Electron Microscopy of Cat Spinal Cord Subject to Circulatory Arrest and Deep Local Hypothermia (15 C)

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Circulatory arrest to the lumbar spinal cord of adult cat was produced by occlusion of the descending aorta and concurrent arterial hypotension. *Local* hypothermia of the cord was induced by paraffin oil at 5 C, which was circulated over the exposed surface of the cord, using the laminectomy wound as a trough. Intramedullary temperature was 15 C at a depth of 5 mm. In 10 control animals oil at 37 or 5 C was circulated over the exposed cords (normal-normothermic and normal-hypothermic controls with 1 and 2 hours hypothermia). Three animals had circulatory arrest and recirculation in normothermia (ischemic-normothermic) and 3 in hypothermia (ischemic-simultaneous hypothermia). Three had circulatory arrest and 15 minutes of recirculation in normothermia followed by 1 hour of hypothermia (ischemic-delayed hypothermia). The medial and lateral portions of the anterior gray horns of the last lumbar spinal segment were studied in the light and electron microscopes. Ischemic-normothermic tissue showed 20% shrinkage in mean areas of neuronal perikarya and massive "watery" swelling of astrocytic cell bodies and processes. Within neuronal perikarya and dendrites, cytoplasm increased in electron density, ribosomes dispersed, Golgi apparatus swelled and mitochondria swelled with loss of matrix density and disruption of cristae. Axons and axon terminals did not increase in size, but mitochondria within these structures doubled in size without loss of matrix density or change in pattern of cristae. Synaptic vesicles were no longer uniform in size, and they were clumped away from the synaptic cleft and diminished in number. Lysosomes were unchanged in appearance and size. Mitochondria of astrocytes underwent approximately fourfold enlargement without loss of matrix density or pattern of cristae. Bundles of astrocytic microfilaments were fragmented, spread apart and diminished in quantity. Oligodendroglia and endothelial cells were unchanged. Normal-hypothermic animals were similar to normal-normothermic except for clefts in rough endoplasmic reticulum of neurons and dendrites. These clefts were formed by a separation of the cisternal membrane from the adjacent row of ribosomal rosettes. Ischemic-simultaneous hypothermia animals had findings identical to normal-hypothermic animals. Ischemic-delayed hypothermia animals were similar to ischemic-normothermic animals except for less swelling of astrocytic processes, greater swelling of astrocytic mitochondria and less alteration of microfilaments. The findings show that ischemia in normothermia brings about alterations in virtually every organelle of the neuronal perikaryon except the lysosome. Simultaneous hypothermia in ischemia prevents the protean alterations of ischemia, whereas hypothermia delayed until after the ischemic episode only slightly modifies the cellular lesions found in ischemic-normothermic animals (Am J Pathol 72:369–396, 1973).

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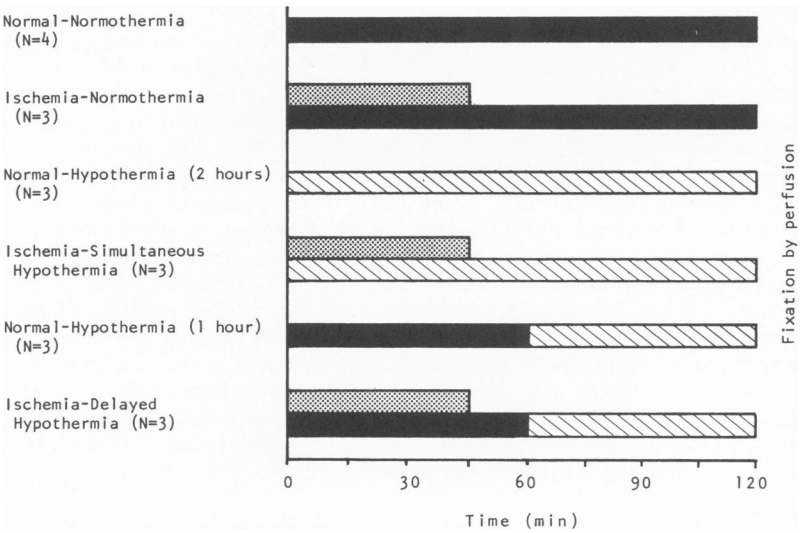
THE CELLULAR MECHANISMS for rendering a tissue nonrevivable after a period of ischemia or anoxia remain elusive in all tissues, but particularly so in the central nervous system, whose vulnerability to this insult exacts a heavy toll in deaths and disablement. In the hope of shedding some light on these mechanisms, the cat's spinal cord was studied by light and electron microscopy after ischemia, ischemia and local hypothermia, and local hypothermia alone.

The literature describing the protection that hypothermia confers in ischemia and anoxia is quite voluminous, going back several centuries. While there has been no recent review, a wealth of material and references may be found in the monographs of Blair,[1] Kayser,[2] and Smith[3] and in the volumes edited by Taylor[4] and by Dripps.[5]

Methods and Materials

A total of 19 normal cats, unselected as to age and sex, but all exceeding 2 kg in weight were used in this study. The animals were allocated to experimental groups in the following manner (Text-figure 1).



TEXT-FIG 1—Experimental design. (Normothermia, *solid bars;* hypothermia, *hatched bars;* ischemia, *dotted bars*).

Lumbar laminectomy was performed on adult cats under pentobarbital anesthesia (30 mg/kg). Animals were mechanically ventilated; their mean arterial blood pressure was continuously monitored (l. carotid) along with intermittent determination of arterial blood pH, pO_2 and pCO_2. Blood loss was precisely mea-

sured and replacement made with dextran 70. Body (rectal) temperature was maintained in the range of 35 to 37 C.

The dura was incised longitudinally, and the exposed spinal cord covered with paraffin oil. The cord was then inspected with an operating microscope for any signs of contusion or other operative trauma. Presence of the slightest suspicion of operative trauma disqualified the animal, and its cord was not processed for electron microscopy.

Arrest of Circulation

Thoracotomy was performed, and the beginning of the descending aorta occluded over 60 seconds by slowly closing a bulldog clamp. The initial dangerous hemodynamic disturbance, consisting of a rapid rise in blood pressure and sometimes followed by a precipitous often fatal hypotension, was mitigated by rapid withdrawal of blood into heparinized syringes, followed by a slower withdrawal of blood until the mean arterial pressure in the carotid artery was 40 to 50 mmHg. The blood vessels on the surface of the spinal cord were kept under direct observation with the operating microscope. For purposes of timing, circulatory arrest was said to begin when blood flow was *seen* to cease in the vessels on the surface of the cord. If blood was withdrawn as described above, flow stopped reproducibly within 5 minutes of aortic occlusion. Preliminary studies showed that if blood was not withdrawn and the mean pressure was permitted to remain high, spinal cord blood flow could not be halted at all by simple aortic occlusion.

After 45 minutes of circulatory arrest the aortic clamp was removed, and shed blood reinfused. Body (rectal) temperature was maintained at 35 to 37 C during reperfusion. Blood flow was observed to return to the cord within seconds, and no regions of failed perfusion could be detected by inspection of the surface vessels. Restored blood flow was continued for 75 minutes, after which the spinal cord was fixed by perfusion.

Hypothermia

Hypothermia was induced locally in the lumbosacral cord by circulating cold paraffin oil along the exposed surface, using the laminectomy wound as a trough.[6] Temperature of the circulating oil was 5 C. A 24 gauge needle thermistor (YSI) was implanted in the third lumbar spinal cord segment (*ie*, about 4 cm from the cord segment taken for morphology), by means of a Kopf stereotaxic drive, 2 mm lateral to the midline to a depth of 5 mm. This placed the tip of the thermistor in the anterior gray matter approximately ventral to the central canal. At this depth the temperature attained was about 15 C. Half-time, until equilibrium temperature was reached, averaged 15 minutes.

Electron Microscopy

Fixation

Fixation was by perfusion of 5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, and 1% purified gum acacia at room temperature. Perfusion was effected by clamping the thoracic aorta, incising it and tying in place a glass cannula. Fixative was driven by a peristaltic pump at an initial rate of 1 liter/min for 1 minute and then slowed to perfuse an additional 4 liters over 1 hour. The time from aortic occlusion to beginning of fixative flow did not exceed 60 seconds in any animal. Washout of blood from the vessels on the exposed surface of the cord was complete within 15 to 20 seconds from the time the perfusion was begun. Paraspinal muscles and peritoneum were then cut away from the lumbar spinal column

and the bone with cord in place was sawn out and the whole immersed in the same fixative at 4 C overnight. The cord was then dissected free, and the last lumbar spinal segment sliced with a razor blade and dissected into anterior and posterior gray matter. Only the medial and lateral portions of the anterior gray horns were used.[7] The final blocks, approximately 0.5 × 0.5 × 3.0 mm, were then postfixed without buffer wash in Dalton's fixative, dehydrated in increasing ethyl alcohol concentrations and cold propylene oxide, and then embedded in Araldite, the long axis of the block representing the cephalocaudad axis of the cord.

If the perfusion was considered successful on purely technical grounds (*ie*, elapsed time between aortic occlusion and beginning of perfusion, elapsed time until complete blood washout) the tissue thus obtained was then carried forward through complete electron microscopy and statistical analysis without any further selection. We believe that the custom of "evaluating the fixation" before deciding if given tissue is "in" or "out" of the study introduces dangerous bias, especially in cases where genuine lesions may be confused with fixation artifacts.

Area Determinations

One-micron thick sections were cut with glass knives and stained with toluidine blue. All neurons whose nuclei were included in the section where photomicrographed from sections from 48 normal-normothermic blocks and from 29 ischemic-normothermic blocks and enlarged to a final linear magnification of 1456, as determined by a stage micrometer. Photographic enlargements were made on sheets from the same box and were processed and dried under identical conditions. The standard deviation of weights of the finished full photographic sheets was found to be less than 1% of the mean weight of these sheets. Photographic images of the cells, 141 normal-normothermic and 103 ischemic-normothermic, were then cut out and weighed to the nearest hundredth gram. The true area of these cellular profiles was then calculated from the mean area per gram and the areal magnification factor.

Thin sections with gray interference color were cut on diamond knives on an LKB Ultratome at the 400 to 500 Å setting of the thermal feed. Sections were mounted on carbon-coated formvar substrates on copper grids and then photographed in a Siemens Elmiskop 1A at an original magnification of approximately 8000. Exact magnification was determined from Fullam replica gratings.

Comparison of fixation obtained in the animals reported here with that of 18 animals in a preliminary series where gum acacia was not used showed that gum acacia contributed nothing to the quality of fixation. It should be pointed out that the procedures of Schultz and Case,[8] whose claims prompted the use of gum acacia, were not followed precisely.

Fixation of the white matter was consistently very poor whether or not gum acacia was used. No observations or conclusions concerning white matter are reported here. In material fixed without gum acacia (preliminary studies), large myelinated axons in white matter showed explosion of myelin lamellae. Myelin sheaths were well preserved only in small axons or in the most superficial subpial white matter.

Results

Light Microscopy

Figures 1 and 2 show light micrographs of a normal-normothermic and an ischemic-normothermic motoneuron. Ischemic-normothermic neurons and blood vessels were surrounded by clear halos not found

in the normal-normothermic controls. The neuronal perikarya of ischemic-normothermic animals contained numerous small vacuoles in their cytoplasm. The cytoplasm and nuclei of such cells were more darkly stained than the surrounding neuropil, whereas the cytoplasm of normal-normothermic neurons usually stood out as less darkly stained than the surrounding neuropil. Ischemic-normothermic neurons appeared to be shrunken compared to controls; this observation was borne out by statistical analysis of weights of cut-outs of these profiles. The areas of 141 normal-normothermic and 103 ischemic-normothermic spinal motoneuron profiles were 1760 ± 1470 sq μ and 1410 ± 1210 sq μ (mean ± SD), respectively; $t = 1.980$ and $P < 0.05$. Ischemic anterior horn neurons shrank by 20%; the probability that this difference arose by chance was less than one in twenty, by Student's t-test.

Electron Microscopy

The normal fine structure of the gray matter of the cat spinal cord observed in this study does not differ from that reported previously by other authors. Apart from explosion artifacts of myelin, two other preparative artifacts were consistently observed. Focal blebs of mitochondria were frequently seen in neurons, but only very rarely in astrocytic mitochondria. Normal dendrites occasionally show fusiform dilation and rarification, usually associated with apparently greater density of neurotubles and neurofilaments in immediately adjoining dendrites and axons. Electron micrographs of material from normal-normothermic controls are presented for comparison with the experimental material in Figures 3, 7, 9, 11 and 15 and are described in the legends to these figures.

Ischemia-normothermia

Figures 4, 5, 6 and 8 are electron micrographs of ischemic-normothermic motoneurons; Figure 10 is that of an astrocyte and Figure 12 is that of a small blood vessel. Some neurons differed only subtly from normal morphology, showing only modest swelling of the Golgi apparatus. Some differed from normal by showing only dispersion of polysomes into individual ribosomes. The characteristic change of neuronal perikarya and dendrites, however, was increased electron density of the cytoplasm, with dense packing of ribosomes and neurotubules and occasional collections of glycogen particles. Embedded in this dense matrix were grossly swollen Golgi vesicles and enlarged vacuous mitochondria. Mitochondrial inner or outer

membranes were parted and the pattern of cristae grossly disorganized. However, mitochondria with virtually normal morphology could always be observed in the same cell. Cisterns of rough endoplasmic reticulum responded variably, sometimes retaining normal shape, with or without increased electron-dense material within the cistern, sometimes showing modest dilation and sometimes apparently disappearing altogether. Lysosomes (dense bodies) retained a normal appearance. Swollen dendrites were not encountered in ischemic material. Large dendrites behaved similarly to neuronal perikarya. Neuronal nuclei lost their normal plump appearance and became irregular in contour, with increased density of chromatin but without gross change in its distribution. The distance between inner and outer nuclear membrane was somewhat increased. There was no apparent change in nuclear pores.

Synaptic vesicles of axon terminals (Figures 6 and 8) displayed the same range of changes observed by Williams and Grossman [9]—*ie*, apparent diminution in number, pleomorphism and clumping away from the synaptic cleft. The size of axon terminals did not change. Mitochondria of axon terminals increased in size, but without loss of matrix density or change in pattern of cristae. Axons and their mitochondria behaved in the same manner as axon terminals. Neurotubules, neurofilaments and smooth endoplasmic reticulum of axons were unchanged.

Astrocytic cytoplasm (Figures 8, 10, 12) underwent massive "watery" swelling. Processes of astrocytes were ubiquitously enlarged and probably ruptured and merged together. Astrocytic microfilaments were greatly spread apart from their normal compact bundles and apparently fragmented. When occasional normal appearing compact bundles were encountered, they always occurred in *unswollen* processes. Astrocytic mitochondria enlarged in size, but usually without any changes in matrix density, pattern of cristae or membrane integrity.

Capillary endothelial cells and their surrounding basement membranes were unchanged. Oligodendroglia were unchanged. Insufficient data were available to evaluate satellite cells, axon hillocks or nodes of Ranvier.

Normal-hypothermia

Specimens from these animals (Figures 13, 14 and 16) were qualitatively indistinguishable from normal-normothermic animals

except in one respect. Neurons of hypothermic cords contained electron-lucent crescent-shaped clefts in aggregates of rough endoplasmic reticulum (the Nissl bodies of light microscopy). In electron micrographs these clefts were typically about 3 μ long and generally less than 1 μ wide at their widest point. They were found in every neuron observed without exception, and were never found in astrocytes, oligodendroglia or in capillary endothelial cells. These clefts had a characteristic morphology which distinguishes them from simple dilation of the cisterns of the rough endoplasmic reticulum. Along the concave face of the cleft an intact membrane of the endoplasmic reticulum, with a more or less normal distribution of ribosomal rosettes nearby, was usually found. Within the cleft, immediately adjacent to the intact face, shreds of the membrane forming the other wall of the cistern could be seen. Occasionally, clear vesicles were observed within the clefts, which appeared to be formed from these shreds. Along the convex side of the clefts were seen normal ribosomal rosettes, which appear to be those torn away from the cistern.

No differences between 1 and 2 hours of hypothermia were observed.

Ischemic-simultaneous Hypothermia

Tissue from animals in which hypothermia was induced *simultaneously* with ischemia were indistinguishable from normal except for the hypothermic clefts described above (Figure 17). Clear vesicles in the hypothermic clefts were more frequent in the ischemic animals than in nonischemic-hypothermic animals.

Ischemic-delayed Hypothermia

Tissue from animals in which induction of hypothermia was *delayed* until 15 minutes after restoration of blood flow (Figure 18) was similar to tissue from animals subjected to ischemia alone, without hypothermia. Certain subtle differences were observed, however, which require quantitative treatment to document more adequately. Consistent differences were restricted to the astrocytes. First, compact and apparently normal bundles of microfilaments were observed in *swollen* processes. Second, there appeared to be a greater tendency toward massive swelling of astrocytic mitochondria with loss of matrix density. Third, preliminary quantitative results indicated that the mean size of astrocytic processes in ischemic-

delayed hypothermia material was only 50% greater than normal, compared to about 400% increase in ischemic-normothermic material.

Our findings are summarized in Table 1.

Table 1—Summary of findings

Ischemia-normothermia
Neuron
Shrinkage and increased electron density of perikaryon
Increased electron density of dendrites
Axon terminals unchanged in size, with loss of synaptic vesicles
Swelling of Golgi apparatus
Dispersal of polysomes into single ribosomes
Swelling with loss of matrix and pattern of cristae of mitochondria of perikaryon and dendrites
Enlargement without loss of matrix or cristae of mitochondria of axons and axon terminals
Lysosomes unchanged
Astrocyte
Massive watery swelling of cytoplasm
Spreading and fragmentation of bundles of microfilaments
Enlargement without loss of matrix or cristae of mitochondria
Normal-hypothermia (1 and 2 hours local hypothermia)
Morphologically normal except for clefts in rough endoplasmic reticulum of neurons
Ischemia-simultaneous hypothermia
Morphologically normal except for clefts in rough endoplasmic reticulum of neurons
Ischemia-delayed hypothermia
Morphology substantially identical to that of ischemic-normothermic animals

Discussion

The spinal cord offers many advantages for such a study as this, since its blood flow can be unequivocally interrupted and its temperature lowered and directly measured without compromising the animal's overall cardiovascular function. The first experiment demonstrating hind limb paralysis subsequent to aortic occlusion is attributed to Nicolas Steno (Niels Stensen) in his *Elementorum Myologiae Specimen*, published in Amsterdam in 1669. Eighteenth, nineteenth and early twentieth century studies have been reviewed by Rexed.[10] In addition to the immense background of earlier literature, the more recent work of Gelfan and Tarlov [11] and of van Harreveld and Schadé [12] rendered physiologic studies of our preparations unnecessary to establish that 45 minutes of circulatory arrest to the spinal cord would produce hind limb paralysis. The ischemic time period chosen for this study could be anticipated to destroy virtually

all small interneurons while damaging only a portion of the large spinal motoneurons of the anterior horn.[13] We have focused our attention upon the anterior horn neurons in the hope of discerning a spectrum of cell changes which would be suggestive of underlying mechanisms. While motoneurons are recognized to be among the least vulnerable neurons of the central nervous system, 45 minutes of total ischemia is sufficient to alter virtually all cells to some degree.

Ischemia

We have attempted to review and analyze all published literature on the ultrastructure of ischemic, anoxic or hypoxic central nervous tissue, both *in vivo* and *in vitro*. From this literature, we selected for discussion reports which are relevant to our experimental design, technics and analysis of data.

There are some respects in which our findings differ from those of other workers because we have had the benefit of quantitative study of our material.[14] Yamamoto, Bak and Kurokawa [15] and van Harreveld and Khattab [16] state that ischemia or anoxia cause swelling of presynaptic endings. This is not borne out from their published pictures and probably reflects the optical illusion which arises when a profile previously seen to be packed with structures is found to be "empty." We have found many empty profiles of axon terminals, which on measurement, proved to be unchanged in mean size. An opposite sort of optical illusion occurs when a structure actually increases in size but is contained within another structure which has undergone yet greater increase. Contrary to what we have found, Garcia, Cox and Hudgins,[17] Bakay and Lee,[18] and McGee-Russell, Brown and Brierley [19] reported no substantial change in the mitochondria of astrocytes. Measurement of the astrocytic mitochondria in their published pictures strongly suggests that they had indeed undergone considerable enlargement, but that this enlargement was overlooked because the mitochondria had not lost matrix density and were floating in the lucent cytoplasm of swollen astrocytic processes.

Different experimental and preparative procedures may account for some of the differences in findings on neuronal mitochondria and cytoplasm. Most workers have found massive swelling of the mitochondria of neuronal perikarya to be a hallmark of ischemia and anoxia. However, this was observed by neither Karlsson and Schultz [20] nor by van Nimwegen and Sheldon.[21] It should be noted that these investigators rendered the brain ischemic by washing the blood from

the brain with saline and then perfusing with fixative at specified intervals thereafter. Both papers report "dumbbell" and ring-form mitochondria but no grossly swollen vacuous ones. The washing fluid itself may be responsible for this difference, in that these workers may have inadvertently rendered the animals hypothermic, thus obscuring the progress of the ischemic lesion. Neither paper states the temperature of the washing fluid. Investigators of ischemia and anoxia must be constantly aware of the decisive effects of temperature, even where they have little interest in temperature as such. In their study of the "no-reflow phenomenon," Olsson and Hossmann [22] flushed the cerebral vasculature with room temperature saline. Olsson and Hossmann attributed a therapeutic vascular effect to saline perfusion in ischemia, which in our view is due exclusively to the effect of lowering the temperature of the brain by the room temperature saline washout.

Increased electron density of cytoplasm of ischemic or anoxic neurons has only been reported by (or can be seen in the published pictures of) workers who have fixed by perfusion with aldehydes, postfixed with osmium and stained with lead and/or uranium ions. Increased cytoplasmic density is not reported by any worker who fixed by immersion or perfusion with an osmium fixative. Immersion fixation in osmium tetroxide is not necessarily synonymous with poor fixation, since the fixation of rabbit retina achieved by Webster and Ames [23] using immersion in an osmium fixative is excellent. We do not feel the question can be answered with the materials at hand.

The central nervous system has special anatomical, vascular and cellular properties, some of which may contribute to its marked vulnerability to ischemia. One of these is the presence of the astrocyte. Virtually all investigators agree that astrocytes swell in ischemia. Occurring within a nondistensible dura and skull, this may contribute to the death of a victim of a massive stroke. Chiang, Kowada, Ames *et al* [24] implicate extravascular compression of capillaries by swollen astrocytic processes in the failure of reperfusion after a period of ischemia (no-reflow phenomenon). There is no evidence at present that astrocytic swelling contributes directly to the cellular vulnerability of neurons to ischemia or anoxia.

In our opinion, it is possible to offer a straightforward mechanistic explanation for the swelling of astrocytes in ischemia and anoxia. Astrocytes have a high resting potential and internal ionic composi-

tion similar to that of neurons, and a high membrane permeability to potassium.[25] Ames [26] has calculated that after failure of the neuronal sodium pump, the earliest event is an exchange of sodium for potassium, causing the neuron's internal sodium concentration to rise and that of its potassium to fall. However, appreciable shifts in chloride and water cannot occur until the bulk of the intracellular potassium has been exchanged for sodium. This calculation is based on the assumption that at equilibrium, there is electrical neutrality and osmotic equilibrium, and that the Gibbs-Donnan relationship, with potassium and chloride as relatively permeant ions, obtains. During the early phase when the neuron is exchanging potassium for sodium, *reciprocal changes in sodium and potassium concentration must be occurring in the extracellular space,* particularly in ischemia which converts the tissue into a "closed system." The swelling of astrocytes can then be explained if we assume one further factor—*ie,* that the sodium–potassium pump of neurons fails sooner than that of astrocytes after oxygen or blood has been cut off. Increased potassium in the extracellular space would then lower the resting potential of the astrocyte membrane, resulting in a net influx of chloride, potassium and water into the astrocyte, causing it to swell. This would be an isoosmotic swelling of astrocytes as described by Bourke *et al.*[27] The key assumption of this hypothesis lies in the time sequence of neuron versus astrocytic pump failure. However, the phenomenon of astrocytic swelling is equally consistent with the assumption that the neuronal membrane is much more permeable to sodium than that of the astrocyte, resulting in a much more rapid exchange of sodium for potassium in the neuron. The more slowly exchanging astrocyte would then be presented with a high extracellular concentration of potassium ion and swell as described above. The assumption of greater relative sodium permeability of neurons is not supported by findings in experimental ouabain intoxication, where the ion pumps of both neurons and astrocytes presumably fail at the same time. If time sequence is the important factor, when both fail simultaneously, one would predict that atrocytic swelling would not be prominent and that neuronal elements would swell as much or more. This, in fact, has been shown by Towfighi and Gonatas,[28] who found that astrocytic swelling was restricted to the boundary of the rapidly expanding "mass lesion" caused by massive dendritic swelling due to intracerebral injection of ouabain.

The role of astrocytic microfilaments in the astrocyte's response to

ischemia is unclear. We observed that intact bundles of astrocytic filaments were never found in swollen astrocytic processes in ischemic-normothermic tissue. When filament bundles were found they were always in nonswollen processes. In addition to the ionic mechanism of astrocytic swelling proposed above, hydrolysis of filamentous protein could contribute to swelling by generation of more osmotically active solute. In ischemic tissue with delayed hypothermia we observed compact bundles within swollen processes. Preliminary statistical studies of this material indicate that the degree of swelling of astrocytic processes was intermediate between that of the normal controls and that of the normothermic-ischemic cords. Lesser swelling in ischemia with delayed hypothermia could reflect the lesser degree of destruction of filamentous protein. This is suggested by the retention of intact fiber bundles in swollen processes of delayed hypothermia cords.

The shrinkage of neurons which we have demonstrated in ischemia can be explained only with difficulty, especially with questions of fixation artifact remaining unanswered. Whatever its basis, the shrinkage reflects a net loss of water by the neurons. Since the cells must remain in osmotic equilibrium with the surrounding structures, this water loss can reflect either an increase in osmotic activity outside the neuron or a decrease in osmotic activity within the neuron or both. Generation of an additional osmotic load by hydrolysis of astrocytic filamentous protein could lead to a dehydration of the neurons to raise their osmotic pressure to equal that of the astrocytes. On the other hand, soluble macromolecules presumably make a substantial contribution to the osmotic pressure within the neuron. Any precipitation or crosslinking of previously soluble macromolecules would result in a *decrease* in the total osmotic pressure within the neuron. Water would then leave the cell unless there were a concomitant decrease in the osmotic activity outside the cell. However, as we have seen, the most likely change to be occurring outside, if any, is an *increase* in osmotic activity outside the neuron. Decreased internal pH, increased unbound calcium ion concentration, and rearrangements of disulfide bridges and sulfhydryl groups due to the shift in the oxidation-reduction potential of the cytoplasm can all be anticipated to lead to precipitation of at least some sensitive proteins. A proportionate amount of water would then have to leave the cell to restore osmotic equilibrium.

Swollen mitochondria have been seen in ischemic neurons by all

workers in this field except Karlsson and Schultz,[20] and van Nimwegen and Sheldon,[21] who, as pointed out above, may have inadvertently induced hypothermia. It is tempting to conclude that the mitochondria are the site of the irreversible lesion of ischemia, forever denying the cell new sources of usable energy. However, a variety of findings should engender caution in acceptance of this hypothesis. There is at least one condition (puromycin intoxication) which causes similar swelling of mitochondria with loss of matrix density and disruption of pattern cristae which has been found to be completely reversible.[29] Histochemical technics have been of limited value so far, since they have only been able to demonstrate the oxidative enzymes. These enzymes are not depressed when the mitochondrion is uncoupled, and in any case have been found to maintain their activity by histochemical assessment for many hours of ischemia.[30]

Caution must be used in drawing conclusions from isolated mitochondria from ischemic tissue for the following reasons: our study clearly demonstrates that there are at least three distinct subpopulations of CNS mitochondria, those of the neuronal perikarya and dendrites, those of axons and axon terminals, and those of the astrocytes. As there is no good bookkeeping procedure for mitochondria, it is difficult to determine if the swollen mitochondria are not lost during homogenization and fractionation. In every neuron examined, unswollen mitochondria could be found whose functional capacities are unknown. Most preparative procedures for isolated mitochondria are at pains to remove the loose "fluffy" coat which occurs when mitochondria are prepared by centrifugation, such as in the method of Ozawa.[31] Yet one would expect that ischemia or anoxia causes precisely those changes in mitochondria which would cause them to sediment with the discarded fluffy coat. These considerations apply to the work on mitochondria isolated from anoxic rabbit brain by Silverstein *et al*[32] and from ischemic rabbit brain by Schutz *et al*,[33] with the additional complication that their preparations may be enriched to unknown degrees by mitochondria from the glial, axonal and axonal terminal, or perikaryal and dendritic subpopulations.

The very large number of unswollen mitochondria in axons, axon terminals and astrocytes also raises some questions about the significance of biochemical measurements of the reappearance of ATP in the whole tissue after the ischemic episode is terminated. The lost contribution of the mitochondria of neuronal perikarya and dendrites may not be detectable in the face of a very large retained contribu-

tion of the mitochondria of axons, presynaptic endings and glia. Yet selective destruction of the mitochondria of neuronal perikarya would clearly be disastrous.

The lysosome hypothesis of the mechanisms of ischemic damage is neither strengthened nor weakened by our findings. We have seen no apparent change in form or size of lysosomes. Our findings do not support those of Becker and Barron [34] or Khattab [35] that lysosomes swell in ischemia. The work of Clendenon *et al* [36] indicates little change in the nonsedimentable lysosomal enzymes during the time period during which the lethal changes appear to occur. In our material, the lysosome was the only organelle whose appearance was *unchanged* by ischemia.

The role of the extracellular space in the production of ischemic CNS lesions remains obscure. Van Harreveld [37] and his co-workers have made a convincing case that the narrow extracellular space seen in normal CNS fixed by conventional means is itself a consistent artifact of fixation. The evidence is now overwhelming [25] that the normal neuron exchanges materials with the blood stream via the extracellular space. Our methods of fixation do not allow any conclusions concerning the fate of the extracellular space in ischemic spinal cord. However, in our opinion, it is possible that the imbibition of extracellular fluid by anoxic cells could lead to an occlusion of the extracellular space thus limiting the exchange between neuron and blood when blood flow or oxygen is resupplied.

Hypothermia (Nonischemic)

Previously reported work on the ultrastructure of hypothermic mammalian central nervous tissue is rather sparse and not in agreement with our own findings. Klatzo *et al* [38] found hypothermia to have no ultrastructural effect on cat periventricular brain tissue. Yamamoto, Bak and Kurokawa [15] found considerable swelling of dendrites and dendritic mitochondria with a greatly widened extracellular space in guinea pig olfactory cortex slices. Pasztor and Hamori [39] found only a subtle darkening at the synaptic junction, which they documented with densitometer tracings. No previous workers have reported clefts in rough endoplasmic reticulum. We are unable to explain the mechanism of their formation or their possible significance. Rosomoff and Gilbert [40] found a 4% decrease in brain volume of dogs undergoing whole body hypothermia to 25 C. If the different components of brain tissue shrink to different degrees, one might

expect that mechanical stresses developing within the tissue could manifest themselves as "tears" at the weakest point. There is no evidence aside from our data that the region between the cisterns of rough endoplasmic reticulum and the adjacent row of ribosomes is mechanically weak.

We have observed no effect of hypothermia on neurotubules, as previous reports of the effects of low temperature on microtubules might lead one to expect.[41–43]

Combined Ischemia and Hypothermia

When hypothermia was instituted at the same time that blood flow was arrested ("simultaneous hypothermia"), the morphology of the tissue was substantially identical to that of hypothermic tissue without ischemia. Our findings do not offer any evidence concerning the mechanisms of hypothermic protection.

When hypothermia was instituted after the end of the ischemic episode and after a brief period of normothermic restitution, little significant difference was found, compared to tissue suffering ischemia alone, with the exception already discussed above. Unfortunately, our hope of finding a therapeutic effect of delayed hypothermia was disappointed.

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Legends for Figures

Fig 1—Normal-normothermic spinal motoneuron of cat. The boundary between the neuropil and the neuron can be seen along most of the neuron perimeter. The neuropil is compact and more densely stained than the adjoining neuronal cytoplasm, which is devoid of lucent vacuoles. The nucleus has a smooth circular profile, the nucleoplasm staining lighter than the surrounding cytoplasm. Nomarski interference contrast optics produce the shadowed effect (Toluidine blue, × 1300).

Fig 2—Ischemic-normothermic spinal motoneuron of cat. The neuron is surrounded by a lucent halo, and its cytoplasm is more darkly stained than the surrounding lucent region. The neuronal cytoplasm is filled with small lucent vacuoles. The nuclear profile is irregular and yet more darkly stained than the surrounding cytoplasm. Stain and optics as in Figure 1 (× 1300).

Fig 3—Normal-normothermic spinal motoneuron. Mitochondria are electron dense, cisterns of Golgi apparatus are flattened, ribosomes of rough endoplasmic reticulum are grouped in rosettes, nuclear chromatin is dispersed and nuclear envelope is smoothly curved. Note pleomorphism of lysosomes. *Star* denotes a lipofuscin granule (× 16,000).

Fig 4—Ischemic-normothermic spinal motoneuron: example of apparently less severe cellular change than shown below in Figures 5, 6, and 8. Cisterns of Golgi apparatus are swollen (*star*), ribosomes are dispersed and no longer found in rosettes, and nuclear envelope follows irregular course. Lysosomes, mitochondria and general lucency of cytoplasmic background do not differ appreciably from normal (× 16,000).

Fig 5—Ischemic-normothermic spinal motoneuron. Mitochondria are enlarged and electron lucent; however, note presence of electron-dense smaller mitochondria. Nuclear chromatin is clumped and of increased electron density; nuclear envelope has irregular contour with widening of space between inner and outer membrane (× 16,000).

Fig 6—Ischemic-normothermic spinal motoneuron. Cytoplasmic background has increased electron density. Ribosomes are not organized in rosettes, but found individually along cisterns of rough endoplasmic reticulum. Most mitochondria of neuronal perikarya are swollen, as are vesicles of Golgi apparatus. Mitochondria of axon terminal retain approximately normal appearance (see text); synaptic vesicles are apparently diminished in number (× 20,000).

Fig 7—Normal-normothermic motoneuron and adjoining neuropil. Adjacent to the motoneuron surface are axon terminals and astrocytic processes. Distal to axon terminals lies a delicate feltwork of astrocytic processes which can be recognized by compact bundles of astrocytic microfilaments and by large mitochondria which characteristically have a homogeneous matrix with poorly defined cristae (× 16,000).

Fig 8—Ischemic-normothermic motoneuron and adjoining neuropil. Astrocytic processes are massively swollen with apparent net loss of microfilaments. Bundles of microfilaments in swollen processes are fragmented and spread apart. Compact bundles of microfilaments (*double arrows*) are found in unswollen processes. Astrocytic mitochondria are increased in size without change in matrix density or pattern of cristae (× 16,000). **Inset**—Enlargement of compact bundles of astrocytic microfilaments (*double arrows*) (× 48,000).

Fig 9—Normal-normothermic astrocyte. The cytoplasm has the normal complement of rough endoplasmic reticulum, microtubules and microfilaments, as well as mitochondria, and is not watery in its general appearance (× 13,000).

Fig 10—Ischemic-normothermic astrocyte. The cytoplasm has lost most of recognizable structures except for prominent mitochondria and a spread-apart, fragmented bundle of microfilaments. Mitochondria are enlarged in size but have not lost their electron-dense matrix (× 11,000).

Fig 11—Normal-normothermic small blood vessel. Lumen is devoid of blood cells. The perivascular investment of astrocytic processes is compact and made up of a multitude of such processes. They contain compact bundles of astrocytic microfilaments, mitochondria and glycogen particles (× 12,000).

Fig 12—Ischemic-normothermic small blood vessel. Lumen is devoid of endothelial blebs or blood cells. The endothelial and perithelial cells appear normal. The perivascular astrocytic investment is massively swollen with electron-lucent cytoplasm and devoid of any organelles except enlarged but normal appearing mitochondria (× 13,000).

Fig 13—Normal spinal motoneuron after 2 hours local hypothermia. Note clefts in Nissl bodies. Stain and optics as in Figure 1 (× 2700).

Fig 14—Large dendrite of same cell as Figure 13. Clefts are also found in Nissl bodies of dendrite. Stain and optics as in Figure 1 (× 1900).

Fig 15—Normal-normothermic dendrite and adjoining neuropil. An aggregation of cisterns of rough endoplasmic reticulum (Nissl body) occupies part of the dendrite. Ribosomal rosettes are arrayed between the parallel cisterns of the endoplasmic reticulum. An invagination of dendrite into two axon terminals forms pseudoinclusions in these synapses. Small collections of glycogen particles are not uncommon in axon terminals of cat spinal cord (× 14,000).

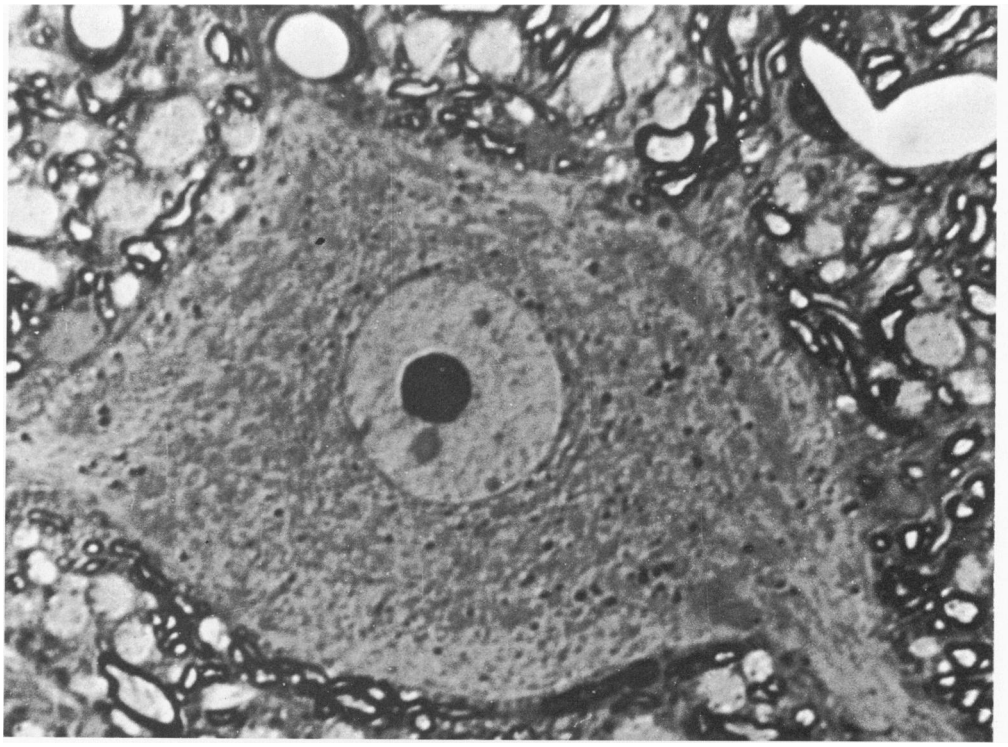
Fig 16A—Normal-hypothermic motoneuron (2 hours local hypothermia). Curved clefts in Nissl body are formed between a cistern and the row of ribosomal rosettes normally adjoining it. The convex aspect of the cleft is formed by the somewhat disorganized row of rosettes. The concave aspect is formed by a fragmented cisternal membrane (*double and triple arrows*) toward the inside of the cleft and an intact cisternal membrane toward the outside (× 14,000). **B**—Enlargement of part of a cleft of rough endoplasmic reticulum indicated by double arrows in Figure 16A (× 40,000).

Fig 17—Ischemic-simultaneous hypothermia. A dendrite contains rough endoplasmic reticulum and hypothermic clefts (× 12,000).

Fig 18—Ischemic-delayed hypothermic spinal motoneuron and adjoining neuropil. Compact bundles of astrocytic microfilaments are retained in swollen astrocytic processes. Astrocytic mitochondria appear swollen compared to those in ischemic-normothermic material (× 16,000).

Key to Figures

M = mitochondrion
G = Golgi apparatus
L = lysosome
N = nucleus
NE = nuclear envelope
RER = rough endoplasmic reticulum
AT = axon terminal
S = synaptic junction
SV = synaptic vesicle
AsP = astrocytic process
AsM = astrocytic mitochondrion
AsMf = astrocytic microfilaments
Gl = glycogen particles
VL = blood vessel lumen
PC = perithelial cell
Cl = cleft of rough endoplasmic reticulum
V = vacuole
Psl = pseudoinclusion



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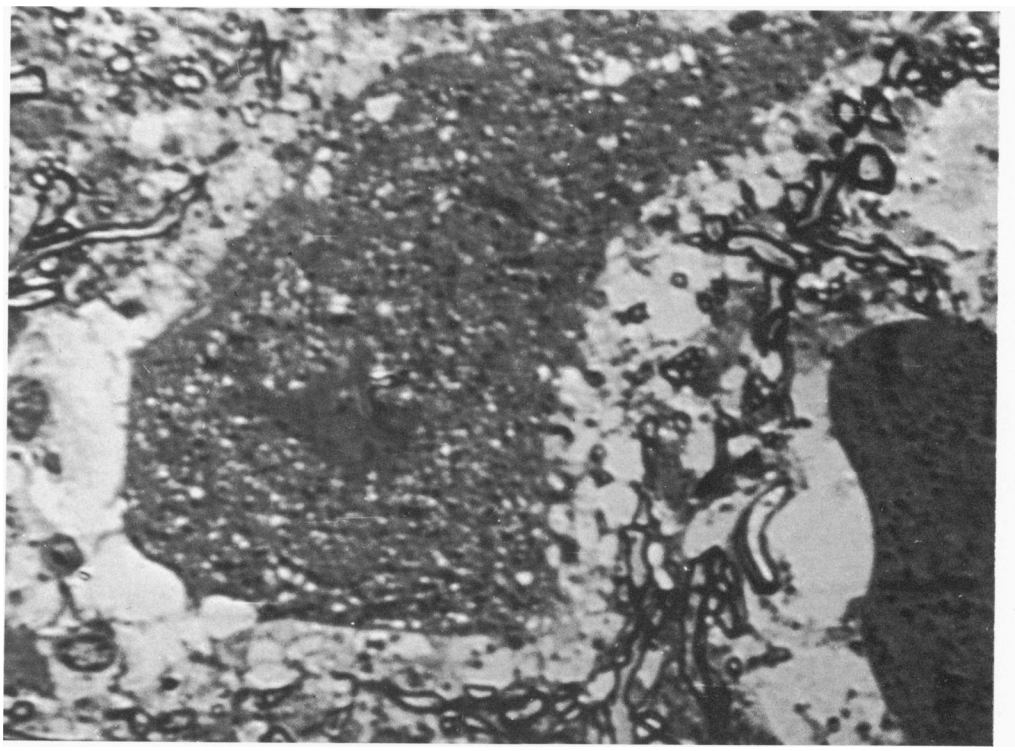

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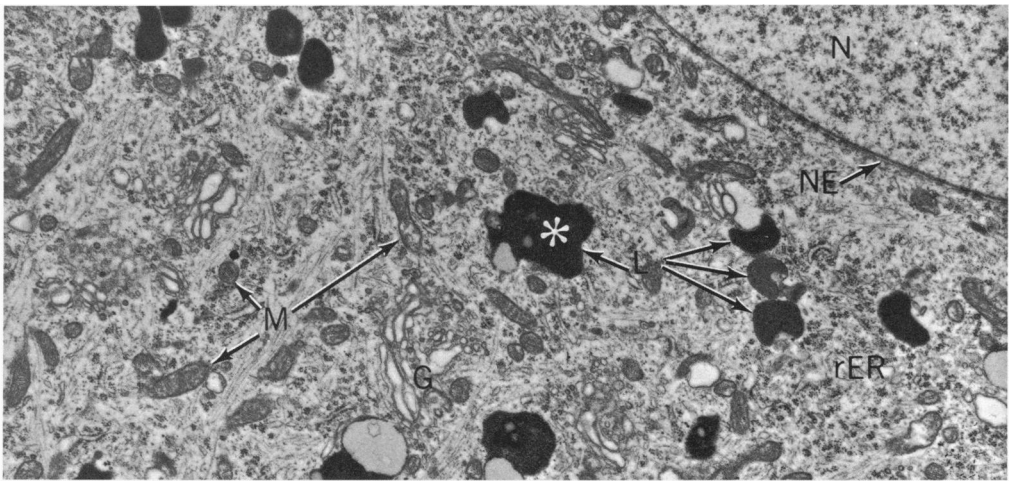

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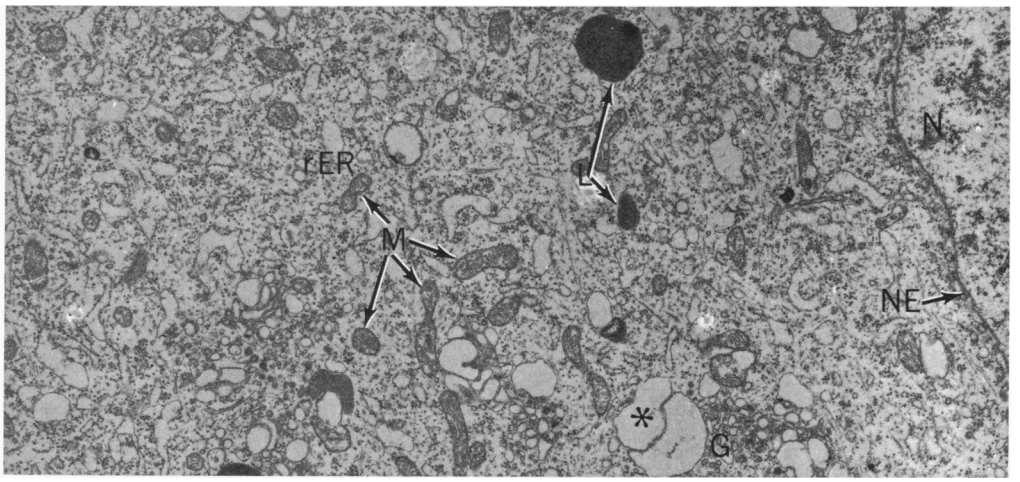

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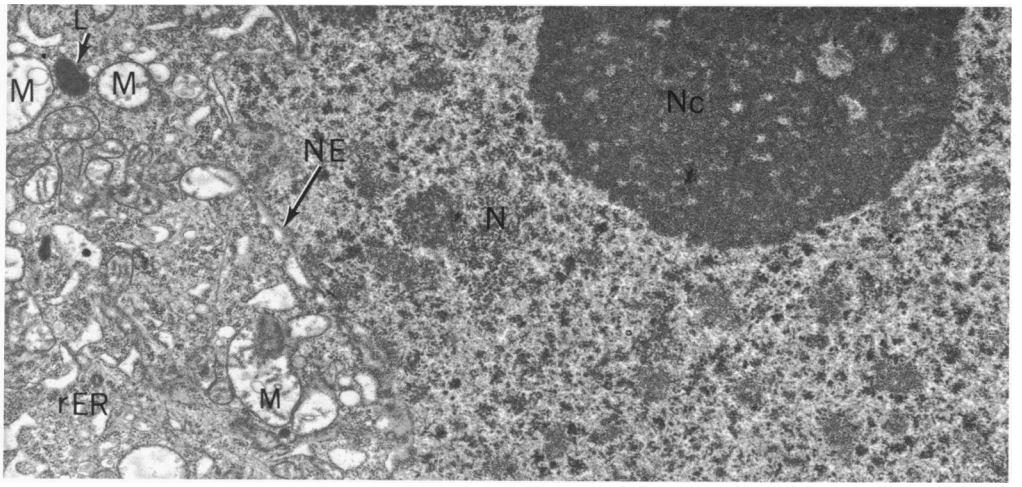

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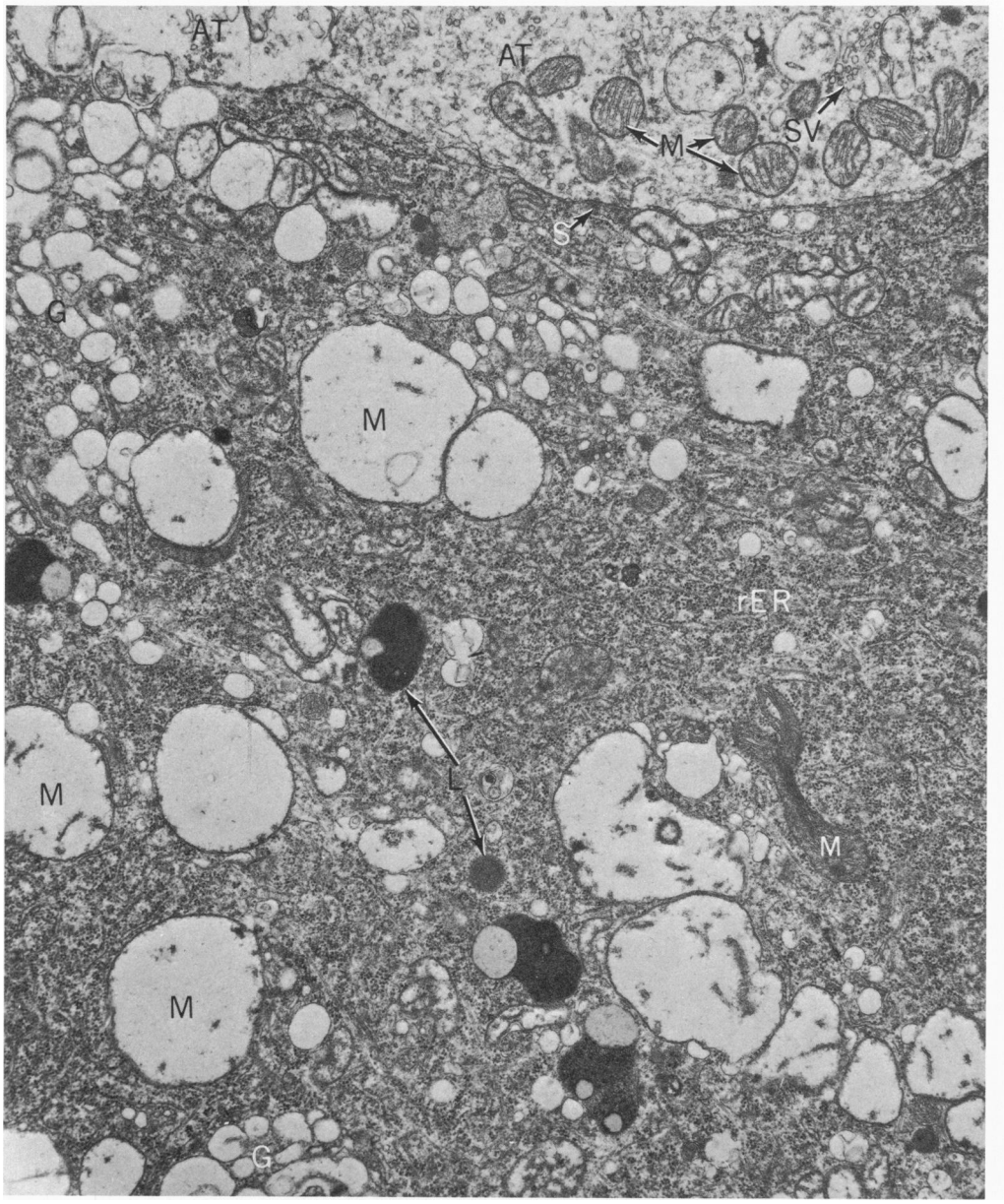

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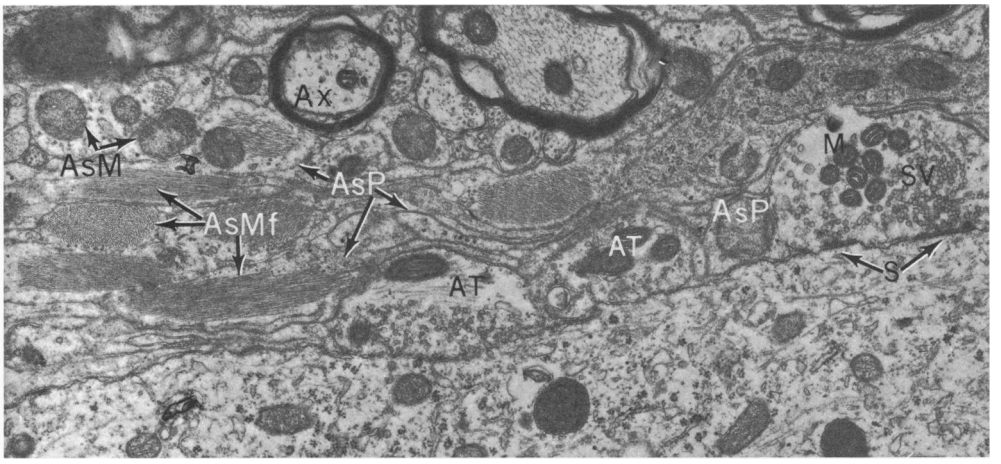
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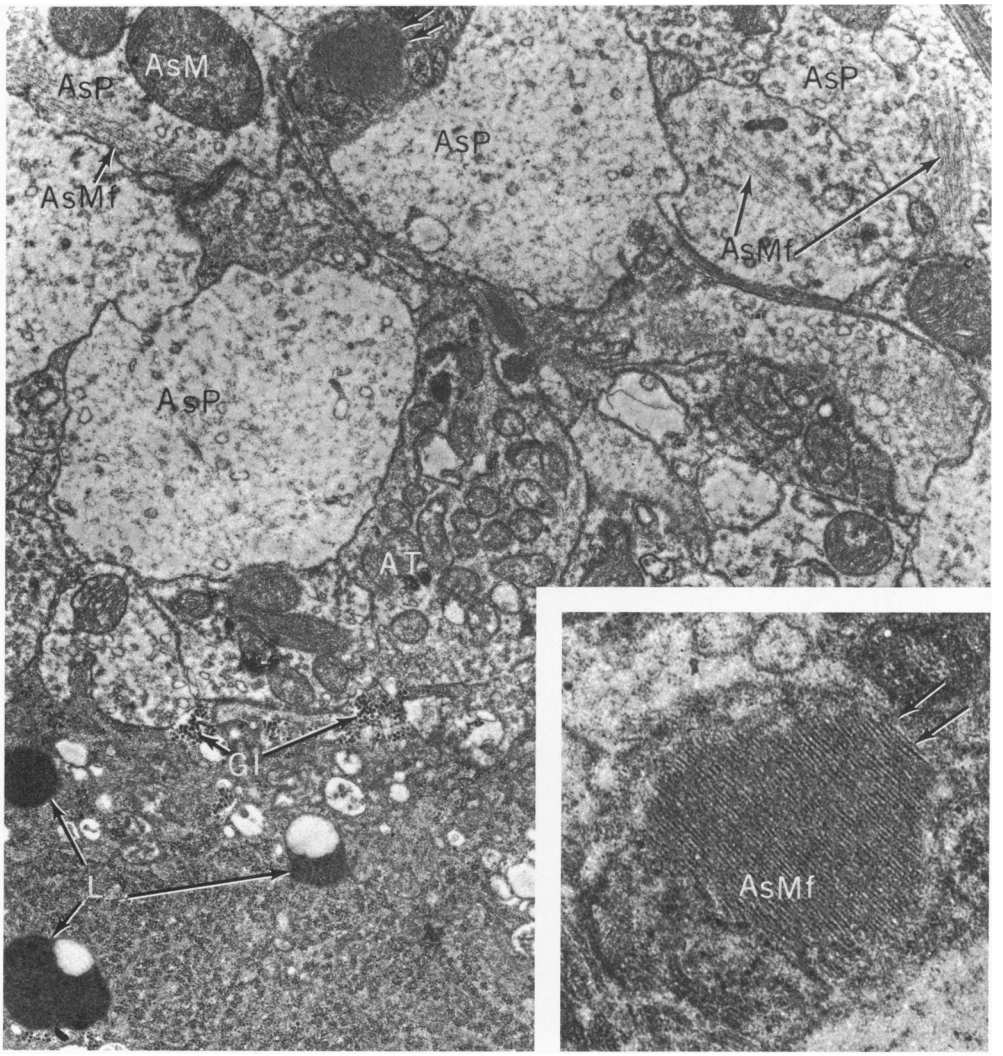
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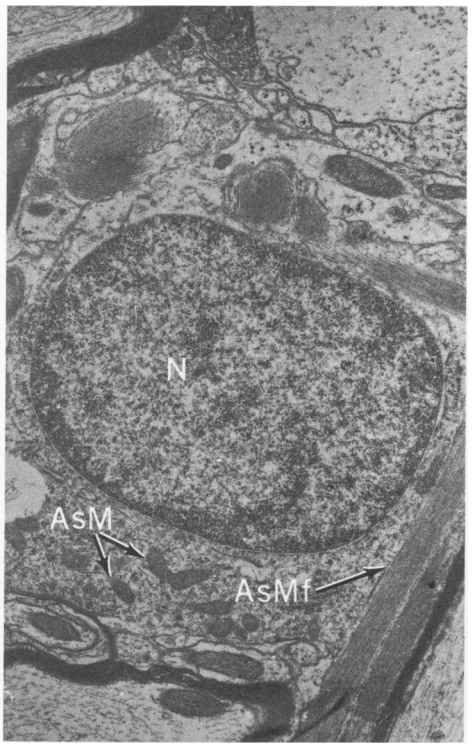

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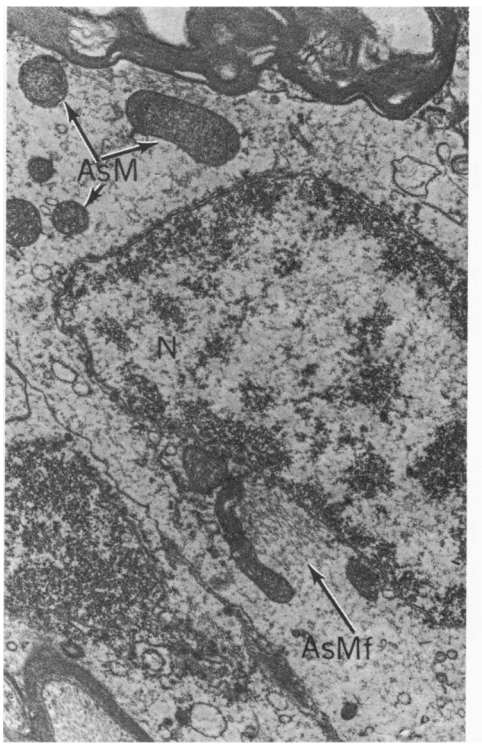

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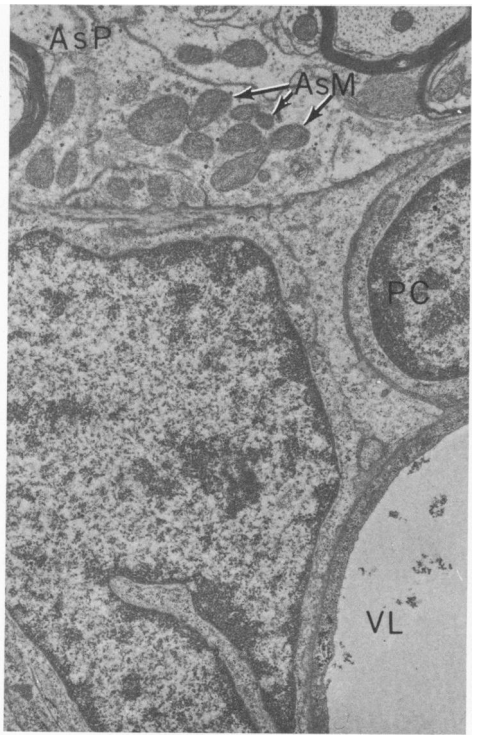

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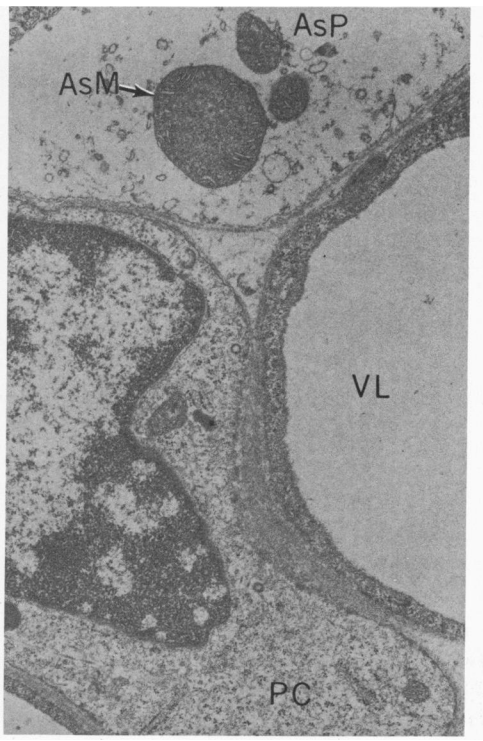

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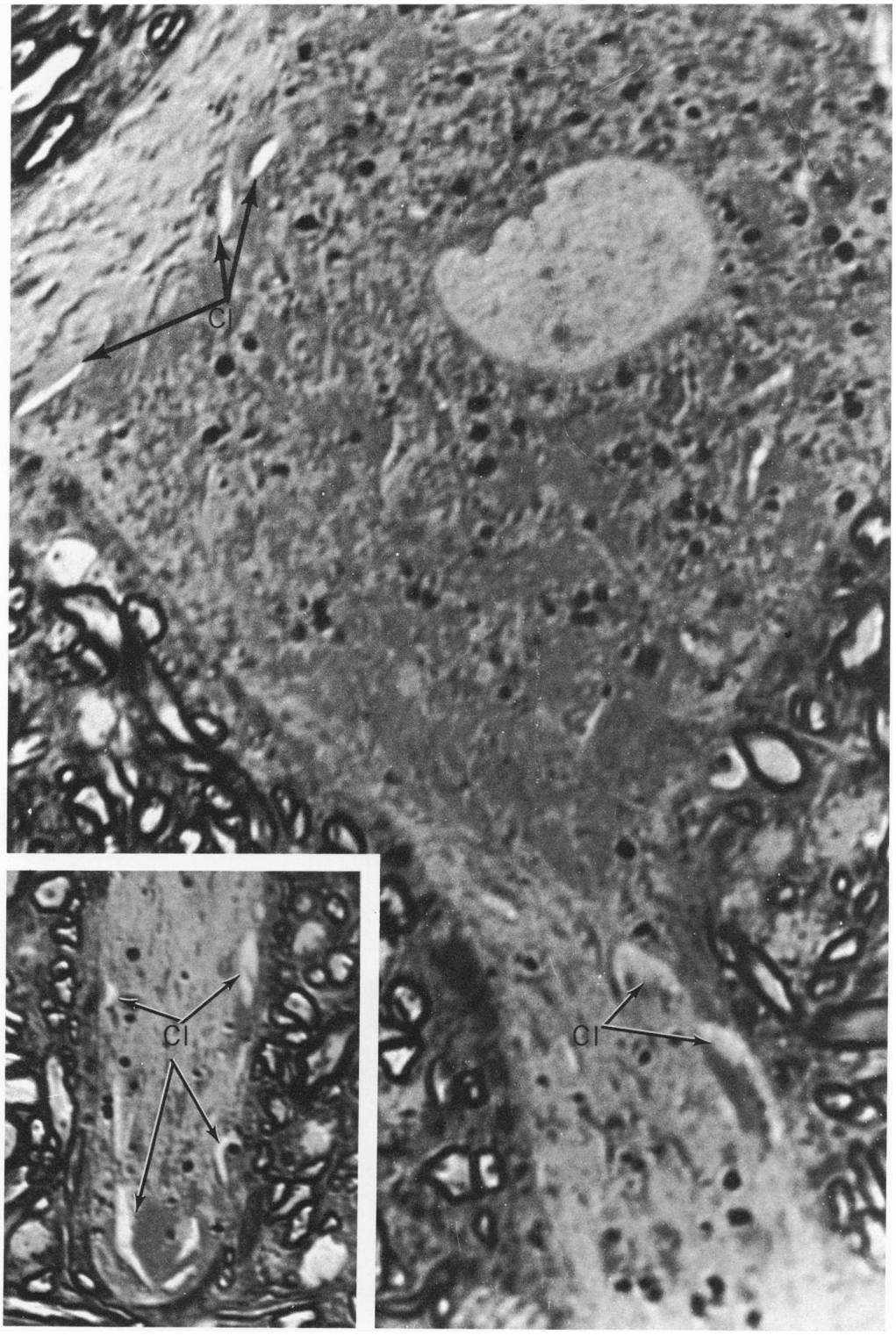

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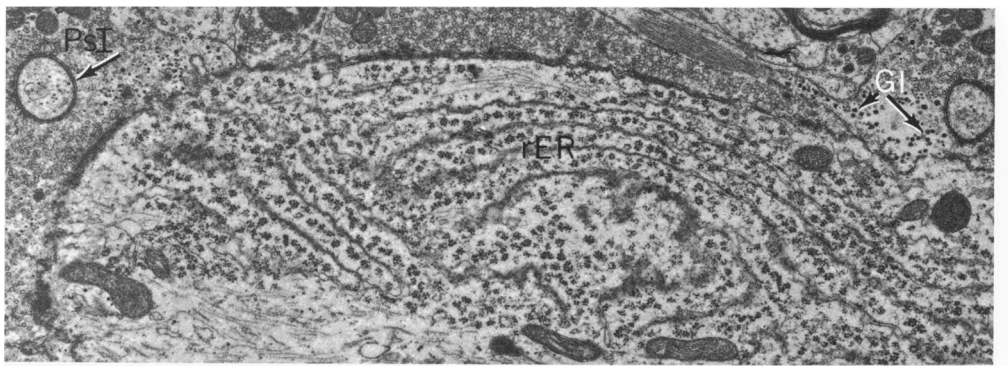

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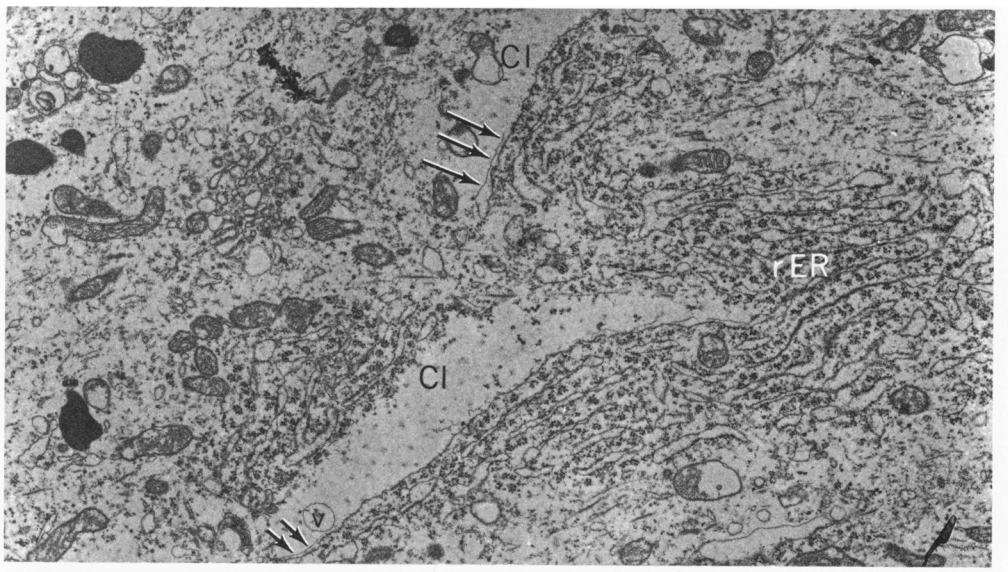

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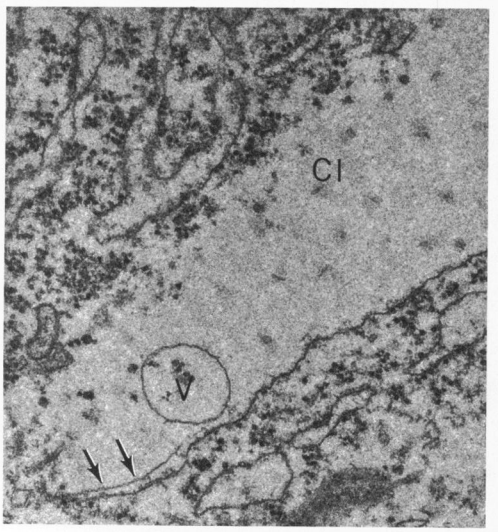

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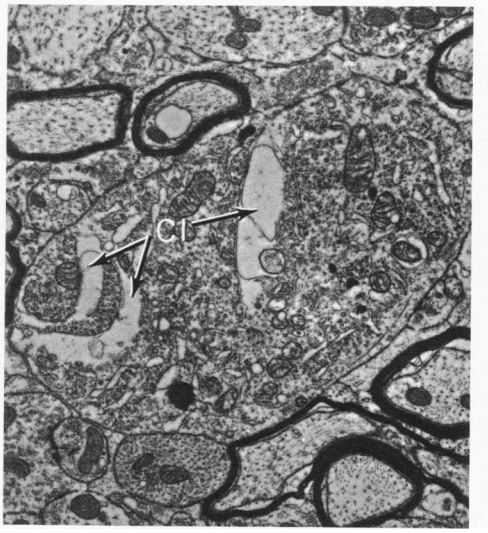

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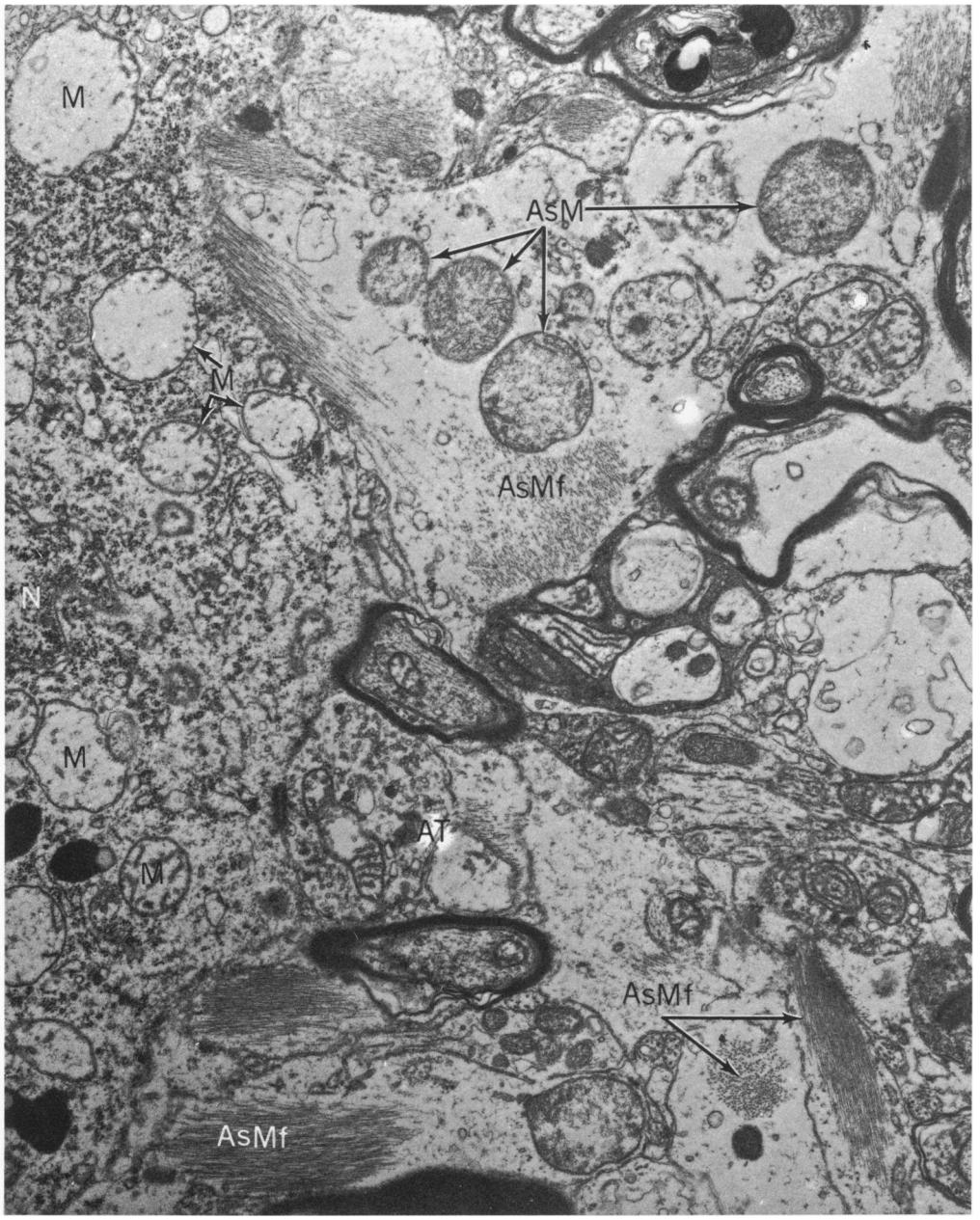

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