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THE RELATION OF AXONAL TRANSPORT OF MITOCHONDRIA WITH MICROTUBULES AND OTHER AXOPLASMIC ORGANELLES

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SUMMARY

Axonal transport of mitochondria was studied in frog sciatic nerves incubated in agents selected for their known or alleged effect on microtubules or axonal flow.

Quantitative data on mitochondria, microtubules, neurofilaments, endoplasmic reticulum, and cross-sectional area of the axon indicate that axonal transport of mitochondria is dependent on microtubules. When more than half of the microtubules are destroyed, the axonal transport of mitochondria is diminished in proportion to the destruction of microtubules.

Axonal transport of mitochondria is not related to neurofilaments and endoplasmic reticulum.

Changes in the cross-sectional area of axons, even upon reduction to half the normal size, do not noticeably affect mitochondrial transport.

Cyanide which blocks oxidative metabolism also blocks axonal transport of mitochondria, but analysis of fine structure indicates that cyanide is destructive to microtubules as well.

INTRODUCTION

Microtubules are thought to play a critical role in axonal transport as destruction of microtubules seems to interfere with axonal flow, especially with fast flow (Kreutzberg, 1969; Schlaepfer, 1971). In these experiments nerves were treated with mitosis-inhibiting agents, known to destroy microtubules, such as vinblastin and colchicine. However, few investigations include quantitative electron microscopic data on axoplasmic organelles, and in these experiments only the microtubules and rarely the neurofilaments were examined (Banks, Mayor, Mitchell & Tomlinson, 1971; Fink, Byers & Middaugh, 1973) because only the former are

presumed to be affected by spindle inhibitors. The possibility that the block of axonal flow may result from alteration of subcellular structures other than microtubules has never been tested in detail.

In the present experiments the effect of four agents upon the axonal transport of mitochondria was tested in segments of frog sciatic nerves *in vitro*. The densities of mitochondria, microtubules, neurofilaments, endoplasmic reticulum and the cross-sectional area of axon in the middle of the nerve as well as the density of mitochondria and the mitochondrial enzyme accumulation at nerve stumps were determined in hope of defining a precise relationship between the axonal transport of mitochondria and the various axoplasmic organelles.

METHODS

American bull frogs, *Rana catesbiana*, weighing 400–500 g, were kept in a cool room at 4° C for variable lengths of time and were allowed to warm up to room temperature for 1–2 hr before experimentation. The frogs were eviscerated under urethane anaesthesia and segments of sciatic nerves, 3 cm in abdominal portions and 5 cm in thigh portions, were prepared for enzyme studies; only abdominal segments were processed for electron microscopic studies. The nerves were incubated *in vitro* in the following test solutions at 20° C; the pH was maintained at 6·8–8·0 by bubbling 95 % O_2 and 5 % CO_2. Each nerve in a given set of experiments was from a different frog.

(1) Frog Ringer solution: NaCl, 110 mM; KCl, 1·9 mM; $CaCl_2$, 1·1 mM; $NaHCO_3$, 1·2 mM; glucose, 11 mM (Van Harreveld & Russell, 1954).

(2) Vinblastin 0·1 mM in frog Ringer (Schlaepfer, 1971).

(3) Colchicine 2 mM in frog Ringer (Kreutzberg, 1969; Hökfelt & Dahlström, 1971).

(4) NaCN 5 mM in frog Ringer (Ochs & Smith, 1971; Heslop & Howes, 1972).

(5) Reverse frog Ringer solution (concentrations of Na^+ and K^+ reversed in frog Ringer NaCl: 1·9 mM; KCl: 110 mM). This solution was used only in the study of the cross-sectional area of axon.

Nerves used for electron microscopy were incubated for 10 hr. Samples from the middle and the stumps of nerve segments were fixed in 1·5 % glutaraldehyde, post-fixed with 2 % osmic acid, embedded in Spur medium and stained with lead and uranium. The sections were examined under a Zeiss EM 9S-2 electron microscope at a final magnification of × 9500. Electron micrographs were enlarged × 3·2. Only fibres cut perpendicular to their axes were selected. The area of axonal cross-section was measured with an Ott precision disk planimeter by tracing the axolemmal membrane. Mitochondria, endoplasmic reticulum, microtubules and neurofilaments were counted per axon: their density was defined as the number of organelles per unit cross-sectional area of axon. Since myelinated fibres show considerable calibre-dependent variation in the ratio of microtubules/neurofilaments (Friede, 1970; Friede & Samorajski, 1970), most studies were done in non-myelinated fibres, which have a fairly uniform calibre range and relatively little variation in microtubule and neurofilament densities. Also, the non-myelinated fibres are more readily penetrated by test agents than the myelinated fibres. Approximately twenty-five axons per nerve were measured at random. In addition twenty myelinated fibres were studied in

a similar manner in nerves incubated in frog Ringer and in vinblastin. For control studies nerves were incubated in frog Ringer solution for 1, 2, 4, 6, 10, 12, 24 and 48 hr and examined under the electron microscope. For histochemical studies cryostat sections were prepared from the stumps, and NAD-disphorase activity was demonstrated, using nitro-BT as an electron acceptor (Friede, Fleming & Knoller, 1963). NAD-diaphorase was used as a marker for mitochondria. Its fairly even distribution in the cytoplasm of Schwann cells provides a point of reference for the evaluation of enzyme accumulation in the axon stumps (Friede, 1959). The enzyme reaction was quantitated by grading the histochemical staining for enzyme activity in axonal balloons. It was rated as absent (0), mild (1), moderate (2) and marked (3).

RESULTS

Electron microscopic study of sciatic nerves incubated in frog Ringer for 1, 2, 4, 8 and 10 hr revealed no apparent structural changes in axons other than degenerative alterations in a few mitochondria (Pls. 1, 2). There was slight swelling of axons and decrease in the number of microtubules in nerves incubated for 12–24 hr. Therefore, all nerves used in the subsequent electron microscopic study were incubated for 10 hr. Further tests showed that the nerves retained normal chronaxie and conduction velocity upon 10 hr incubation with all media except cyanide.

Electron microscopic analysis of axoplasmic changes in the middle of incubated nerve segments

The cross-sectional areas of the axons and the densities of mitochondria, microtubules, neurofilaments and endoplasmic reticulum were measured in 100 axons each (pooled from four frogs) incubated in frog Ringer, colchicine, vinblastin and NaCN. The mean values for each set of experiments were tested with the means of the other experiments by the Student t test (two tails); $P < 0{\cdot}05$ was considered statistically significant.

Axon calibres. The average cross-sectional area of non-myelinated axons in nerves incubated in frog Ringer, vinblastin and colchicine was fairly constant, the differences being slight and statistically insignificant. A significant increase in the cross-sectional area was found in nerves incubated in cyanide (Pl. 3, Table 1). The rate of increase was calculated as follows.

$$\frac{\text{Mean of the cross-sectional areas in nerves incubated in cyanide}}{\text{Mean of cross-sectional areas in nerves incubated in frog Ringer, vinblastin and colchicine}} = \frac{2{\cdot}254\ \mu m^2}{(1{\cdot}533\ \mu m^2 + 1{\cdot}564\ \mu m^2 + 1{\cdot}688\ \mu m^2):3} = 1{\cdot}4.$$

Because of the swelling of axons incubated in cyanide, all data for organelle density for this agent were corrected by a factor of 1·4. The correction did not significantly affect the interpretation of the experimental results.

TABLE 1. Cross-sectional area of axons (μm^2) and densities of mitochondria (mitochondria/μm^2) in the middle of axons after 10 hr incubation cross-sectional area

Incubation solution	Mean	S.D.	t	P	Significance (compared with frog Ringer)
Frog Ringer	1·533	0·918			
Vinblastin*	1·564	1·242	0·063	> 0·9	—
Colchicine*	1·688	1·322	0·851	< 0·4	−
Cyanide*	2·254	2·202	3·021	< 0·001	+
		Mitochondria			
Frog Ringer	0·414	0·648			
Colchicine†	0·686	0·971	2·333	< 0·02	+
Vinblastin†	1·069	1·613	3·770	< 0·001	+
Cyanide†	1·648	3·640	3·699	< 0·001	+

* The differences in the cross surface areas of axons between cyanide and vinblastin ($P < 0·01$) and between cyanide and colchicine ($P < 0·05$) are statistically significant.

† The difference between the densities of mitochondria in axons incubated in colchicine and vinblastin ($P < 0·01$) and the difference between those incubated in cyanide and colchicine ($P < 0·02$) are statistically significant; the difference between those incubated in cyanide and vinblastin is statistically insignificant.

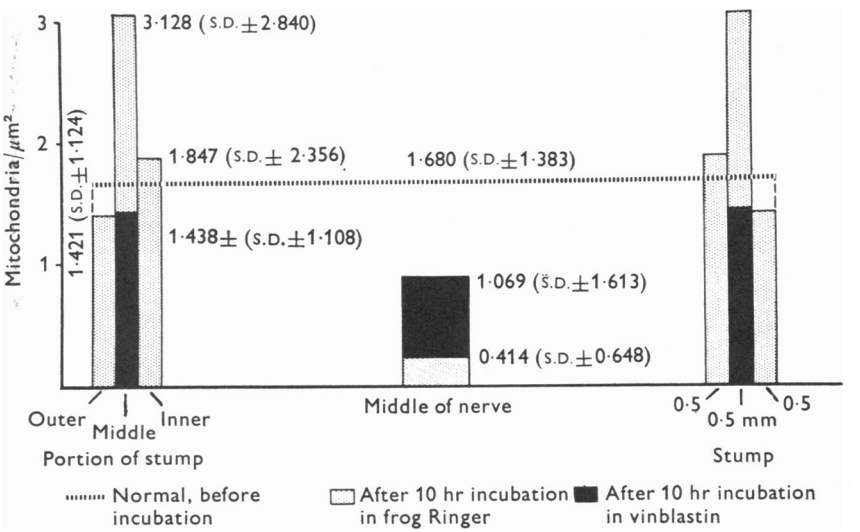
Mitochondrial density. Counts of mitochondria included all organelles containing either an outer membrane and cristae or an outer membrane and an inner membrane with granules or dots. Cystic structures resembling mitochondria but lacking these features were excluded from the counts, although their configurations left little doubt that they were degenerative forms of mitochondria.

The mitochondrial densities in the middle of nerve segments after 10 hr incubation in different solutions are shown in Table 1.

To determine whether the incubation agents had any direct effect on mitochondria the ratio of mitochondria with degenerative changes to all mitochondria was determined: vinblastin, 0·25 (mean) $\pm$0·32 (S.D.); frog Ringer, 0·32 $\pm$ 0·40; colchicine, 0·36 $\pm$ 0·36; cyanide, 0·38 $\pm$ 0·37. The degenerative changes are not necessarily to be considered specific for the incubation solutions. Some mitochondria may be destroyed or lysed by the incubation agents without displaying degenerative configuration, while others may undergo spontaneous changes. However, the figures do provide

a rough estimate for the effect of the incubation solution on mitochondria. Colchicine and cyanide incubation show a slightly greater and vinblastin a slightly lesser mitochondria degeneration than frog Ringer. The differences are slight and statistically not significant.

Since frog Ringer is the most physiological incubation solution, and since incubation in cyanide, vinblastin or colchicine shows no better preservation of mitochondria than frog Ringer, the higher mitochondrial density in the middle of nerves incubated in the latter agents (Table 1) indicates a retention of mitochondria due to an inhibition of mitochondrial transport to the stumps. This interpretation was verified by comparing mitochondrial densities in the middle of the nerve segments with corresponding changes in the stumps (Text-fig. 1 and Pl. 4).



Text-fig. 1. Incubation of nerve segments in frog Ringer causes a redistribution of mitochondria with an increase at the stumps and a decrease in the middle of the nerve segment. Incubation in vinblastin abolishes this effect.

Relation of mitochondrial density and axon calibre. The differences in the cross-sectional area of axons after 10 hr incubation in frog Ringer, vinblastin and colchicine were small and statistically not significant; yet, the densities of axonal mitochondria all differed significantly from each other (Table 1). This shows that mitochondrial translocation was not affected by changes of cross-sectional area. Further, the cross-sectional area of twenty-five axons incubated in 'reverse' Ringer was markedly decreased (mean of the cross-sectional area 0·774/μm^2, S.D., 0·224); whereas, that of

axons incubated in cyanide was increased. Yet, the densities of axonal mitochondria were elevated for both (mean density of mitochondria in axons incubated in 'reverse' Ringer, 0·536/μm^2; S.D. 1·120). This is further evidence that mitochondria density can change independently of the changes in axonal calibre.

TABLE 2. Densities of microtubules, neurofilaments and endoplasmic reticulum (organelles/μm^2) in the middle of axons after 10 hr incubation

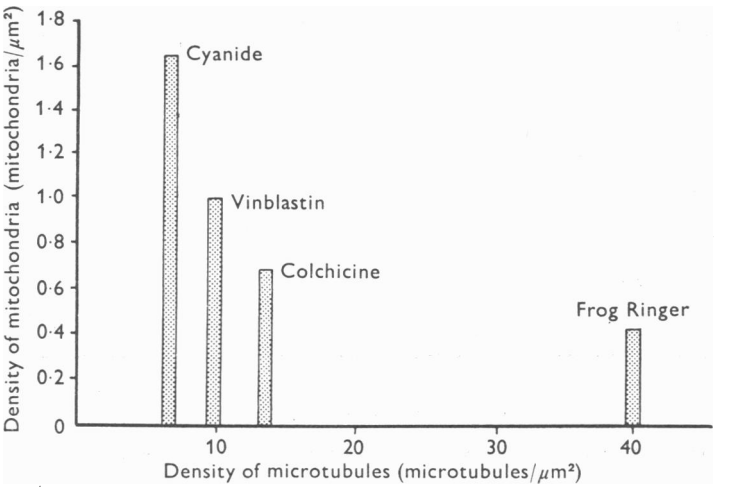
Incubation solution	Mean	S.D.	t	P	Significance (compared with frog Ringer)
		Microtubules			
Frog Ringer	39·44	16·30			
Colchicine*	13·42	9·68	14·024	< 0·01	+
Vinblastin*	9·86	7·73	16·674	< 0·01	+
Cyanide*	7·45	19·55	12·805	< 0·01	+
		Neurofilaments			
Frog Ringer	109·18	31·43			
Cyanide	112·07	84·99	0·306	< 0·8	−
Vinblastin	130·41	55·16	3·341	< 0·01	+
Colchicine	136·04	63·03	3·813	< 0·01	+
		Endoplasmic reticulum			
Frog Ringer	2·588	2·031			
Colchicine	1.321	1·634	4·859	< 0·001	+
Vinblastin	1·936	1·868	2·362	< 0·02	+
Cyanide	1·962	2·433	1·972	< 0·05	+

* The difference in the densities of microtubules in axons incubated in colchicine and vinblastin ($P < 0·005$) is statistically significant. The difference in those incubated in vinblastin and cyanide ($P < 0·02$) is insignificant.

Relation of mitochondrial and microtubular densities. Colchicine and vinblastin were found to destroy microtubules (Table 2). Many para-crystals were found, most aggregated to form a highly regular, hexagonal honeycomb structure (Pl. 5). The para-crystal formations appeared to be related to the destruction of microtubules, and it was more pronounced for vinblastin than for colchicine and more microtubules were destroyed by vinblastin. Cyanide was a more potent agent in destroying microtubules than either vinblastin or colchicine, but it did not produce paracrystal formation in the axoplasm.

Correlation of Tables 1 and 2 (shows Text-fig. 2) that the greater the loss of microtubules the greater the retention of mitochondria in the middle of the nerves. It may be emphasized that the increments in the retention of mitochondria and those in the degrees of destruction of microtubules from

frog Ringer to colchicine and from colchicine to vinblastin are all statistically significant. The great increment in the retention of mitochondria from vinblastin to cyanide is, however, not statistically significant because of the large scatter.

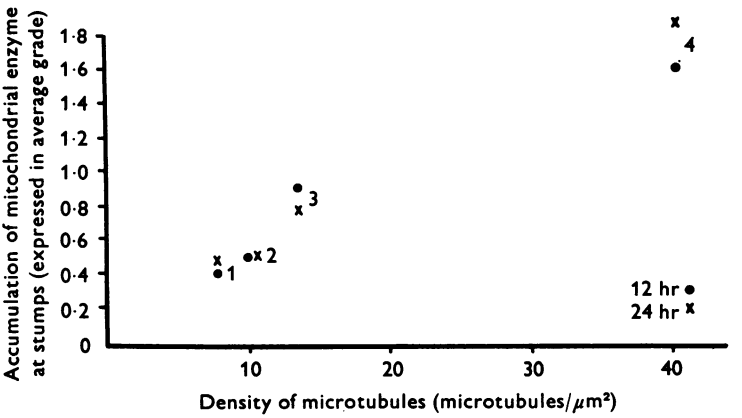


Text-fig. 2. Relation between the density of mitochondria to that of microtubules. The greater the destruction of microtubules the greater the retention of mitochondria in the middle of the nerve segment.

Relation of mitochondrial and neurofilament densities. The destruction of microtubules in nerves treated with vinblastin and colchicine was associated with an increase in neurofilaments (Table 2). However, the loss of microtubules was much greater than the increase of neurofilaments. Further, the decrease of microtubules in nerves treated with cyanide occurred without a corresponding increase in neurofilaments. Hence, the decrease in microtubules and the increase in neurofilaments were independent of each other. Comparison of Tables 1 and 2 shows that the retention of mitochondria is independent of density of neurofilaments.

Relation of mitochondrial and endoplasmic reticulum densities. Dilate cystic profiles were more frequent in nerves incubated in vinblastin or colchicine than in frog Ringer; they were especially prominent after cyanide incubation. Their configurations suggested their derivation from dilated tubules or cisternae of endoplasmic reticulum. Also, a concurrent decrease of identifiable endoplasmic reticulum was noted in axons incubated in these solutions (Table 2). Any dilated cystic or tubular profiles with a diameter of more than 1000 Å and any dense core vesicles were excluded from the present statistics; whereas, all the cisternal, tubular and vesicular components of endoplasmic reticulum were included. The data

show a significant decrease in endoplasmic reticulum upon incubation in colchicine, vinblastin and cyanide. Comparison of Tables 1 and 2 shows no relation between the retention of mitochondria and the total amount of endoplasmic reticulum.



Key to the numbers: 1 Cyanide 2 Vinblastin 3 Colchicine (2×10^{-3} M) 4 FR

Text-fig. 3. Relation between the accumulation of mitochondrial enzyme at stumps and microtubule density.

TABLE 3. Densities of mitochondria (mitochondria/μm^2) and microtubules (microtubules/μm^2) in the middle of myelinated axons after 10 hr incubation

Incubation solution	Mean	S.D.	t	P	Significance (compared with frog Ringer)
		Mitochondria			
Frog Ringer	0·427	0·425			
Vinblastin	0·719	0·421	2·194	< 0·05	+
		Microtubules			
Frog Ringer	32·84	13·57			
Vinblastin	15·84	6·15	5·194	< 0·01	+

Effect of various agents on accumulation of mitochondrial enzyme at axon stumps. In Text-fig. 3 the degree of microtubular destruction (Table 2) is compared with the degree of enzyme accumulation at nerve stumps, which is expressed as the mean value for 34 to 62 gradings of the intensity of NAD-diaphorase reaction. An inverse relationship between enzyme accumulation and microtubular density is evident. The findings are in agreement with and complementary to the findings shown in Text-fig. 2.

All the aforementioned organelle counts refer to unmyelinated fibres.

In addition, mitochondria and microtubules were counted in twenty myelinated fibres each incubated in frog Ringer and vinblastin. The destruction of microtubules corresponded to a retention of mitochondria in the middle of segments of myelinated fibres similar to that observed for unmyelinated fibres (Table 3).

DISCUSSION

The speed and mode of axonal transport of mitochondria is a matter of controversy. Weiss & Pillai (1965) assumed that mitochondria are transported en bloc along with the slow axonal flow; Zelená (1968) and Zelená, Lubińska & Gutmann (1968) reported rapid movement of mitochondria. Banks, Mangnall & Mayor (1969) gave a rate of 14 mm/day; Karlson & Sjöstrand (1971), 6–12 mm/day; Partlow, Ross, Motwani & McDougal (1972), 2–31 mm/day. The difficulties in assessing mitochondrial flow lie mainly in the lack of reliable markers. Certain oxidative enzymes may be used as mitochondrial markers (Banks *et al.* 1969; Partlow *et al.* 1972), but the mitochondria of Schwann cells constitute by far the largest share of the total mitochondrial population of the nerve and they may, in addition, be altered by experimental techniques of ligature or of injury to the nerve. Isotopic tracers (Jeffery, James, Kidman, Richards & Austin 1972; Elam & Agranoff 1971), on the other hand, possess low specificity and may be subject to redistribution among other axoplasmic organelles upon release from mitochondria.

In the present study direct counts of mitochondria were considered the most reliable technique. Such counts per random cross-section of nerve fibre may be criticized because mitochondrial density is thought to vary along myelinated axons with a crowding at nodes; however, Berthold (1968) showed that mitochondrial density is essentially uniform along the internodes of myelinated fibres. Further, an increased mitochondrial density at nodes has not been demonstrated for non-myelinated fibres on which the present study is mainly based.

The effect of colchicine and vinblastin on the axonal transport of mitochondria has been controversial. Mitochondrial accumulation was demonstrated in axons proximal to sites of injection of vincristin (Schlaepfer 1971). Jeffrey *et al.* (1972) reported inhibition of axonal transport of labelled mitochondrial protein by colchicine. The direct effect of these agents upon the mitochondria and upon the organelles other than microtubules was not studied. Conversely, colchicine (at least in submaximal dosage) had no effect on the transport of mitochondrial enzymes in Kreutzberg's (1969) experiments, but no electron micrographs were taken to determine whether a substantial number of microtubules had been

destroyed at the dosage used. Text-figs. 2 and 3 indicate little, if any, effect on the axonal transport of mitochondria when only a small fraction of the microtubules is destroyed. On the basis of electron microscopic studies, Banks *et al.* (1971) concluded that vinblastin and colchicine have little effect upon movement of mitochondria. The mitochondrial changes were estimated subjectively in terms of the number of mitochondria in electron micrographs of axon stumps. However, most nerves show a gradient in mitochondrial density toward the swollen stumps, and the stumps of the individual nerve fibre are not aligned; even at the same level of a given stump, the density of mitochondria varies greatly from one fibre to another, a fact reflected in the great standard deviations for the present data (Text-fig. 1). Therefore, unless the electron micrographs are taken at a very narrow level of stumps, and unless a large number of axons is examined, an electron microscopic assessment of the accumulation of mitochondria at stumps could yield misleading results.

Our studies indicate that colchicine and vinblastin destroy microtubules and block the axonal transport of mitochondria. The exact ratio of the retention of mitochondria and of the destruction of microtubules cannot be calculated, but examination of Text-figs. 2 and 3 suggests that loss of one third to one half of the total number of microtubules had little effect on the axonal transport of mitochondria. Possibly more microtubules are available than required for the transport of mitochondria, or a compensatory mechanism operates to maintain the transport. When less than half of the microtubules are left, the impairment of axonal transport of mitochondria and the destruction of microtubules appear to be in proportion. These observations are consistent with a close topographic association of mitochondria with microtubules (Martinez & Friede, 1970; LaFountain, 1972).

The destruction of microtubules by colchicine and vinblastin may be associated with an increase in the number of neurofilaments (Wisniewski, Terry & Hirano, 1970); hence, axonal flow may be blocked either by the destruction of microtubules or by the increased numbers of neurofilaments (Fink *et al.* 1973; Byers, Fink, Kennedy, Middaugh & Hendrickson, 1974) or by the blocking of the oxidative metabolism by lidocaine, an agent frequently used in these experiments (Cullen & Haschke, 1974; Sakabe, Maekawa, Ishikawa & Takeshita, 1974; Novotny & Bianchi, 1967). Our data indicate that the increase in neurofilaments is independent of the destruction of microtubules (Table 2). Also, the axonal transport of mitochondria was not related to the numbers of neurofilaments (Tables 1, 2), and no increase of other axoplasmic components which might conceivably obstruct axonal transport was observed.

It is conceivable that the endoplasmic reticulum which extends from

cell body to nerve ending may serve as a pathway for the axonal transport of mitochondria, or else that changes in the axon calibre may impair axonal transport by widening or narrowing the axonal channel. Our data indicate that axonal transport of mitochondria is independent of endoplasmic reticulum and axonal calibre, even when the latter is reduced to half of the normal.

Ochs & Hollingsworth (1971), Ochs & Ranish (1971), and Ochs & Smith (1971) reported blockage of axonal transport by agents blocking oxidative metabolism such as cyanide and dinitrophenol. There was no examination of fine structure. Our data show severe loss of microtubules in nerves treated with cyanide (and similar observations were made also for dinitrophenol). Hence, the blockage of axonal transport may result from the blockage of oxidation, or the destruction of microtubules or from both. Anaerobic metabolism seems to plan an important role in the axonal transport in cold-blooded animals (Heslop & Howes, 1972). In conclusion, while energy appears to play an important role in the axonal transport of mitochondria, studies of the role of energy in axonal transport require a careful examination of fine structure to establish whether the agents have any unexpected additional effects on axoplasmic organelles.

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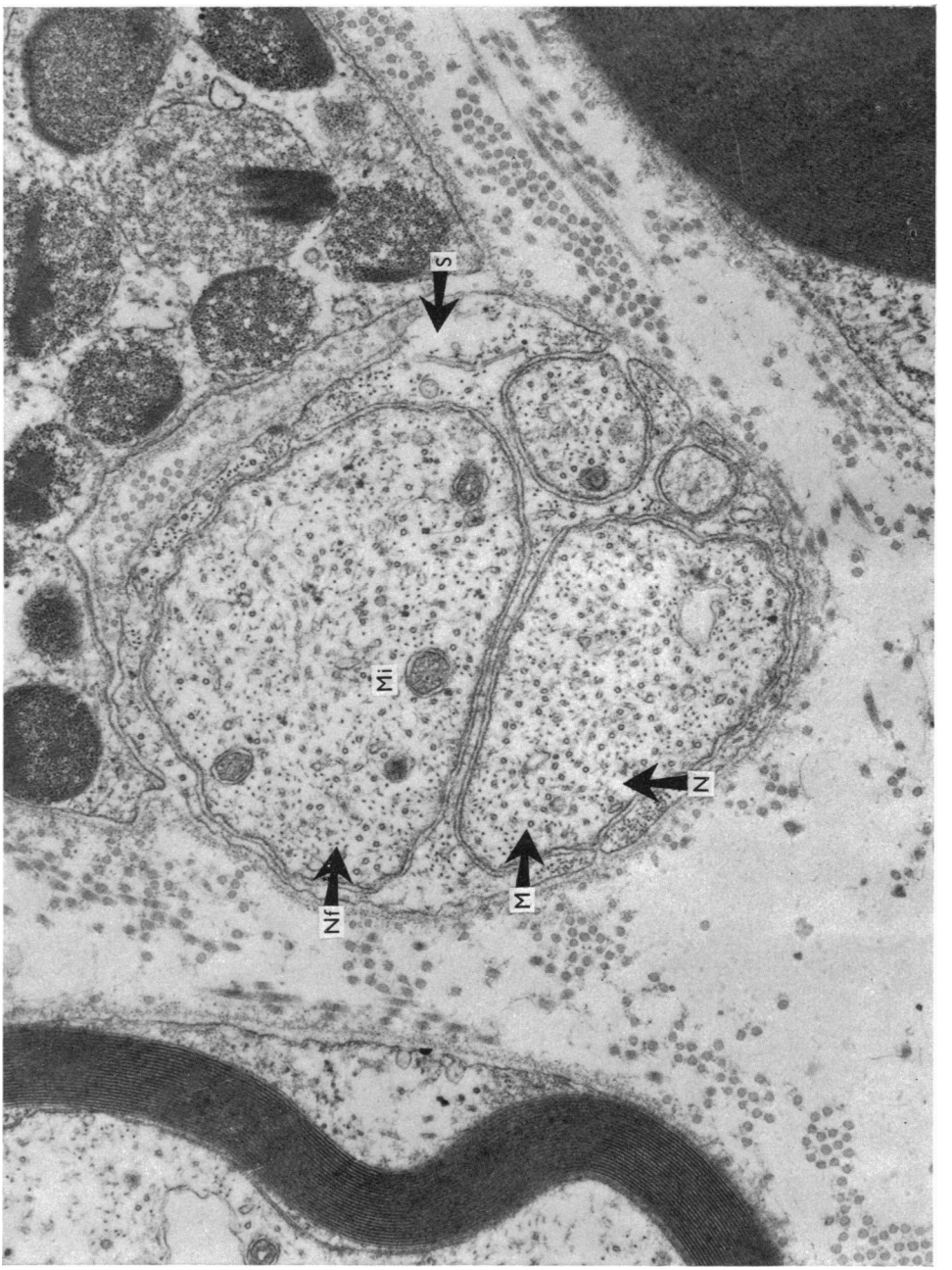
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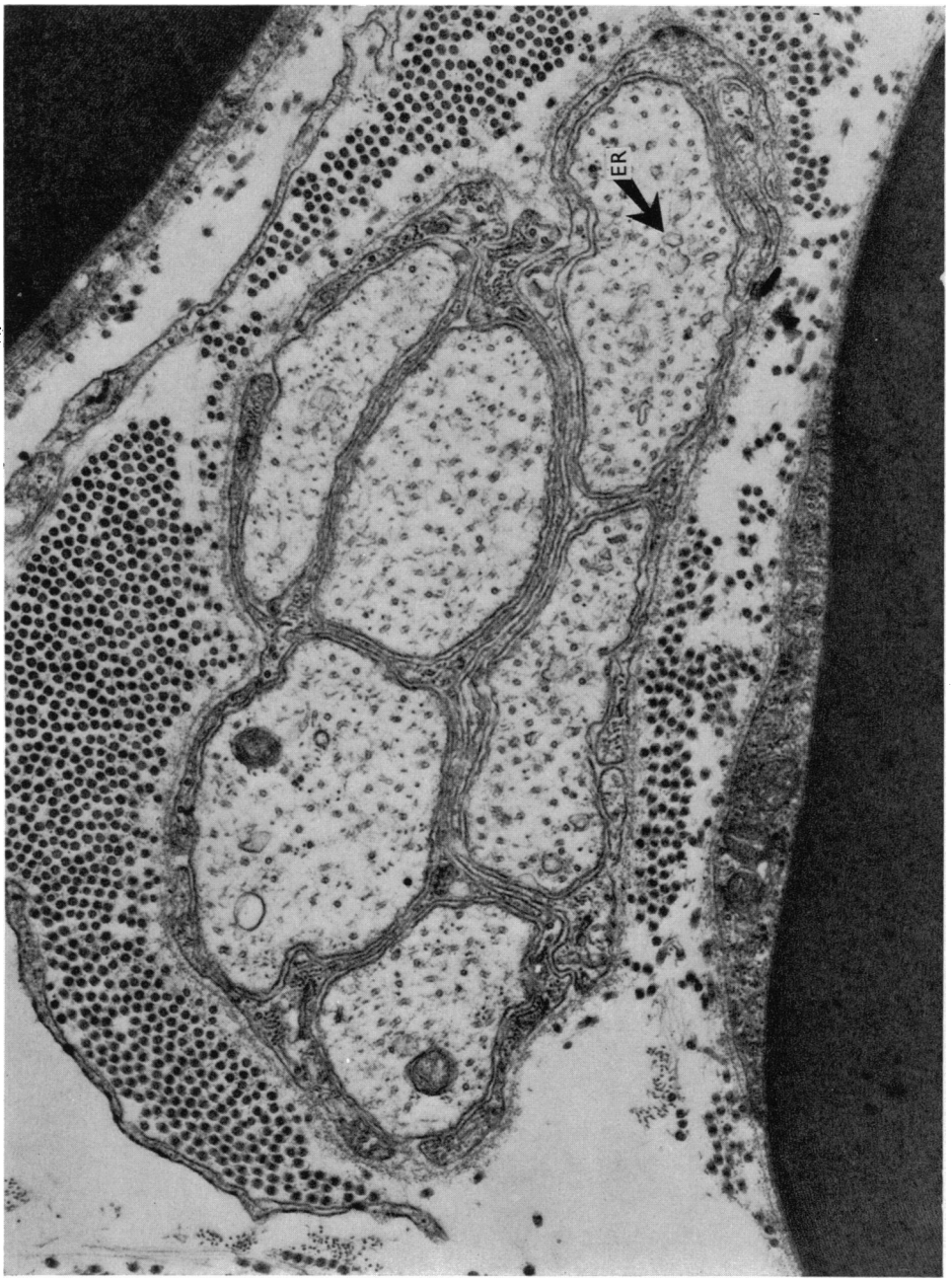

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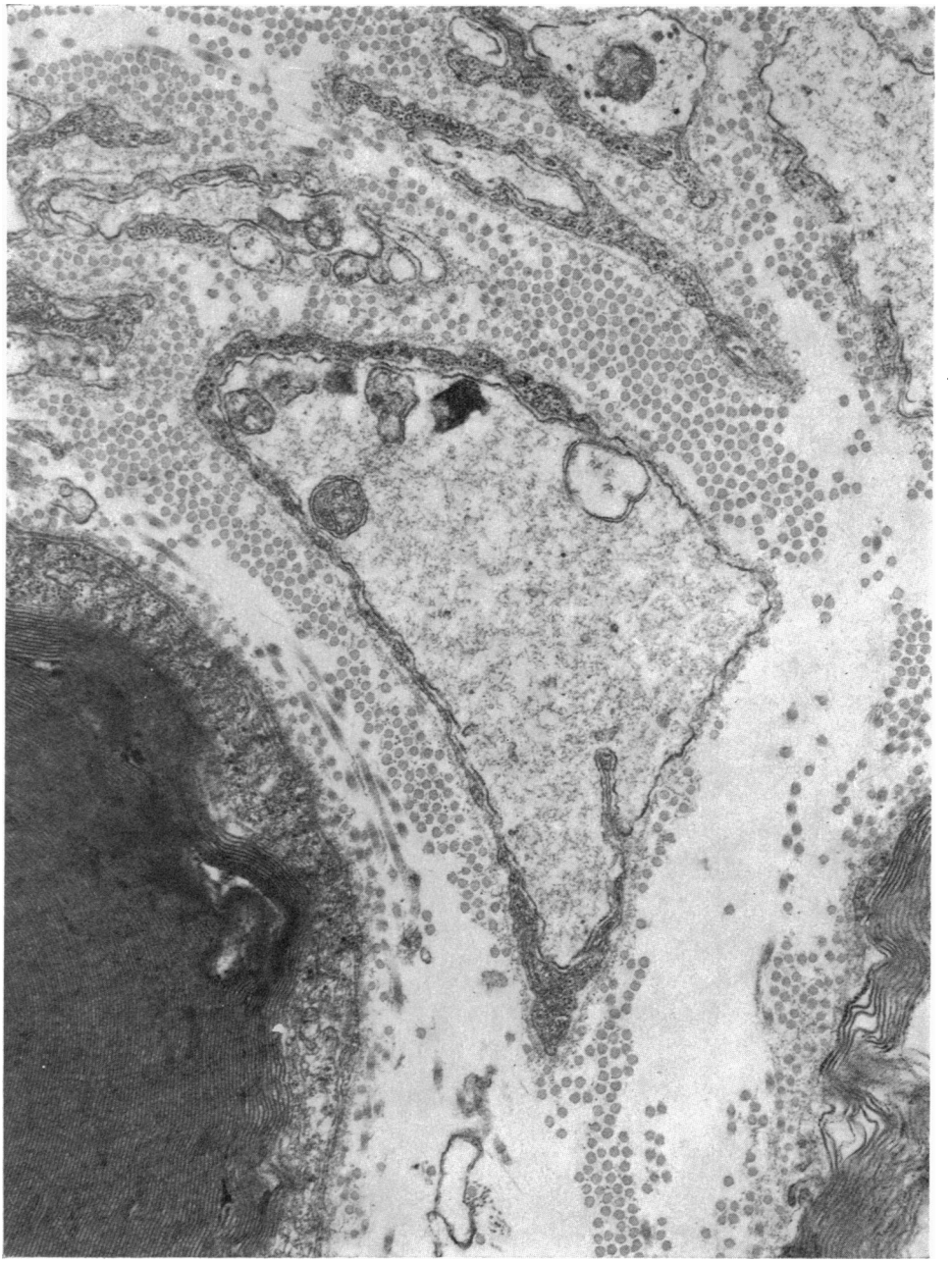

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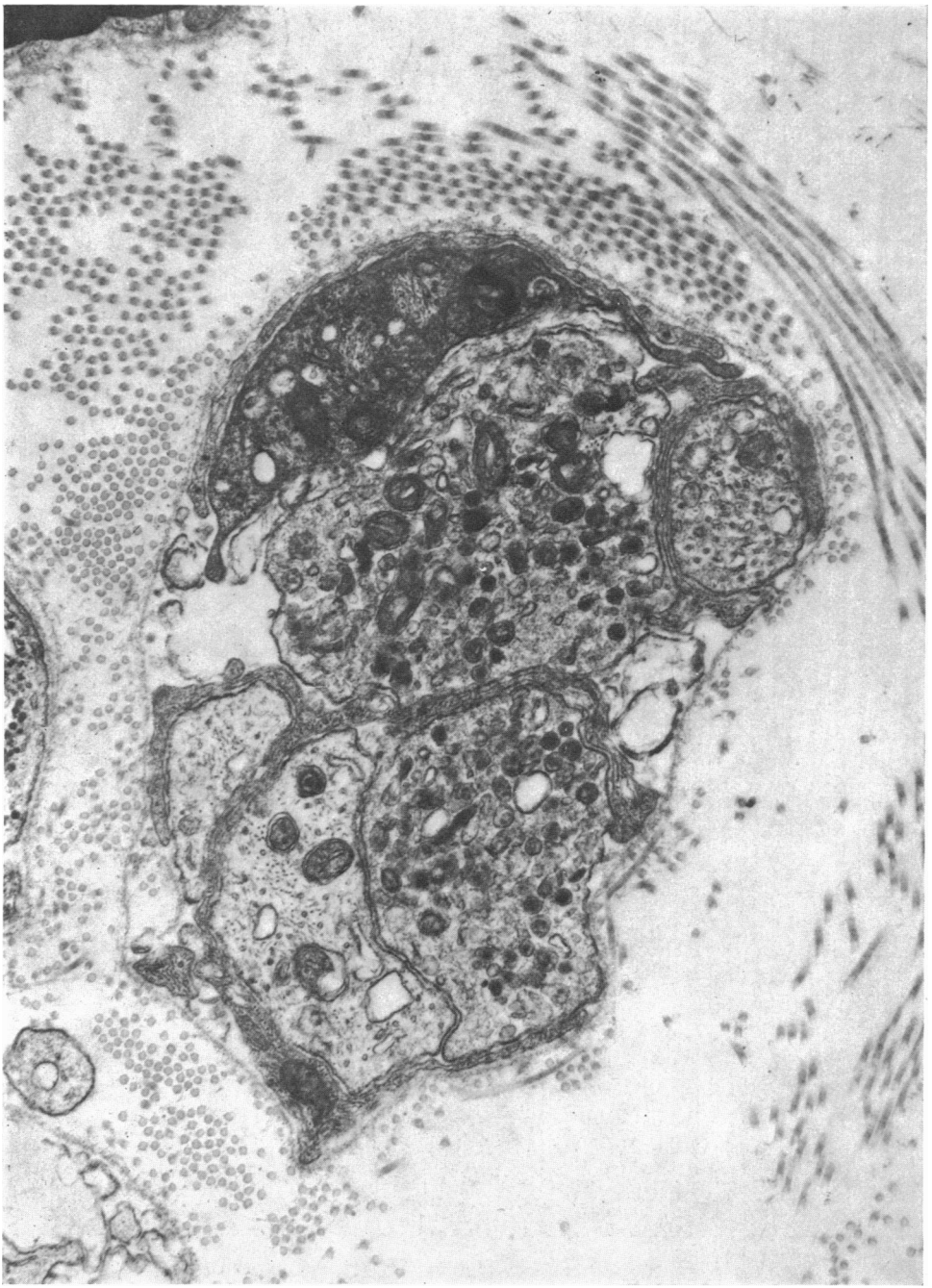

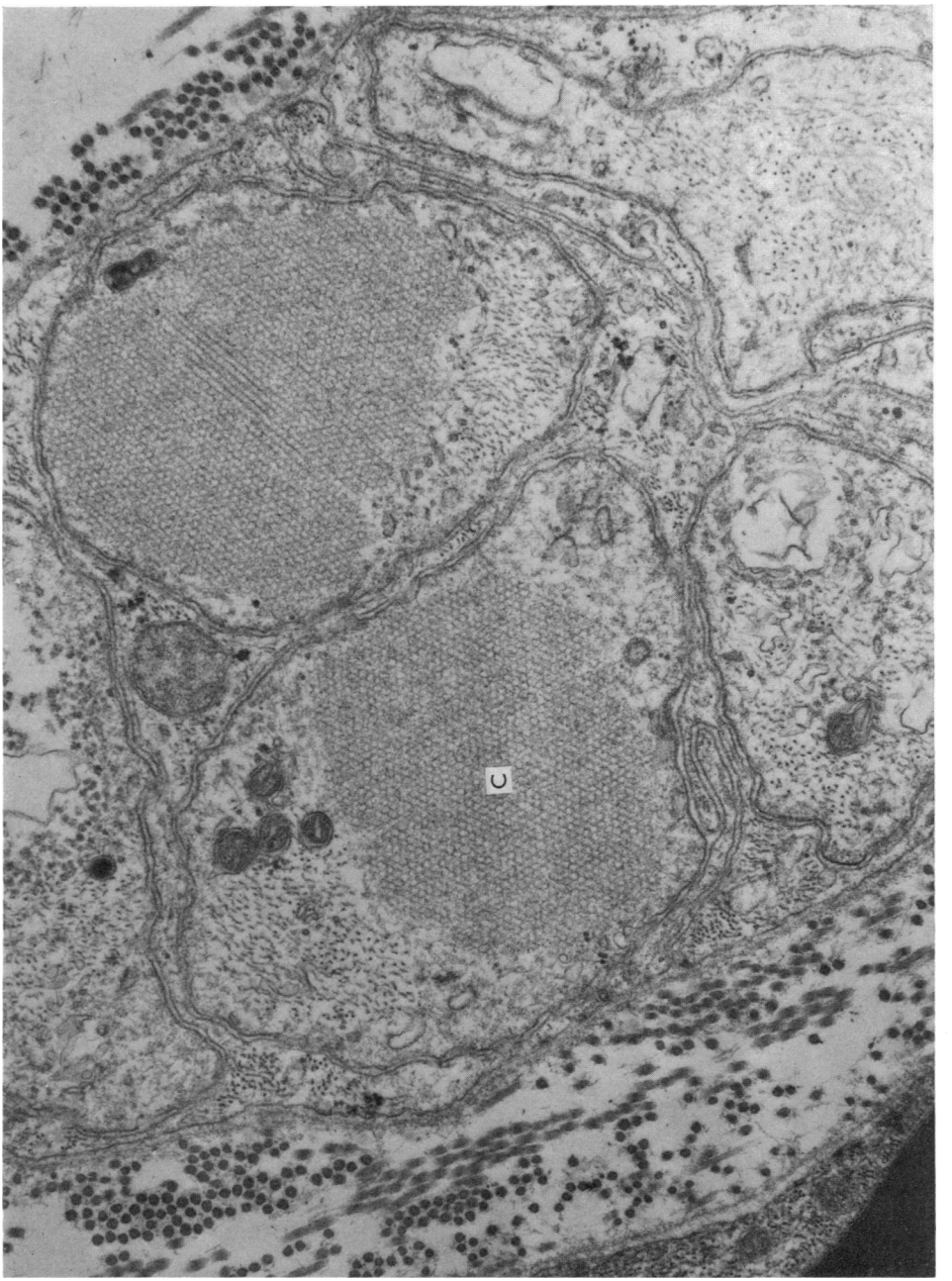

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EXPLANATION OF PLATES

PLATE 1

Normal sciatic nerve before incubation. N, nerve fibre; Nf, neurofilaments; S, Schwann cell; M, microtubule; Mi, mitochondria.

PLATE 2

Middle of nerve-segment after 10 hr incubation in frog Ringer. The microtubules, neurofilaments, and endoplasmic reticulum (ER) are intact; mitochondria are decreased in number.

PLATE 3

Middle of nerve segment after 10 hr incubation in cyanide. There is near complete destruction of microtubules and retention of mitochondria.

PLATE 4

Nerve stumps after 10 hr incubation in frog Ringer solution. There is marked accumulation of mitochondria at stumps.

PLATE 5

Middle of nerve segment after 10 hr incubation in vinblastin. There is marked decrease of microtubules with an associated retention of mitochondria. The neurofilaments are increased in number. Also, paracrystal formation (C) is noted.