THE ISOLATION OF NERVE ENDINGS FROM BRAIN: AN ELECTRON-MICROSCOPIC STUDY OF CELL FRAGMENTS DERIVED BY HOMOGENIZATION AND CENTRIFUGATION

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INTRODUCTION

It has been suggested that synaptic vesicles, seen in great profusion in electron micrographs of many different types of nerve ending as small circular profiles about 0·05μ in diameter (Sjöstrand, 1953; De Robertis, 1958), are storage particles for transmitter substances, among them acetylcholine, and provide a morphological basis for the quantization of transmitter release detected electrophysiologically at the motor end-plate (del Castillo & Katz, 1956; Palay, 1956). The isolation of these vesicles as a distinct fraction containing transmitter substance would be valuable evidence for this theory. It would in addition provide a useful *in vitro* preparation for the study of transmitter release and the action of drugs and toxins on this process.

Hebb & Whittaker (1958) and Whittaker (1959) have succeeded in isolating from sucrose homogenates of forebrains of rabbit, guinea-pig and other species a particulate fraction (fraction *B*, Whittaker, 1959) distinct from nuclei, mitochondria and microsomes, and containing most of the bound acetylcholine (Hebb & Whittaker, 1958; Whittaker, 1959), hydroxytryptamine (Whittaker, 1959) and noradranaline (Chruściel, 1960) of the tissue. Preliminary electron-microscopic studies by K. M. Smith and G. J. Hills (reported by Whittaker, 1959) suggested that the particles of this fraction might be derived from synaptic vesicles.

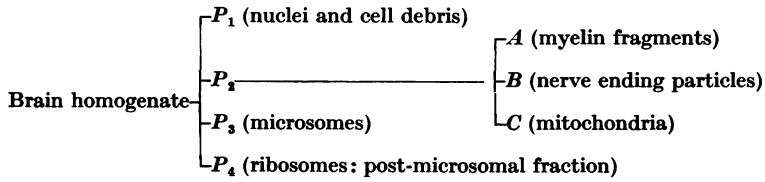
Fraction *B* is one of three obtained by the density gradient separation of a crude mitochondrial (P_2) fraction of brain. The other two are an unidentified, lighter, inactive fraction (*A*) and a denser fraction (*C*) identified on biochemical and morphological evidence as mitochondria.

The primary object of the present work was to investigate the morphology of the particles of fraction *B* in greater detail using improved techniques of fixing, staining and embedding which have proved particularly useful in electron microscopic work on the central nervous system (Gray, 1959). The conditions necessary for obtaining reproducible electron micrographs were worked out and a general study was made of all the fractions obtained by the homogenization and centrifugation procedure. The results (briefly reported at the IV International Neurochemical Symposium, Varenna, 12–17 June 1960 (Whittaker, 1961) and by Gray & Whittaker, 1960) show that fraction *B* consists mainly of particles derived from nerve endings, apparently by a pinching or tearing-off process. They are packed with synaptic vesicles and are

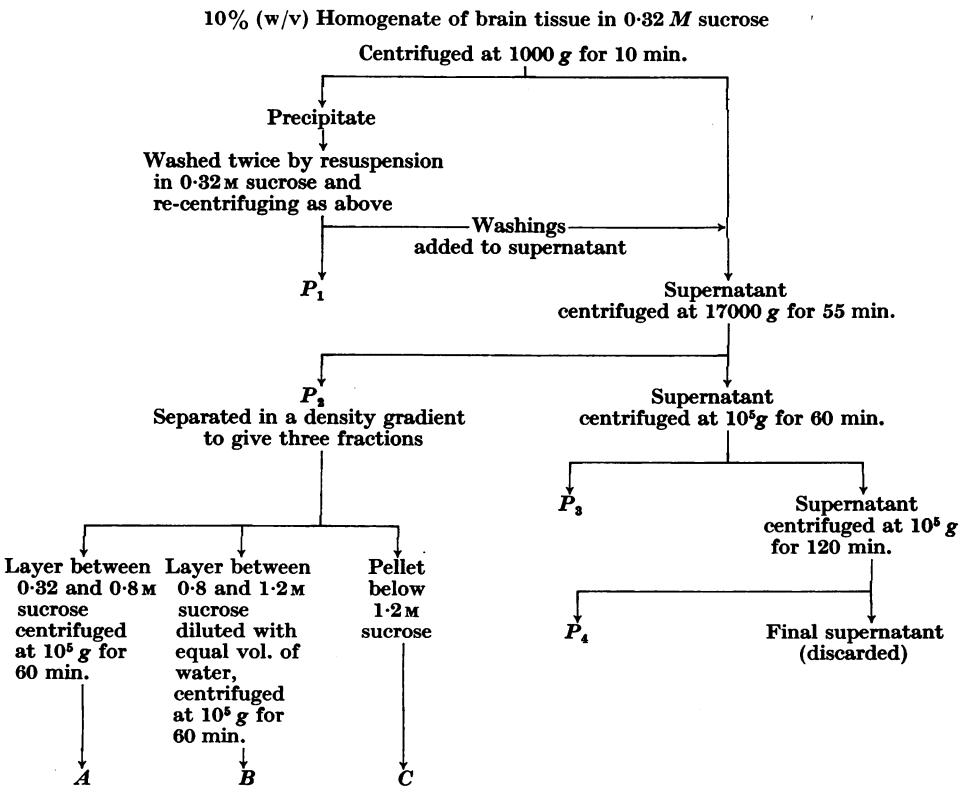
extremely fragile, particularly after treatment with osmium tetroxide; this is believed to account for their less organized appearance in the earlier electron micrographs. Fraction *A* has been found to consist largely of myelin fragments and the identification of the particles of fraction *C* as mitochondria has been confirmed. The appearance of the nuclei and cell debris (P_1) and the microsome fraction (P_3) were much as might be predicted from the results of other workers with the corresponding fractions from other tissues.

METHODS

Preparation of subcellular fractions. Preparations were made at 0–4° C. from guinea-pig brain (brain stem transected between the superior and inferior colliculi) according to the scheme outlined in Text-figs. 1 and 2. The procedure was essentially



Text-fig. 1. Nomenclature used in describing subcellular fractions.



Text-fig. 2. Scheme summarizing preparation of subcellular fractions.

as described by Whittaker (1959) with the following modifications. The difference in diameter between the pestle and mortar of the Perspex and glass homogenizer (Aldridge, Emery & Street, 1960) was 0·025 cm., not 0·025 mm. as previously stated in error. Eserine was omitted from the suspension medium (0·32 M sucrose unless otherwise stated). A Servall type SS-34 refrigerated angle-head centrifuge was used for the separation of the P_2 fraction in later experiments. After suspension in 0·32 M sucrose, P_2 was separated into the *A*, *B* and *C* fractions by centrifuging samples (5–7 ml./tube) at 25,000 rev./min. for 2 hr. into a discontinuous density gradient consisting of 0·8 and 1·2 M sucrose (10 ml. of each each/tube) in the SW 25 head of the Spinco model L preparative ultracentrifuge. In this way material derived from up to 10 g. brain could be separated in a single run. Control experiments showed that maximum separation was obtained in this time. The top and intermediate bands of particulate material were separated using a tube cutter and after dilution of the middle fraction to approximately 0·45 M with respect to sucrose by the addition of an equal volume of water, both fractions were centrifuged at 40,000 rev./min. for 1 hr. in the no. 40 head of the Spinco ultracentrifuge to yield particulate fractions *A* and *B*. The pellet at the bottom of the density gradient was fraction *C*.

The microsomal (P_3) fraction was conveniently prepared at the same time as the sedimentation of fractions *A* and *B*. In some experiments a post-microsomal fraction (P_4) was obtained by centrifuging the supernatant from P_3 for a further 2 hr. For enzyme tests and acetylcholine assays, carried out as described by Whittaker (1959), pellets were suspended in 0·45 (fraction *B*) or 0·32 M sucrose (other fractions). The use of 0·32 M sucrose for resuspending fraction *B* was found to cause a small loss of acetylcholine, as though it were hypo-osmotic. This might be accounted for by the penetration of sucrose into the particles during their passage through the density gradient.

Preparation of fractions for electron microscopy. In the standard procedure, pellets of particulate material were treated like blocks of whole tissue, fixed and stained in osmium tetroxide and phosphotungstic acid and embedded in Araldite (Gray, 1959). Portions of pellets were placed in ice-cold 2 % (w/v) OsO_4 in 0·45 M (fraction *B*) or 0·32 M sucrose (other fractions) containing 0·04 M veronal-HCl buffer, pH 7·4 and cut into small cubes of side *c.* 1 mm. After 3 hr. at 0° the pellets were briefly washed with ice-cold water and dehydrated with 25, 50 and 70 % (all v/v) aqueous ethanol (20 min. in each solvent). They were usually stored in 70 % (v/v) aqueous ethanol at 0° till the following day. They were then further dehydrated in 95 % (v/v) aqueous ethanol and anhydrous ethanol, stained for 3 hr. in 1 % (w/v) ethanolic phosphotungstic acid, washed in two changes of anhydrous ethanol and embedded in a mixture of Araldite casting resin M, hardener 964 B, dibutyl phthalate and accelerator 964 C (50:50:1·5:0·8), obtained from Messrs Ciba (ARL) Ltd., Duxford, Cambridge. The resin was hardened at 70° for 24 hr. Care was taken to sample the blocks at from 4 to 10 levels.

In some experiments an osmium tetroxide-saline fixation medium buffered with veronal acetate similar to that described by Sjöstrand (1957) was used. This contained (all w/v final concentration) 1 % OsO_4, 0·546 % NaCl, 0·029 % KCl, 0·012 % $CaCl_2$, 0·39 % sodium acetate and 0·59 % sodium veronal, adjusted to

pH 7·4 with HCl. In others, particles were fixed at 0° while in suspension (Sjöstrand, 1957; Palade & Siekevitz, 1956). The pellet of osmicated particles obtained by centrifuging was dehydrated, stained with phosphotungstic acid and embedded as in the standard procedure.

RESULTS

The morphology of the particles in fraction B

The particles of this fraction are seen in Pl. 1, figs. 1 and 2. Most of them contain a number of small vesicles (sv), identical in size and appearance with synaptic vesicles, surrounded by a thin outer membrane (tm). The clue to their identity was given by particles of the type indicated by the nerve-ending particle (ne) in fig. 1, which resemble cortical nerve endings as seen in whole tissue sections (Pl. 1, fig. 3, cf. Gray, 1959). Within the particle can be seen a small mitochondrion (m) in addition to the synaptic vesicles, and adhering to a region of the periphery is a portion of post-synaptic membrane (psm) showing the characteristic thickening which is a common feature of synapses in the C.N.S.

Further examples of complete nerve ending particles are seen in Pl. 2, figs. 5–7, and Pl. 3, figs. 8–11. Some of these are selected from electron micrographs of the parent fraction (P_2) in which they are mixed with mitochondria and fraction *A* particles (*vide infra*). A variety of different types of post-synaptic attachments can be seen. In Pl. 3, fig. 10, a band (*b*) of material can be seen in the synaptic cleft (cf. Gray, 1959, Pl. 1, fig. 2). Some of the membrane fragments seen in the *B* fraction micrographs (e.g. mem in Pl. 1, figs. 1, 2) may be detached post-synaptic membranes (compare with Pl. 3, fig. 11) or post-synaptic membranes of nerve-ending particles lying outside the plane of section.

Nerve ending particles prepared from sucrose have more densely packed vesicles than intact nerve endings (compare Pl. 1, fig. 1, with Pl. 1, fig. 3). Some particles are extremely dense, with partial or complete fusion of vesicles into a black mass. These 'black bodies' (bb in Pl. 1, fig. 1; Pl. 2, fig. 4) are nevertheless still recognizable as nerve endings from the post-synaptic attachment (psm, Pl. 2, fig. 4). We suspect that 'black body' formation is a degenerative change induced by mechanical pressure, as more particles of this type are seen on the edges of blocks than in the middle, and perhaps also by exposure to hyper-osmotic sucrose.

Table. 1 *Distribution of bound acetylcholine in subcellular fractions of guinea-pig brain*

	Suspension medium	
	0·15 M NaCl	0·32 M sucrose
Acetylcholine content of homogenate (μm-moles/g. tissue)	5	12
Distribution (% total activity) in fractions:		
P_1	67	22
P_2	33	63
P_3	0	15

Nerve ending particles prepared from saline or hypo-osmotic sucrose. Particles with less tightly packed vesicles, looking more like nerve endings in whole tissue sections, could often be seen in sucrose preparations fixed in osmic-saline (Pl. 3, figs. 8, 9) and were fairly common in P_2 fractions prepared from homogenates made in 0·9% (w/v) sodium chloride (Pl. 2, figs. 5, 7) or slightly hypo-osmotic (0·2 M) sucrose. However, judged by the lower bound acetylcholine content of such homogenates (Table 1), the more 'normal' looking endings might have been survivors from a population containing a larger proportion of damaged or disintegrated particles than preparations made in 0·32 M sucrose. This possibility might be checked

Table 2. *Threshold concentrations of electrolytes for coacervation of particles of* P_2 *and* B *fractions*

Serial dilutions of electrolyte solutions (1 drop) in 0·32 M sucrose were mixed with the fractions suspended in 0·32 M sucrose (1 drop) on a glass slide and examined microscopically. The concentration required for just detectable clumping was recorded.

Electrolyte	Charge on		Threshold concentration for clumping (mM)	
	cation	anion	P_2	*B*
NaCl	1	1	20	20
KCl	1	1	20	—
$MgCl_2$	2	1	2	2
$CaCl_2$	2	1	0·5	0·5
$AlCl_3$	3	1	0·3	0·3
$FeCl_3$	3	1	0·2	1·9
K_2SO_4	1	2	25	25
Na_2SO_4	1	2	17	—
$K_3Fe(CN)_6$	1	3	50	50
Na_3PO_4	1	3	33	—

by a statistical comparison of electron micrographs of the different types of preparation, but this would present obvious difficulties and has not been attempted. With the saline preparation, there was a further disadvantage arising from the coacervating effect of the electrolyte. Coacervation increases the range of effective size of each type of particle, thus rendering separation by differential centrifugation less efficient and causing particles to sediment at lower speeds than in sucrose. Taking acetylcholine as a marker of the fraction *B* particles, this effect is seen in Table 1 which shows that much more is recovered in the low speed P_1 fraction in saline than in sucrose. Table 2 shows that the particles of the P_2 and *B* fractions behave as though negatively charged, in that cations are more effective in causing coacervation the greater their charge, while the effect of anions shows no such charge dependence. Thus some degree of morphological alteration may be an unavoidable penalty of separating nerve-ending particles in relatively pure form and good yield.

Effects of variation in technique of preparation for electron microscopy. Fixation in aqueous osmium tetroxide at 0° or in iso-osmotic osmium tetroxide above 0° was found to bring about extensive disintegration of the *B*-fraction particles. Omission of the phosphotungstic acid stage greatly reduced contrast. Potassium permanganate could not be used as a fixative in the presence of sucrose, which it oxidises.

Preparations fixed while in suspension in sucrose (Sjöstrand, 1957) showed good

preservation of particles (Pl. 3, figs. 8, 9, 11). However, if the osmicated pellet was dehydrated by resuspension in alcohols as in earlier work, extensive disintegration of the outer thin membranes took place (Pl. 4, fig. 12), producing an appearance similar to that published previously (Whittaker, 1959).

Other fractions

Nucleus and cell debris fraction (P_1). Electron micrographs of this fraction (Pl. 4, fig. 13; Pl. 5, figs. 16–19) show large numbers of neuronal and glial nuclei, together with tissue fragments, portions of blood vessels, myelin fragments, mitochondria and vesicular masses (Pl. 5, fig. 17) that are unlike synaptic vesicles or other known structures. The characteristic basement membrane (bm) and endothelial cells (end) of a fragment of blood vessel are seen in Pl. 5, fig. 16, and a nucleus of an endothelial cell surrounded by a remarkably intact cytoplasm and basement membrane in Pl. 5, fig. 19. The nucleoli (nucol) can be readily distinguished in saline preparations (Pl. 4, fig. 13), but in electron micrographs of sucrose nuclei (Pl. 5, figs. 18, 19), nuclear granules appear uniformly dispersed. Occasionally slightly denser aggregates could be observed, indicating that probably the nucleolus disintegrates into small particles. Plastic sections (2–4μ thick) were cut from the blocks of particles prepared in sucrose and saline respectively and were examined by phase-contrast microscopy. As was expected, nucleoli are conspicuous in the 'saline' nuclei (Pl. 4, fig. 14). In 'sucrose' sections (Pl. 4, fig. 15), after partly dissolving away the plastic with xylene to increase contrast, the nucleolus is occasionally just visible, surrounded by a pale margin.

Fraction A (*from* P_2). The particles of fraction *A* consist mainly of myelin fragments (Pl. 6, fig. 20). In high magnification the myelin period can be recognized. These myelin fragments are presumably derived from axons, and occasionally fragments of more highly organized axonal material can be recognized, especially in saline preparations, as in Pl. 6, fig. 21, where the neurofilaments (nf) are preserved.

Fraction C (*from* P_2). Pl. 7, figs. 22 and 23, show two micrographs of the *C* fraction demonstrating its almost pure mitochondrial origin, which is consistent with its high succinic dehydrogenase content. The mitochondrial cristae are clearly seen, though almost all mitochondria show varying degrees of degeneration.

Microsomal and post-microsomal fractions. The microsomal fraction consists, as would be expected, of large numbers of vesicles of varying sizes (Pl. 8, figs. 24, 25). In the lower regions of pellets, large vesicles and occasional small nerve-ending particles (ne) are seen (Pl. 8, fig. 24), the latter probably accounting for the small amount of bound acetylcholine always found in this fraction (Whittaker, 1959). The upper regions of pellets appear as tightly packed masses of ribosomes and small vesicles, some of which could conceivably be isolated synaptic vesicles, (Pl. 8, fig. 25). The post-microsomal fraction consists almost entirely of ribosomes (Pl. 8, fig. 26).

DISCUSSION

Few previous electron-microscopic studies of subcellular fractions from brain have been made. Toschi (1959), Hanzon & Toschi (1959) and Albertsson, Hanzon & Toschi (1959) have demonstrated the presence of ribosomes in brain fractions, partially attached to membranes. Petrushka & Giuditta (1959) have drawn attention

to the presence, in electron micrographs of the so-called mitochondrial fraction, of myelin fragments, microsomes and other unidentified structures, in addition to mitochondria. Their x and y particles may be derived from nerve-ending particles.

The main result of the present work is the identification of the fraction *B* particles as derived from nerve endings. The high concentration of these particles in this fraction and their almost complete absence from other fractions confirms the effectiveness of the density gradient technique as a means of isolating identifiable subcellular structures in good yield and purity. It will now be possible to subject nerve endings to direct chemical and pharmacological analysis in isolation from the rest of the central nervous system and to examine the morphological changes that accompany the release of transmitter substances from them *in vitro*. It is intended to cover various aspects of these topics in subsequent papers.

The manner of formation of the nerve ending particles is not known, but can be inferred from some observations by Gray (1959) on whole tissue sections. At the edges of blocks (Pl. 5, fig. 18, of his paper) nerve endings are seen which have pulled away from their dendrites carrying with them the thickened region of the post-synaptic membrane. The dendrites disintegrate in the region of shear, but the nerve endings, which are evidently much more robust, retain their individuality. During homogenization, then, the nerve-endings may be torn or pinched off from their axons taking with them their post-synaptic attachments. The membrane presumably seals off at its point of rupture from the axon to form a continuous structure round the contents of the nerve ending in much the same way as the endoplasmic reticulum is thought to be pinched off in the formation of microsomes (Palade & Siekevitz, 1956). A similar self-sealing property has been assumed to operate in pinocytosis. The continued attachment of the post-synaptic membranes during homogenization implies the existence of a cementing substance. That the synaptic cleft is not an empty space is clear from Pl. 3, fig. 10, where a band of low density material can be seen in the cleft.

Although the nerve endings are more resistant to liquid shear forces than other parts of the neurone they are highly labile structures, especially after osmication. Thus osmicated nerve-ending particles unlike osmicated mitochondria break up on resuspension in aqueous ethanol. Failure to appreciate such differences in stability between subcellular particles during preparation for electron microscopy could lead to an oversimplified idea of the composition of subcellular fractions.

The identification of the particles of the *B* fraction as isolated nerve endings explains several hitherto puzzling observations. Thus, it was difficult to account for the sedimentation of particles of the size indicated by the earlier electron micrographs along with mitochondria in the P_2 fraction except on the assumption that prior to exposure to hypertonic sucrose they were very much denser than mitochondria, an explanation which was not borne out by measurements of sedimentation rate (Whittaker, unpublished). Some succinic dehydrogenase is always present in the *B*-fraction even after long centrifugation: this is now explained by the enclosure of mitochondria inside the *B*-fraction particles. In so far as the *B*-fraction particles represent organized portions of neurones, they must contain cytoplasm; thus a bimodal distribution of cytoplasmic enzymes in the final supernatant and in the *B* (or P_2) fractions would be predicted. A small proportion (8–28 %) of a number of

predominantly cytoplasmic enzymes are in fact recovered in a fraction equivalent to our P_2 fraction (Johnson, 1960). The distribution of only one of these, lactic dehydrogenase, has been studied in density gradients; it is associated with particles lighter than mitochondria. The particle bound fraction of another, aldolase, is partly released by mild disruptive treatments (Brunngraber & Abood, 1960) similar to those known to lead to the breakdown of particles of fraction *B*. Probably both these findings will be found to apply to the particle-bound fraction of the other cytoplasmic enzymes also.

Sucrose appears to exert a damaging effect on subcellular structure. The more delicate structures such as nucleoli and neurofilaments are not readily seen in sucrose preparations and the nerve ending particles appear more condensed. Holt (1961) has found that immersion of liver slices in sucrose causes a shift of non-specific esterase from lysosome-like bodies to the cytoplasm suggesting that esterase-containing subcellular particles are ruptured by sucrose. This damaging effect of sucrose on cell membranes may be linked with its ability to supress the coacervating effect of electrolytes on subcellular particles thus making their separation by differential centrifugation possible.

Electron micrographs of nerve ending particles have been published by de Robertis, de Iraldi, Rodriguez & Gomez (1961) in a paper which appeared after the completion of our manuscript. Their observations confirm our initial findings (Gray & Whittaker, 1960).

SUMMARY

1. A fraction prepared by differential centrifugation and density gradient separation from sucrose homogenates of guinea-pig brain, and containing most of the bound acetylcholine of the tissue, has been found to consist almost entirely of 'pinched-off' nerve endings.

2. These are seen in electron micrographs to consist of masses of tightly packed synaptic vesicles enclosed within a thin outer membrane. Sometimes mitochondria are seen enclosed with the synaptic vesicles, and a thickened portion of the post-synaptic membrane may remain adherent to a portion of the periphery.

3. Another fraction has been identified as consisting mainly of myelin fragments, while nuclei, basement membranes, neurofilaments, mitochondria, microsomes and ribosomes have all been identified in other fractions.

4. The mode of formation of the nerve ending particles and the value for future study of an almost pure preparation of nerve endings in isolation from the rest of the central nervous system are discussed.

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EXPLANATION OF PLATES

List of abbreviations

b	synaptic cleft material	ne	nerve-ending particle
bb	'black bodies' (degenerated nerve-endings)	nf	neurofilaments
		nuc	cell nucleus
bm	basement membrane	nucol	nucleolus
end	endothelial cell of blood vessel	psm	thickened portion of post-synaptic membrane
lu	lumen of blood vessel		
m	mitochondrion	sv	synaptic vesicles
mem	detached post-synaptic membrane	tm	thin surface membrane of nerve-ending particle
my	myelin sheath fragment		

Micrographs were made by the standard procedure as described in the 'Methods' section unless otherwise stated. All figures are electron micrographs except figs. 14 and 15 which are phase-contrast light micrographs.

Plate 1

Figs. 1, 2. Fraction *B*. Note synaptic vesicles (sv) and mitochondria (m) enclosed within membrane (tm) to form a nerve ending particle (ne) which may have a thickened portion of the post synaptic membrane (psm) adherent to it. Isolated membranes (mem) may represent psm's. 'Black bodies' (bb) are probably condensed nerve ending particles.

Fig. 3. Whole tissue section showing nerve ending for comparison.

Plate 2, 3

Fig. 4. Portion of Fraction *B* (spun out of layer from density gradient without prior dilution) showing numerous 'black bodies' with attached psm's.

Figs. 5–11. Nerve ending particles, showing less tightly packed vesicles of particles in P_2 fraction prepared from saline homogenates (figs. 5, 7), various types of psm and material (b) in synaptic cleft (fig. 10). Figs. 8, 9, and 11 are from electron micrographs of a P_2 fraction in which osmium fixation was carried out in suspension using the saline osmium tetroxide fixative buffered with veronal acetate.

Plate 4, 5

Fig. 12. Fraction *B*. Dehydration was carried out by resuspending the osmicated pellet in aqueous ethanol solutions of increasing ethanol content. The surface membranes of the particles have disintegrated.

Figs. 13, & 14. Nuclei with preserved nucleoli from saline preparation (in this case P_2). Fig. 13, electron micrograph. Fig. 14, phase-contrast light micrograph.

Figs. 15–19. Nuclear and cell debris (P_1) fraction. Fig. 15. Sucrose preparation (phase-contrast). Nucleoli are just visible in a few nuclei. Fig. 16. Blood vessel fragments with characteristic basement membrane and endothelial cells. Fig. 17. Portions of neuron nuclei and vesicular masses unlike synaptic vesicles. Fig. 18. Portion of nucleus showing nuclear membrane and large myelin fragment. Fig. 19. Endothelial nucleus with remarkably intact cytoplasm and basement membrane.

Plate 6

Fig. 20. Fraction *A*. consisting mainly of myelin fragments. Note characteristic myelin lamellae.

Fig. 21. Axonal neurofilaments in P_2 fraction from saline homogenate.

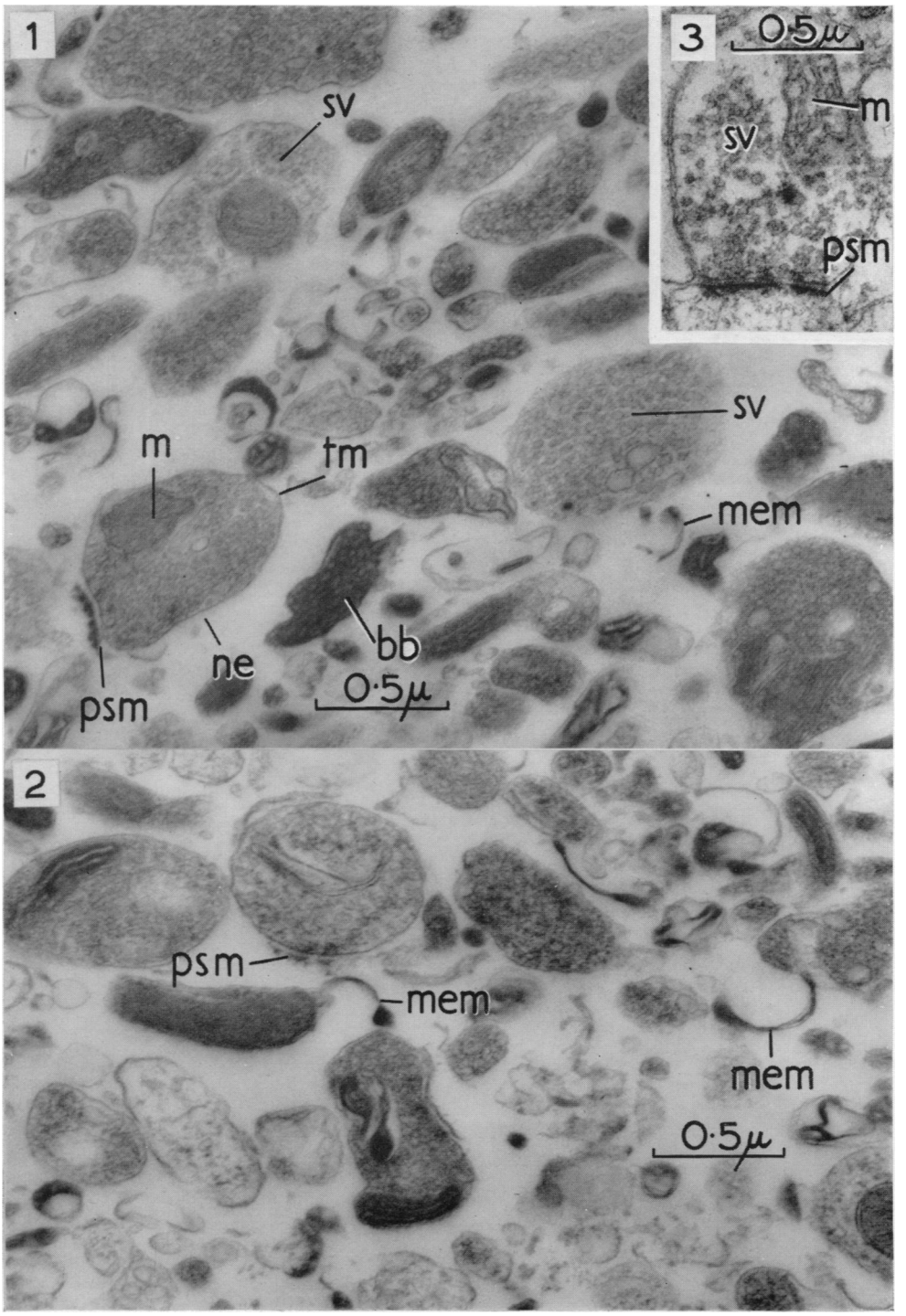
Plate 7

Figs. 22, 23. Fraction *C*, consisting of mitochondria. Note cristae mitochondriales with swelling in some and shrinkage in other mitochondria.

Plate 8

Figs. 24, 25. Two regions of microsomal (P_3) pellet. In fig. 24 note complex masses of large and small vesicles and small nerve ending particles, and in fig. 25 small vesicles and ribosomes.

Fig. 26. Post-microsonal (P_4) fraction. Note almost pure mass of ribosomes.



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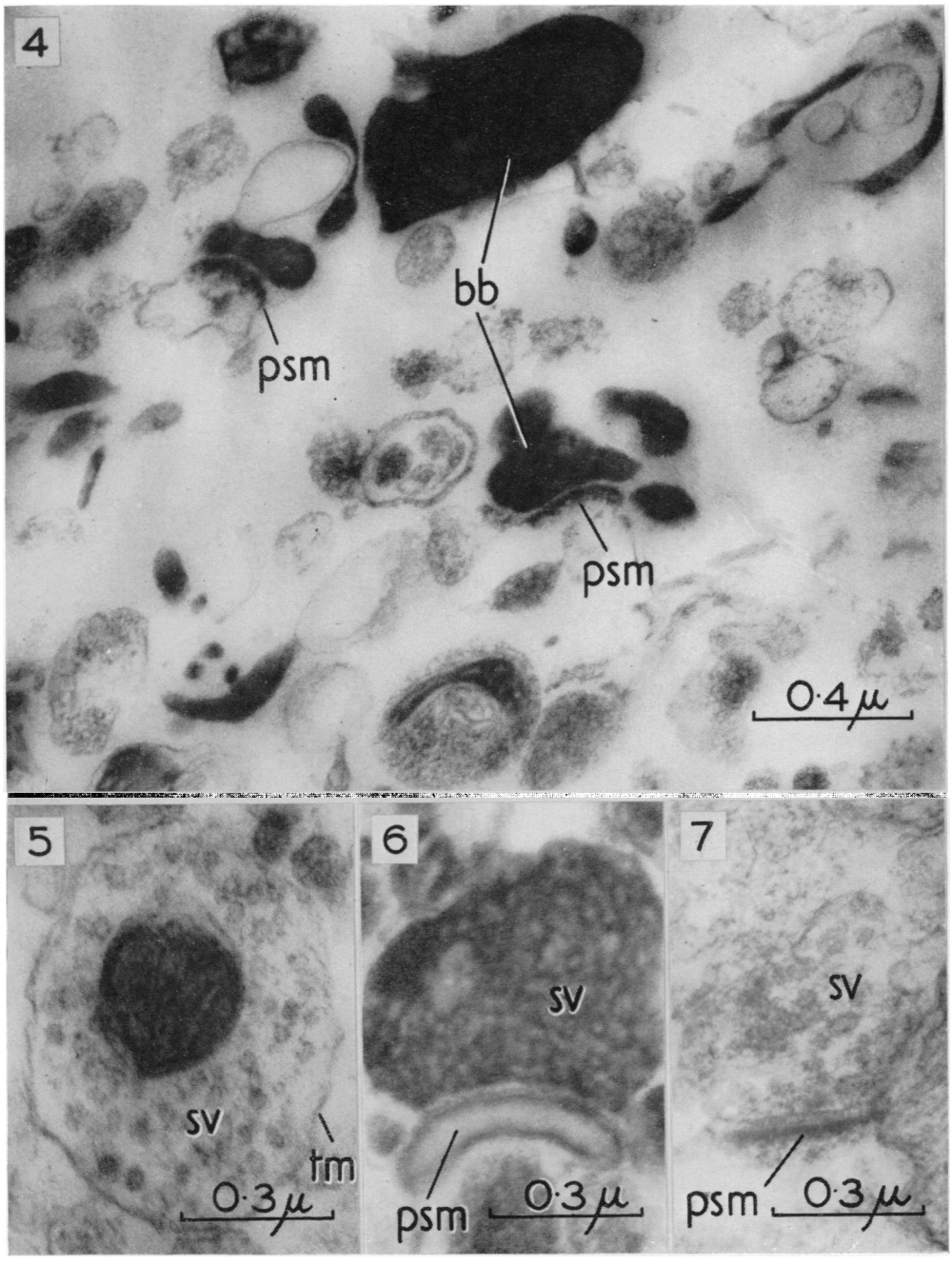

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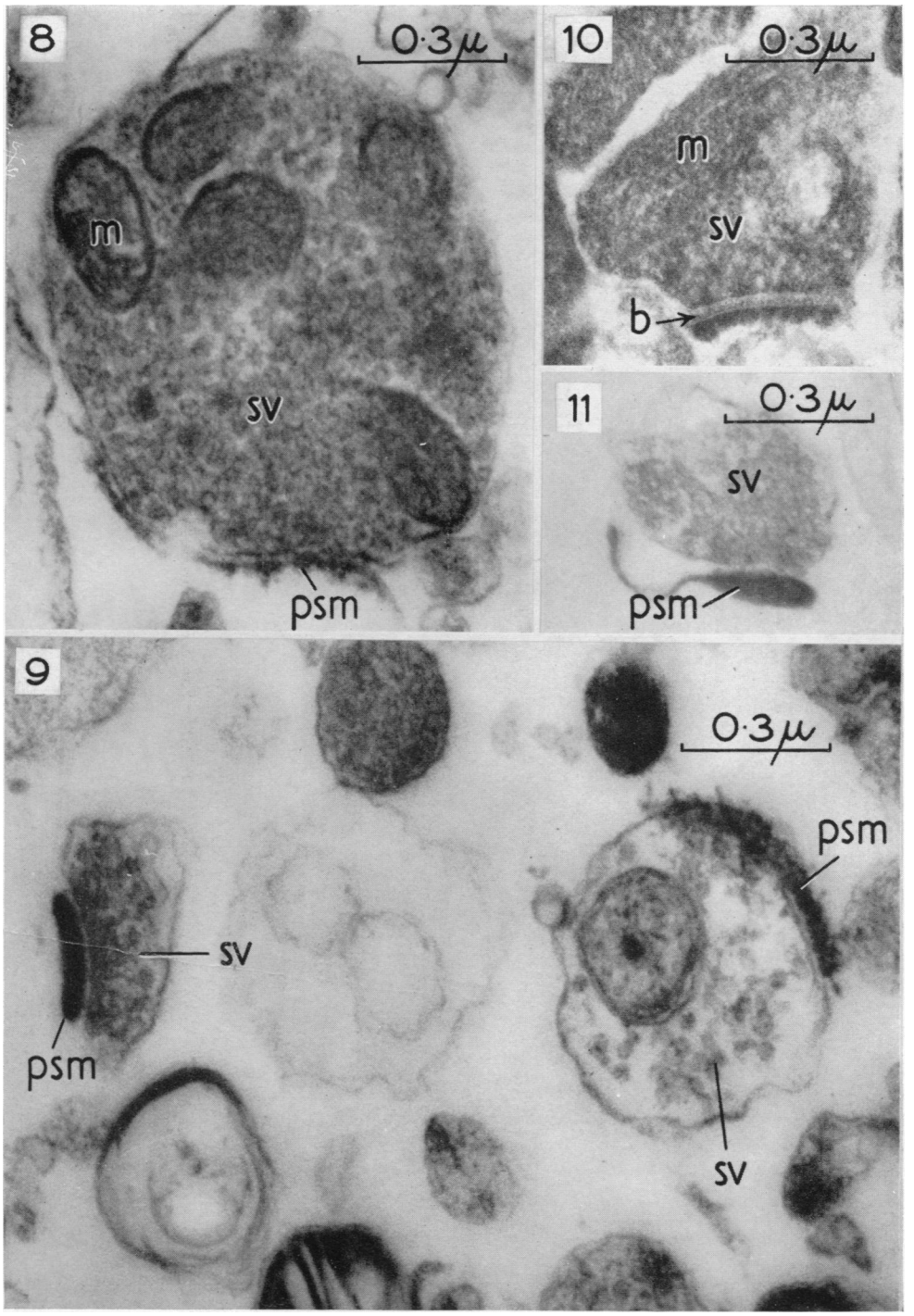

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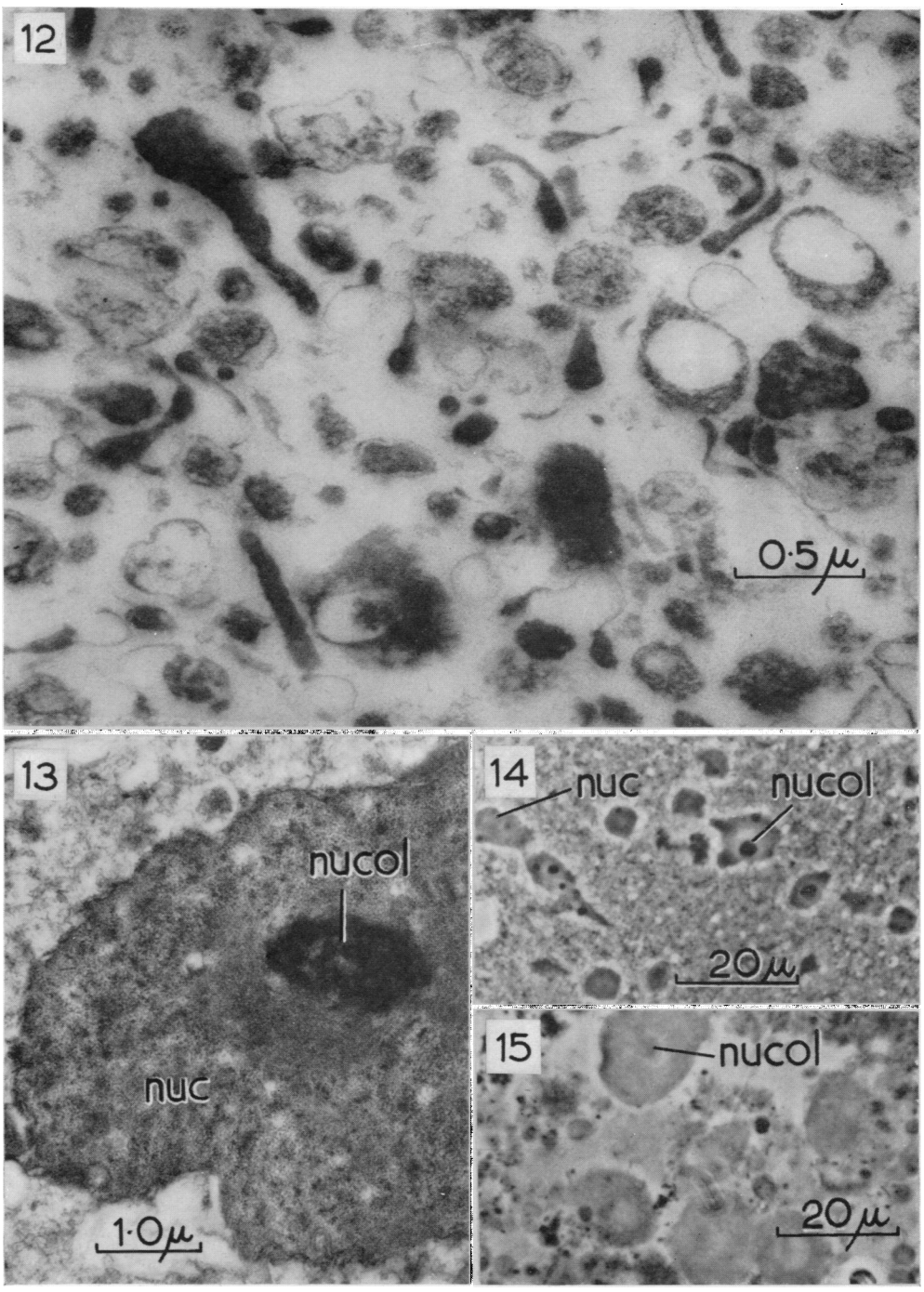
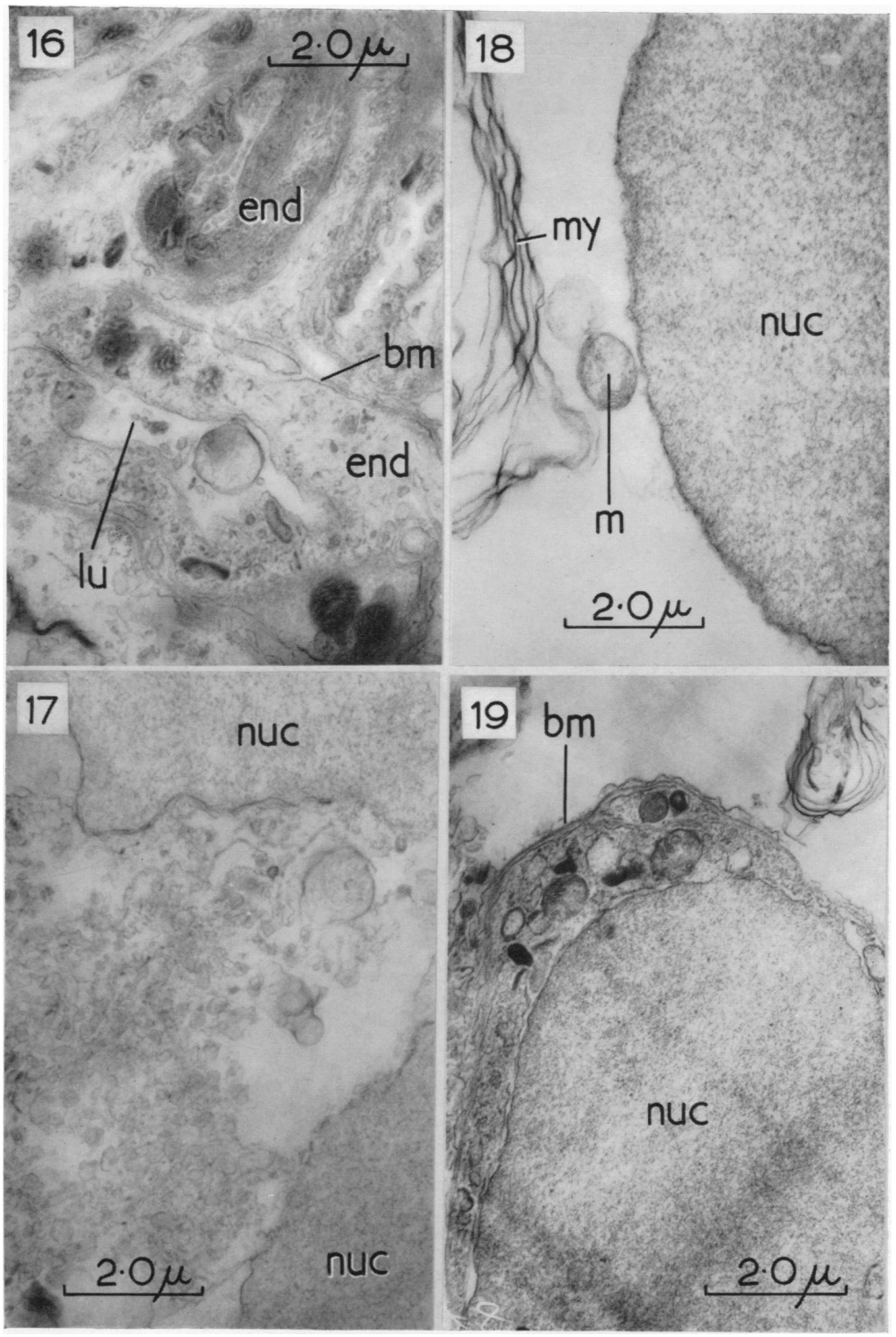
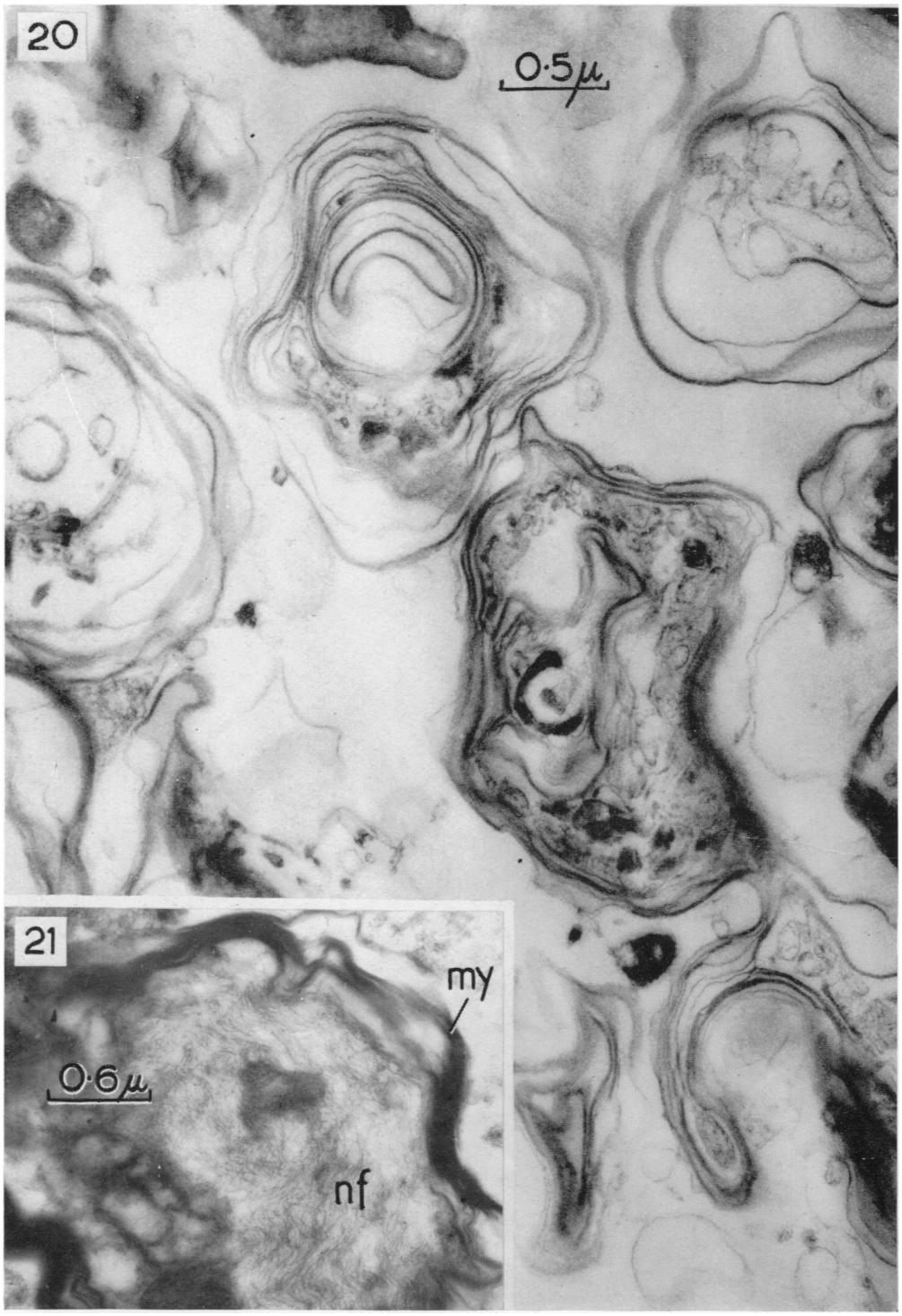


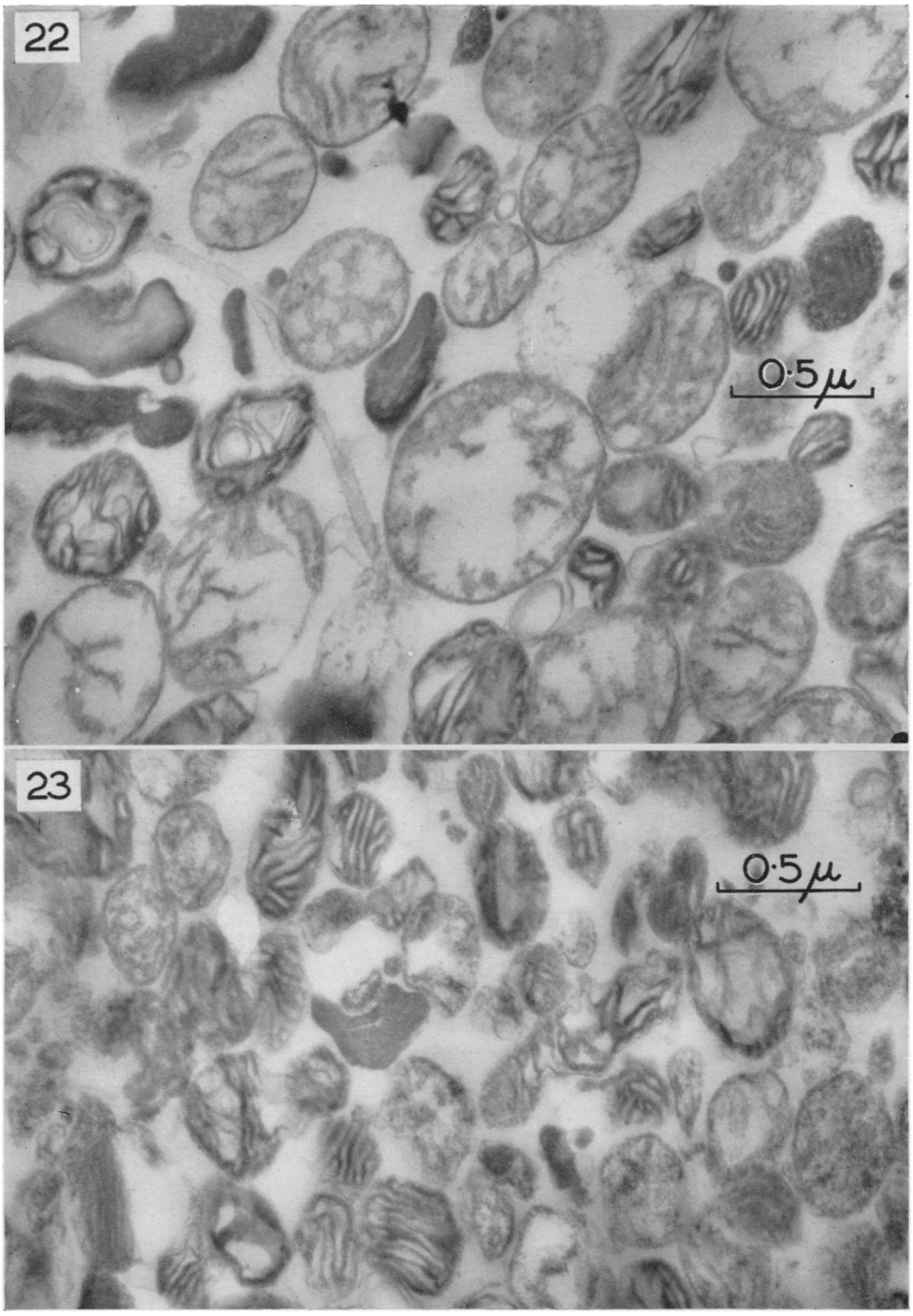
GRAY AND WHITTAKER—ISOLATION OF NERVE ENDINGS FROM THE C.N.S.



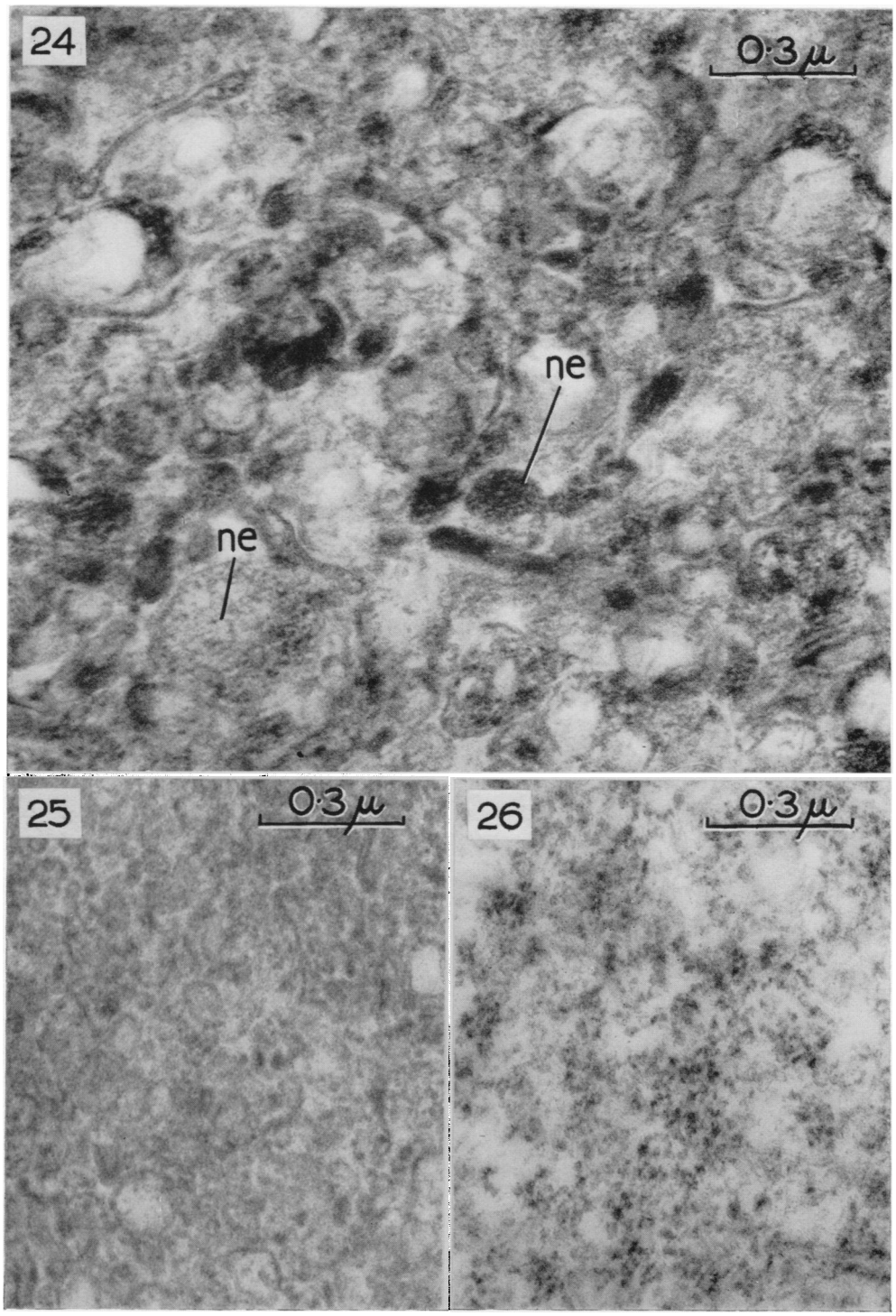
GRAY AND WHITTAKER—ISOLATION OF NERVE ENDINGS FROM THE C.N.S.



GRAY AND WHITTAKER—ISOLATION OF NERVE ENDINGS FROM THE C.N.S.



GRAY AND WHITTAKER—ISOLATION OF NERVE ENDINGS FROM THE C.N.S.



GRAY AND WHITTAKER—ISOLATION OF NERVE ENDINGS FROM THE C.N.S.