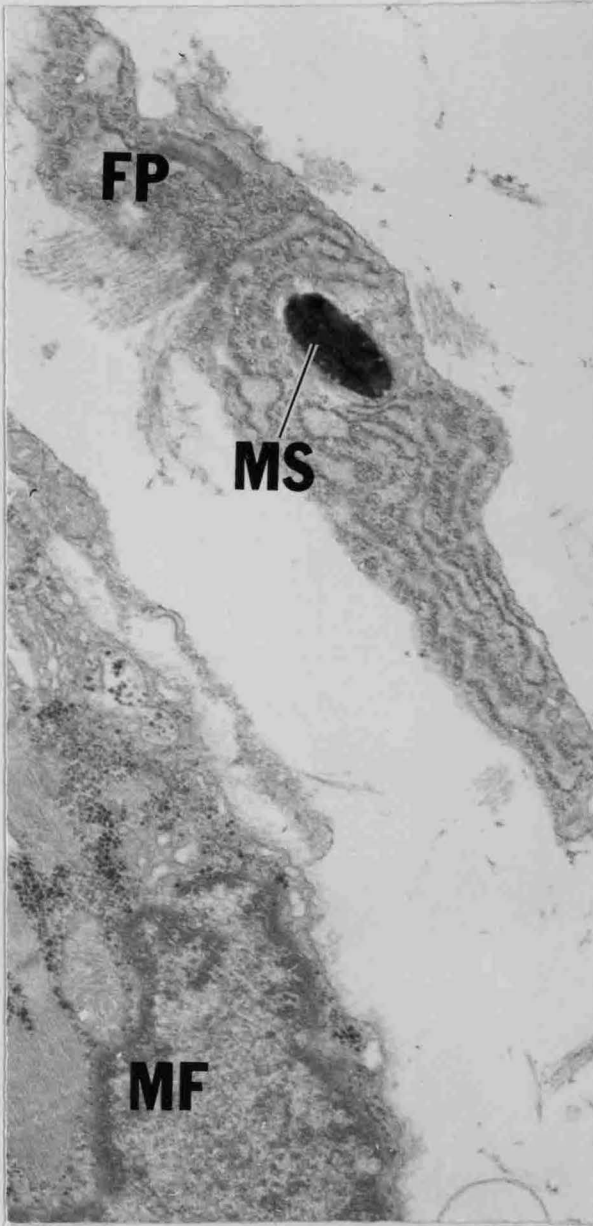
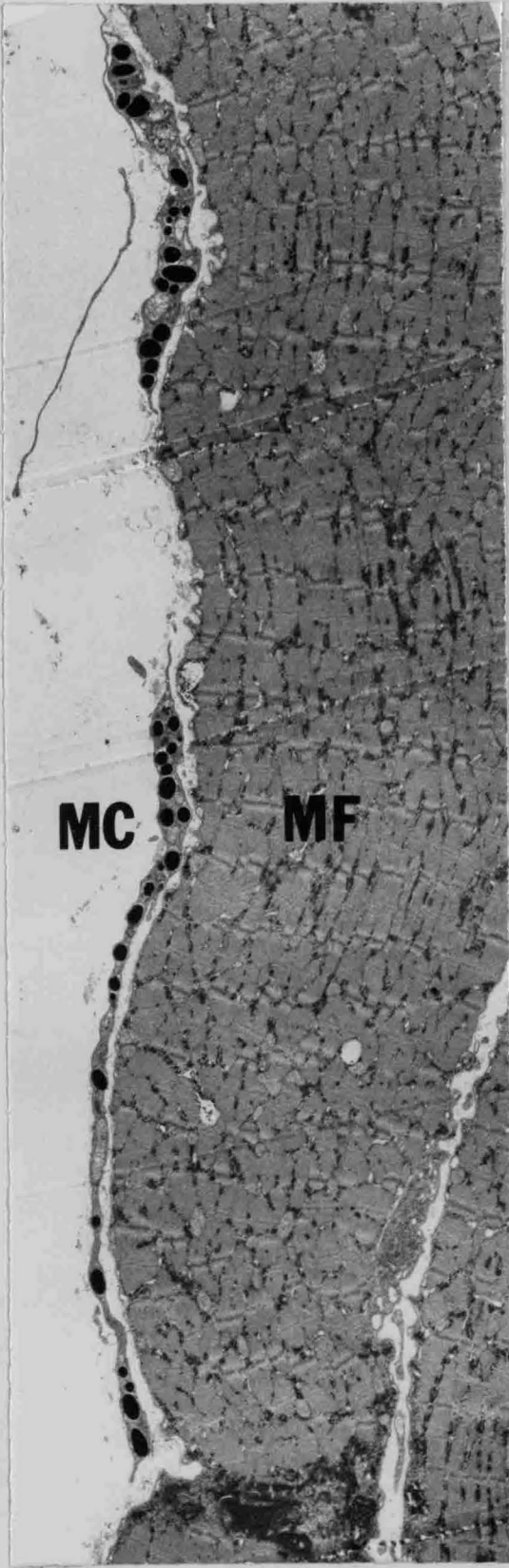


Figure 10. A. Electron micrograph of gastrocnemius endomysium showing apposition of melanocyte to muscle cell. X 13,000.

B. Enlargement showing finer detail of melanocyte to muscle cell junction. X 20,000. Melanocyte, MC; Muscle Fiber, MF.

**A**



**B**

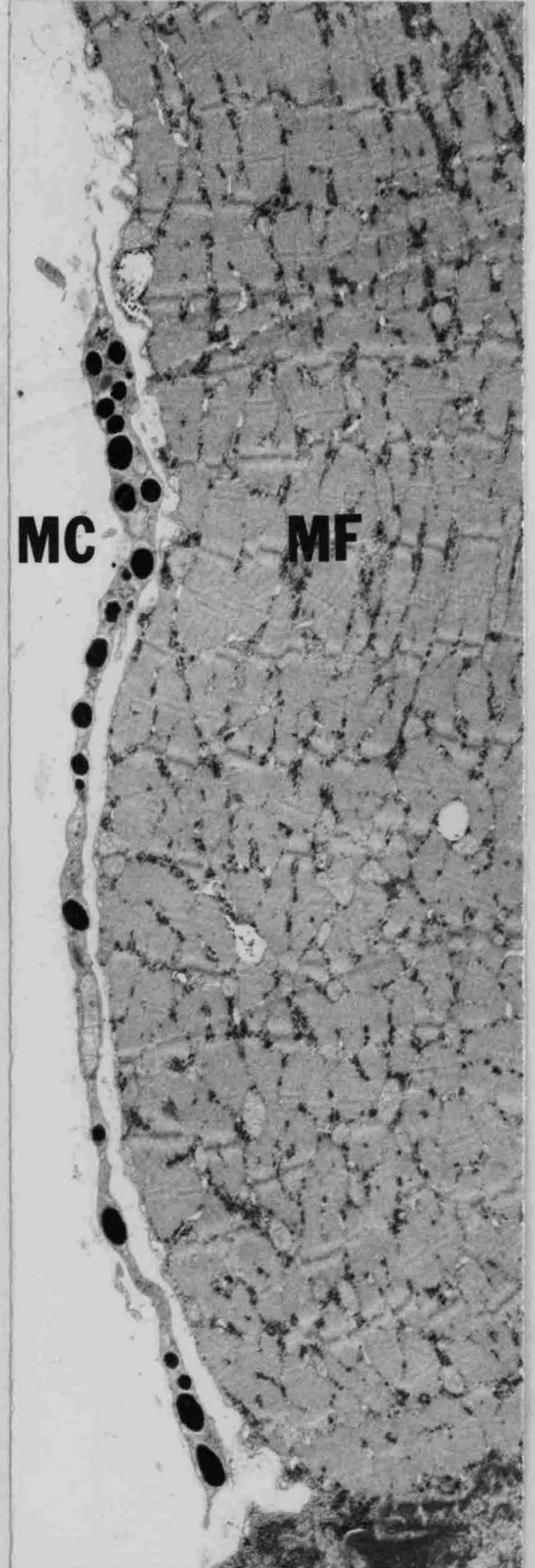




Figure 11. Electron micrograph of gastrocnemius endomysium showing relationship of melanocyte cytomembrane to the sarcolemma. Note fibroblast process which covers the melanocyte dendrite and sarcolemmal protrusions and vesicles. Fibroblast Process, FP; Melanocyte, MC; Muscle Fiber, MF; Sarcolemmal Protrusions, SP; Vesicles, V. X 52,000.

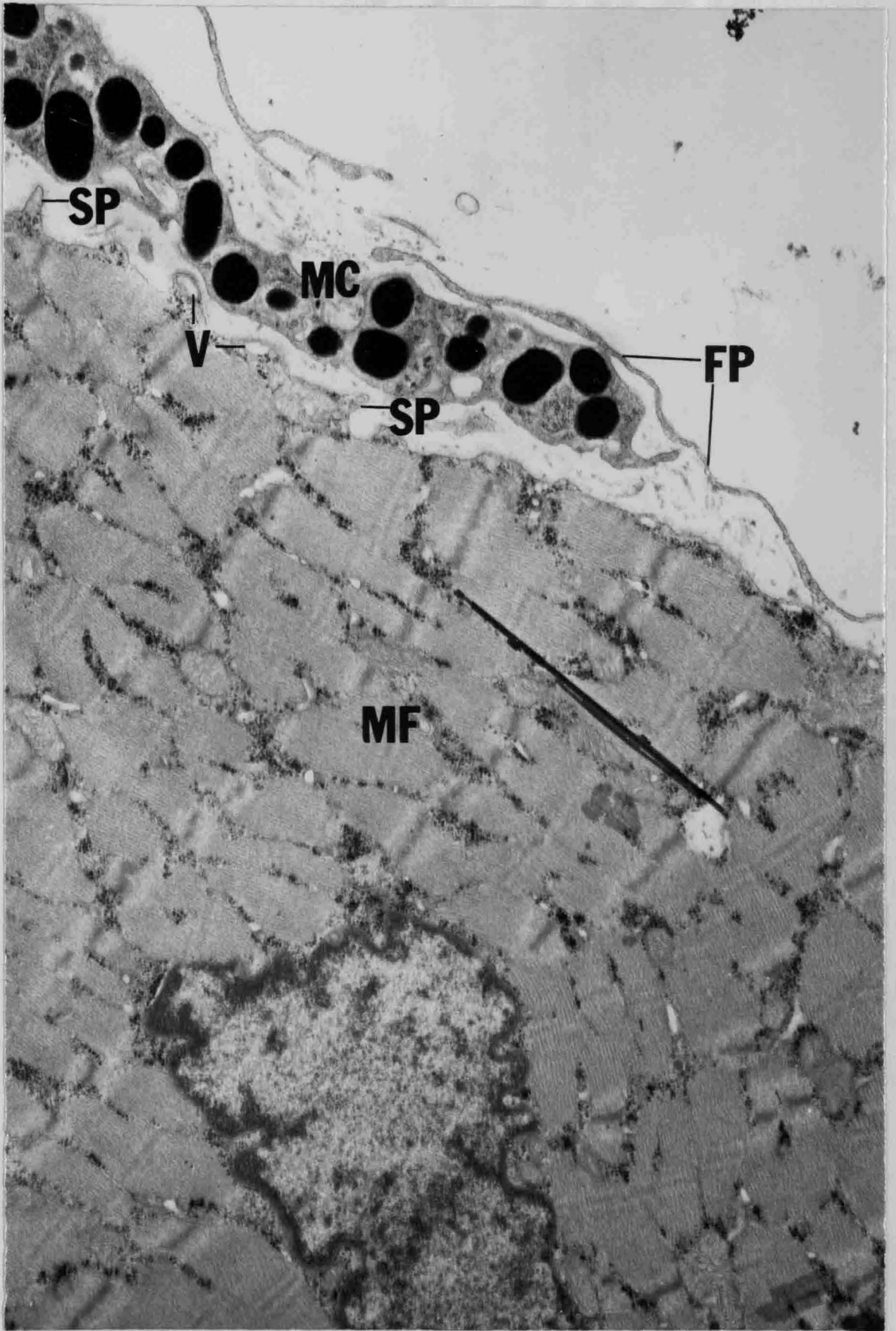


Figure 12. Enlargement of Figure 11 showing protrusions and vesicles of sarcolemma in area of melanocyte perikaryon. Melanocyte, MC; Melanosome, MS; Muscle Fiber, MF; Protrusions, P; Vesicles, V. X 96,000.



## VITA

Charles August Gomer III was born in Durham, North Carolina on December 27, 1948. He received his elementary and secondary education at The Gilman School, Inc. in Baltimore, Maryland where he was graduated in June, 1967. He began his undergraduate studies at the University of North Carolina at Chapel Hill in September, 1967 and received the degree of Bachelor of Arts in International Studies in June, 1971. He undertook postbaccalaureate studies at Hampden-Sydney College from August, 1973 to June, 1975.

In August, 1975 he entered the Graduate School at The University Of Richmond to pursue studies leading to a Master of Science degree in biology. He was elected a member of Beta Beta Beta Biological Honor Society in the fall of 1975.

He is married to the former Adelaide Hinton Park of Ithaca, New York and has a daughter Aletia Park Gomer.