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From the Electron Microscope Laboratory and the Biochemistry Department, Agricultural Research Council, Institute of Animal Physiology, Babraham, Cambridge, England

THE USE OF THE NEGATIVE STAINING METHOD FOR THE ELECTRON-MICROSCOPIC STUDY OF SUBCELLULAR PARTICLES FROM ANIMAL TISSUES

By

R. W. HORNE and V. P. WHITTAKER

With 18 Figures in the Text

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The effect of surrounding small particles with electron-dense material has been described by HALL (1955) and HUXLEY (1956) when examining preparations in the electron microscope by positive staining methods. In recent years, the negative staining (negative contrast) method of BRENNER and HORNE (1959) has proved extremely useful for investigating the fine structure of viruses and bacterial cells in the electron microscope (HORNE 1961). In this method, particulate material, with or without prior fixation, is mixed with 1—2% aqueous potassium phosphotungstate previously adjusted to pH 7.4 and sprayed onto specimen grids with an atomizer in the form of fine droplets of 1—10 μ in diameter. These evaporate under suitable conditions to leave an electron dense glass of phosphotungstate in which the particles are embedded and shown up as areas of relatively low electron density. Various types of fixation procedure can be used in combination with the method, material may be alternatively applied to grids by means of a micropipette and various other polyacid anions may be used in place of phosphotungstate. The advantages of the method as compared with histological preparations are: the much greater speed with which specimens can be prepared for examination and the potentially much higher resolution, which enables full advantage to be taken of the best electron-optical resolution of modern electron microscopes (VALENTINE and HORNE 1962).

The electron microscope is now being increasingly used to identify and control the preparation of subcellular fractions derived from tissue homogenates by centrifugation procedures, but a serious limitation is the time taken to process, by conventional histological methods, the large number of fractions which may be prepared in the course of a single experiment. There are also many problems in the structure of cell organelles which are difficult to resolve from studies of thin sections. We therefore decided to investigate the applicability of the negative staining method to subcellular preparations.

The studies now to be reported have been made with particulate fractions derived from homogenates of brain tissue, since the morphology of these fractions has recently been investigated in detail by thin sectioning (GRAY and WHITTAKER 1960, 1962). The choice of brain was felt to be particularly suitable because during homogenization, nerve endings are torn away from their axon terminations and their post-synaptic attachments to form nerve ending particles about 1 μ

in diameter which can be separated from other subcellular particles by a combination of differential centrifugation and density gradient separation. These nerve ending particles contain organized cytoplasm and thus provide a means of evaluating the negative staining method in relation to structures bounded by a cell membrane.

Material and Methods[1]

Preparation of subcellular fractions. Homogenates of guinea-pig fore-brains in 0.32 M sucrose and subcellular fractions derived therefrom were prepared as described by WHITTAKER (1959) and GRAY and WHITTAKER (1962). Three main fractions were obtained: P_1 by centrifuging the initial homogenate at 1000 *g* for 10 min; P_2 by centrifuging the supernatant from the first centrifugation at 15,000 *g* for 60 min; P_3, by centrifuging the supernatant from the second centrifugation at 105,000 *g* for 60 min. The P_2 fraction was further separated into *A*, *B* and *C* fractions by layering it, after resuspension in 0.32 M sucrose, over a discontinuous density gradient consisting of a layer of 0.8 M sucrose floating on a layer of 1.2 M sucrose and centrifuging at 100,000 *g* for 45 min; the *A* fraction consisted of material floating on 0.8 M sucrose, the *B* fraction of material floating between 0.8 and 1.2 M sucrose and the *C* fraction of material passing through the 1.2 M sucrose layer to the bottom of the tube. All operations were carried out at 0—4°. Previous work (GRAY and WHITTAKER 1962) has shown that P_1 consists mainly of nuclei and large myelin fragments, *A* contains smaller myelin and membrane fragments, *B* mainly nerve endings, *C*, mitochondria and P_3, microsomes.

Negative staining of fractions. The following methods were used unless otherwise stated. (I) Particulate material suspended in ice-cold 1 or 2% aqueous phosphotungstic acid adjusted to pH 7.4 with 1 N KOH was quickly sprayed onto grids using a modified Vapofrin nebulizer (HORNE 1961). (II) Particles were suspended in 0.32 M or 0.45 M (fraction *B*) sucrose and the suspension diluted with phosphotungstate with or without prior fixation at pH 7 for 10 min using formaldehyde (0.5 to 5%) or osmium tetroxide (0.01 to 0.5%); suspensions were then sprayed onto grids or applied by means of a micropipette. The following day the grids were inspected in the Siemens Elmiskop I high-resolution electron microscope (type ÜM-11) at 80 KV.

Results

Conditions for obtaining negative staining

Under appropriate conditions of particle, phosphotungstate and sucrose concentrations, all the various types of subcellular particles previously seen in thin sections could be identified by negative staining. Due allowance had to be made for the different appearance of the particles when seen as a whole and not in section. They seemed well preserved and not unduly collapsed; indeed the height above the grid of the larger particles often exceeded the depth of focus (about 0.5 μ at the specimen) of the microscope. Interest mainly centred in the myelin, nerve ending particles and mitochondria of the P_2 fraction. Comparison of the particles seen in the morphologically fairly homogeneous fractions *A*, *B* and *C* with those seen in P_2 was helpful in making identifications. Most of the work was done with the P_2 fraction since the nerve ending particles and mitochondria of this fraction are less damaged than those of the *B* and *C* fractions (GRAY and WHITTAKER 1962) owing to its shorter time of preparation and the absence of exposure to hypertonic sucrose.

It was necessary to determine, by trial and error, the optimum procedure for each fraction and type of particle. If the concentration of phosphotungstate was too high relative to the amount of subcellular material in the final mixture, the

[1] We are grateful to Dr. E. G. GRAY for providing the unpublished electron micrographs reproduced in Figs. 1, 3 and 7, and to Mr. L. F. WISE for skilled technical assistance.

phosphotungstate did not spread properly, or crystallization took place in the beam either at scattered nuclei or structures, giving the appearance of low-resolution positive staining. Best results with the P_2 fraction were obtained with a final phosphotungstate concentration of 0.75—1.5% and particles equivalent to 50—100 mg brain tissue/ml. Sucrose or inorganic salts interfered with the even deposition of phosphotungstate in the droplets, forming large patches of low electron density. However, interference was usually only severe in the centre of droplets where the depth of phosphotungstate deposition was in any case too great for effective observation of structure, and there were usually useful areas round the edges of droplets where particles were seen embedded in a thin evenly distributed film of phosphotungstate. If sucrose was eliminated, as when well packed pellets were resuspended in sucrose-free phosphotungstate, even spreading of phosphotungstate throughout the droplets was obtained, but hypotonic damage to the particles resulted. In the case of nerve ending particles, slight hypotonic damage (Figs. 4, 9) was useful for revealing details of internal structure; mitochondria, however, were more seriously disrupted. A final concentration of 0.08 M sucrose after dilution with phosphotungstate was found to be a good compromise; provided the particles were transferred rapidly to the grids after dilution, there was little or no hypotonic damage under these conditions.

Fixatives. As fixatives, both formaldehyde and osmium tetroxide were tried. After fixation, particles became more fragile; thus fixation was usually carried out by adding the fixative (neutralized to pH 7) in 0.32 M sucrose to the particles suspended in sucrose and then diluting with phosphotungstate just before transfer to the grids. Osmicated nerve ending particles were too fragile to be sprayed onto the grids without damage and had to be transferred by micropipette. The use of osmium tetroxide as a fixative did not reverse the negative staining, but some loss of contrast occurred (Fig. 5). The effect of the fixatives on the penetration of phosphotungstate into the particles differed with different particles and is discussed below.

Morphology of the particles of the P_2 fraction as seen by negative staining

In Figs. 1—8 a comparison is made between the appearance of the three main types of subcellular particles of the P_2 fraction, myelin fragments (Figs. 1, 2), nerve endings (Figs. 3—6) and mitochondria (Figs. 7, 8) in negative staining and in thin section.

Myelin. The periodic structure of myelin is clearly visible in negative staining (Fig. 2). Fig. 15 shows a densimetric recording of a highly enlarged segment of a myelin fragment. Peaks corresponding to lines of negative staining are seen; these presumably represent the hydrophilic regions between apposed 'unit membranes', the dense lines (tall peaks) to the major dense lines in positive staining and the faint lines (smaller peaks or shoulders) to the intraperiod lines of positive staining (ROBERTSON 1961).

It was noted that the distance between the centres of dense lines of negative staining was usually constant for the first few periods at the edge of the myelin particles and then often increased as one continued towards the centre. This

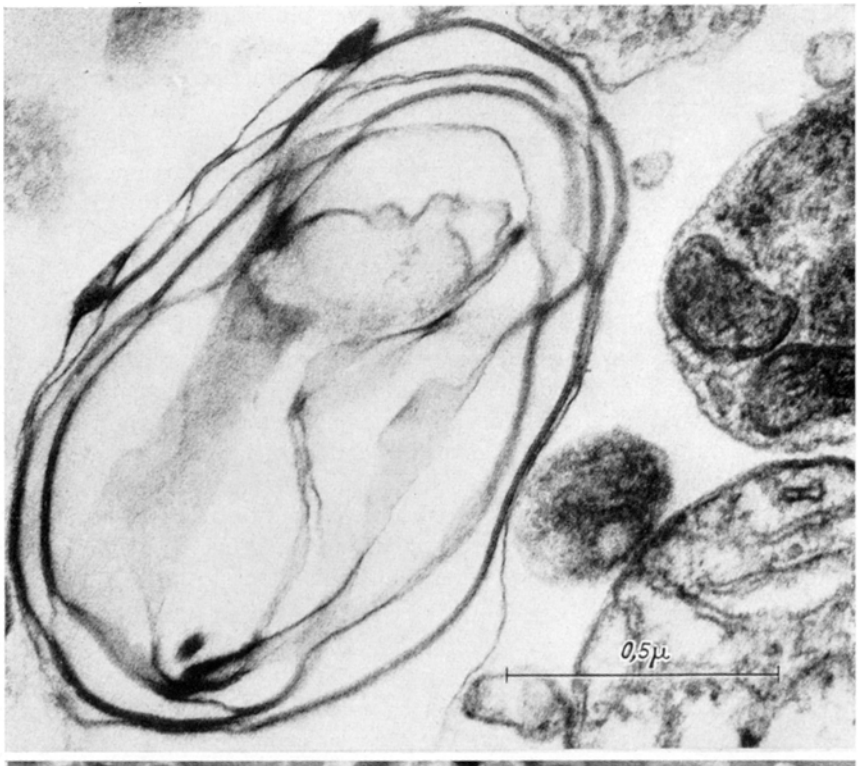

Fig. 1

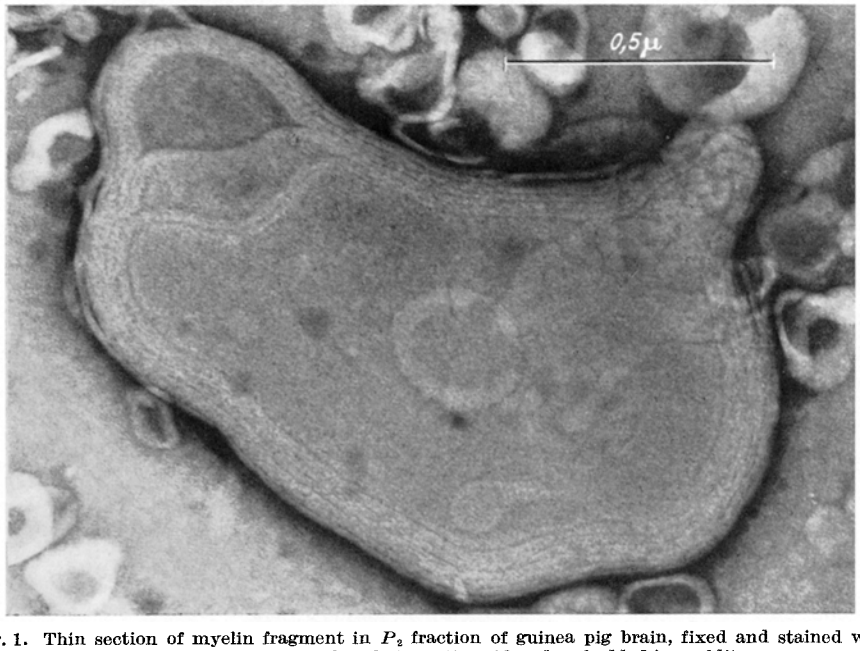

Fig. 2

Fig. 1. Thin section of myelin fragment in P_2 fraction of guinea pig brain, fixed and stained with osmium tetroxide and phosphotungstic acid and embedded in araldite

Fig. 2. Myelin fragment in P_2 fraction, negatively stained by method I

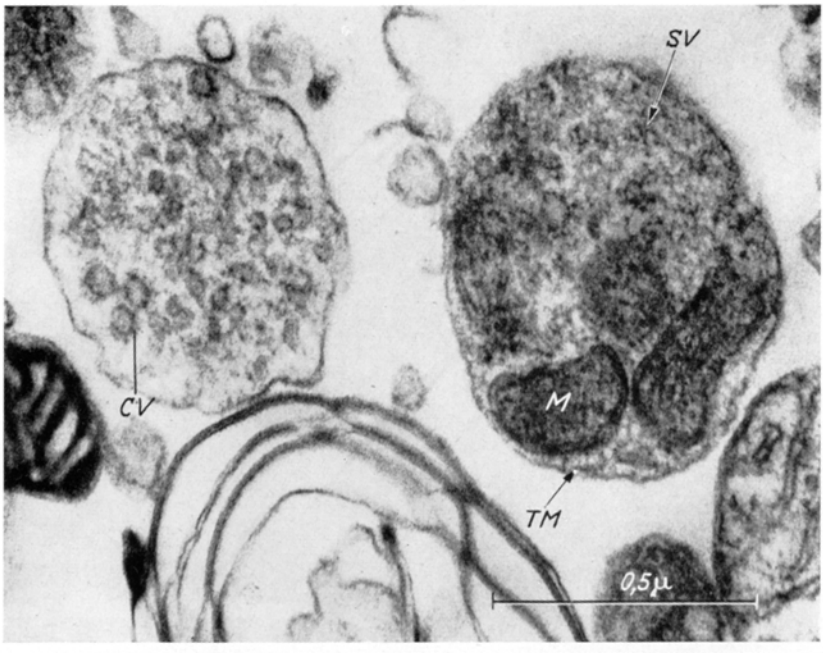

Fig. 3

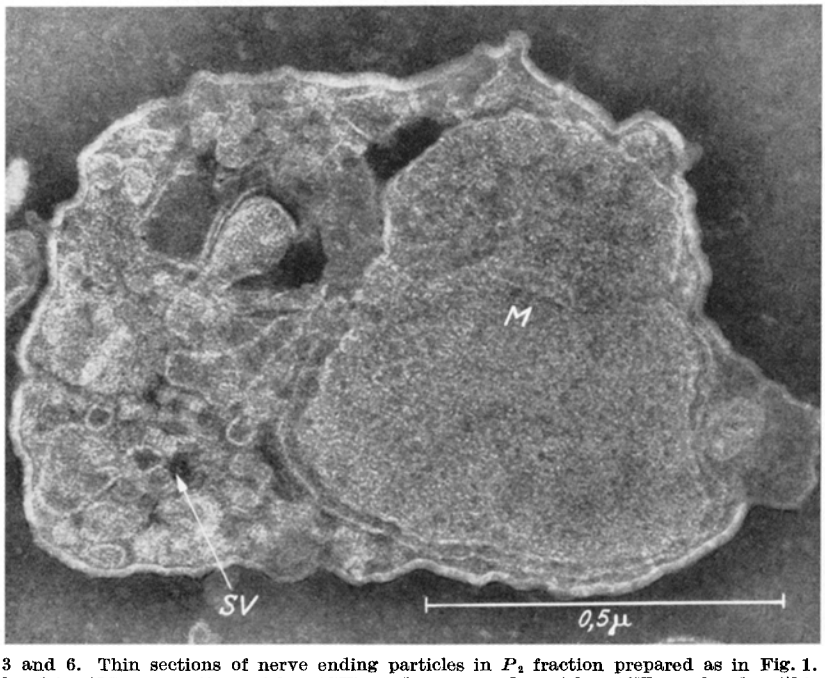

Fig. 4

Figs. 3 and 6. Thin sections of nerve ending particles in P_2 fraction prepared as in Fig. 1. Note mitochondria (*M*), synaptic vesicles (*SV*) and compound vesicles (*CV*) enclosed within thin membrane (*TM*)

Fig. 4. Nerve ending particle of P_2 fraction, negatively stained by method I. Abbreviations as in Fig. 3

increase is probably due to a curving over of layers which are no longer seen edge-on. The minimum repeating period varied from one preparation to another,

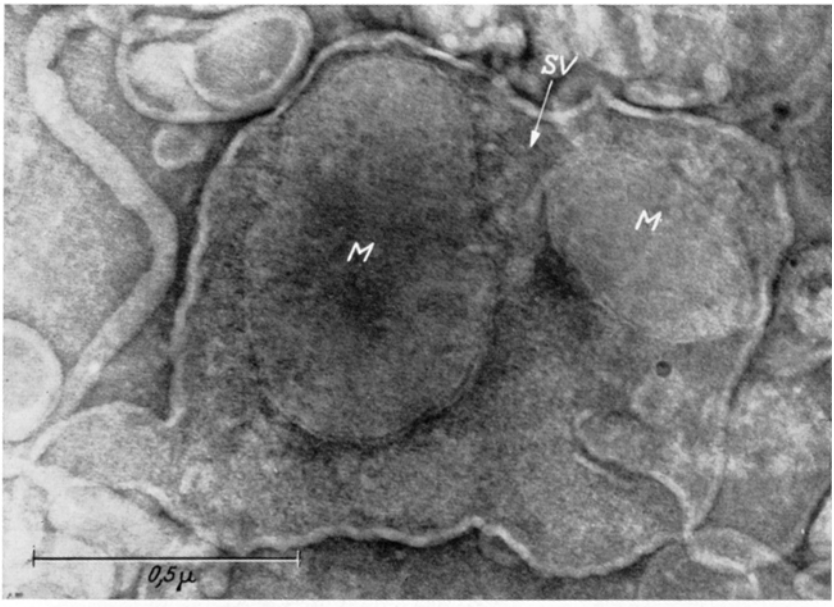

Fig. 5

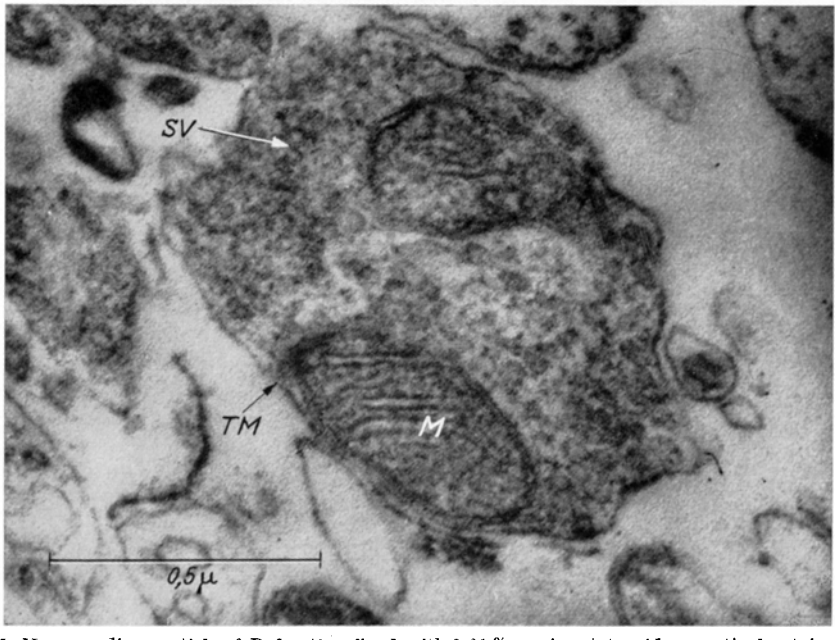

Fig. 6

Fig. 5. Nerve ending particle of P_2 fraction fixed with 0.01 % osmium tetroxide, negatively stained by method II and pipetted onto grids. Note outlines of mitochondria (*M*) and occasional synaptic vesicles (*SV*) perceived through outer membrane, also loss of contrast as compared with Fig. 4 due to osmium

Fig. 6. See Fig. 3

and was about 140—170 Å; the average value is thus somewhat below the value obtained by Finean (1958) from X-ray diffraction on fresh specimens and suggests that some shrinking may occur on drying.

Nerve ending particles. In thin sections, nerve ending particles are recognised (Figs. 3, 6) as thin-walled bags containing large numbers of synaptic vesicles

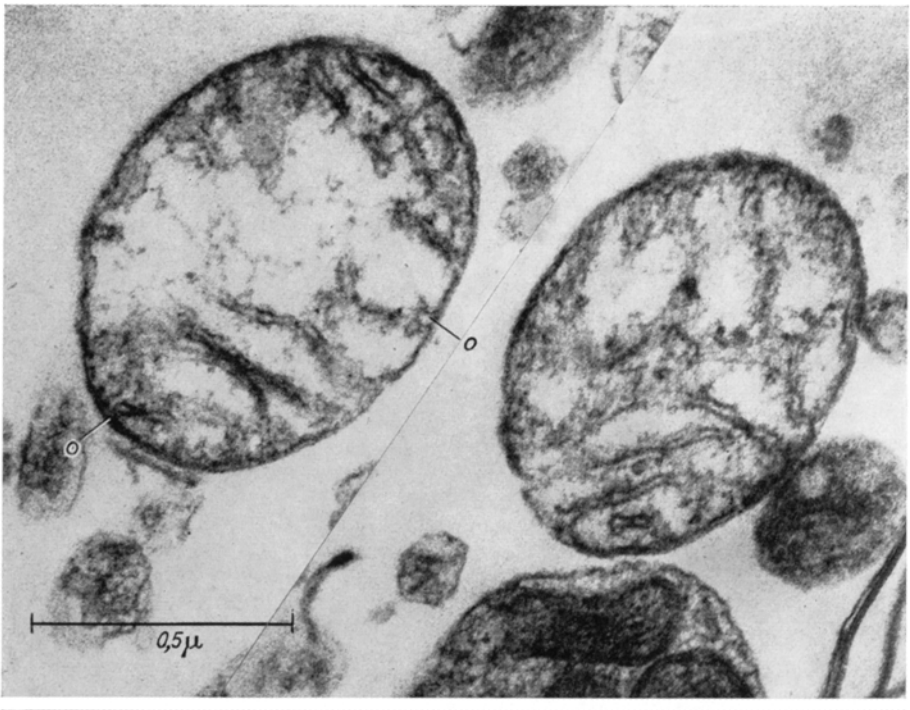

Fig. 7

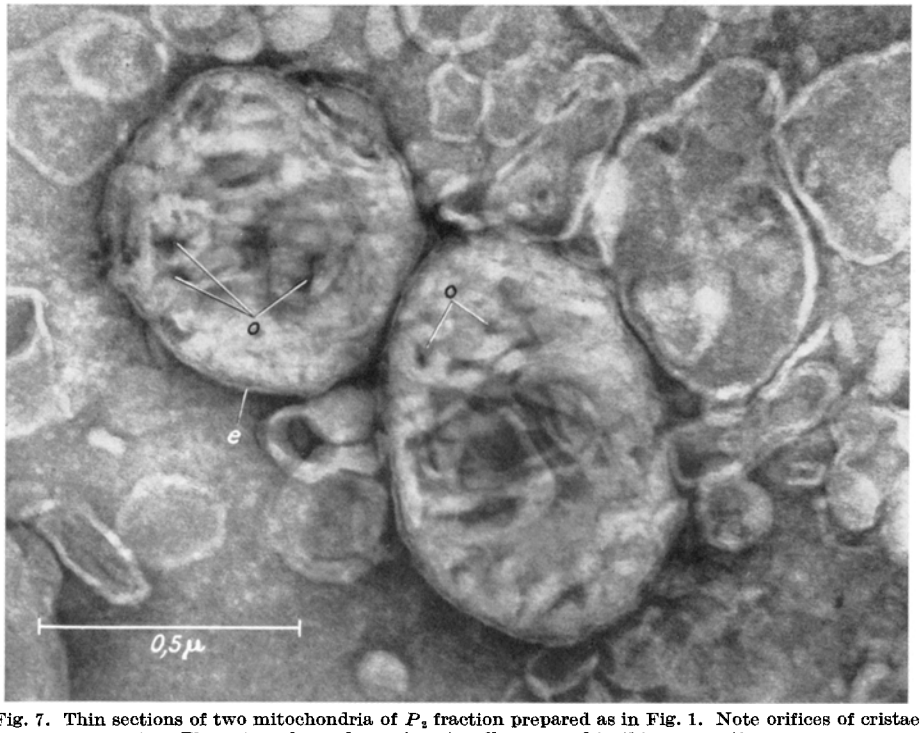

Fig. 8

Fig. 7. Thin sections of two mitochondria of P_2 fraction prepared as in Fig. 1. Note orifices of cristae at *o*. The external membrane is not well preserved in this preparation

Fig. 8. Two mitochondria of P_2 fraction fixed in 0.5 % osmium tetroxide in 0.32 *M* sucrose and negatively stained as in Fig. 5. Note clearly defined external membrane (*e*) and orifices of cristae (*o*) opening on to upper surface of the matrix

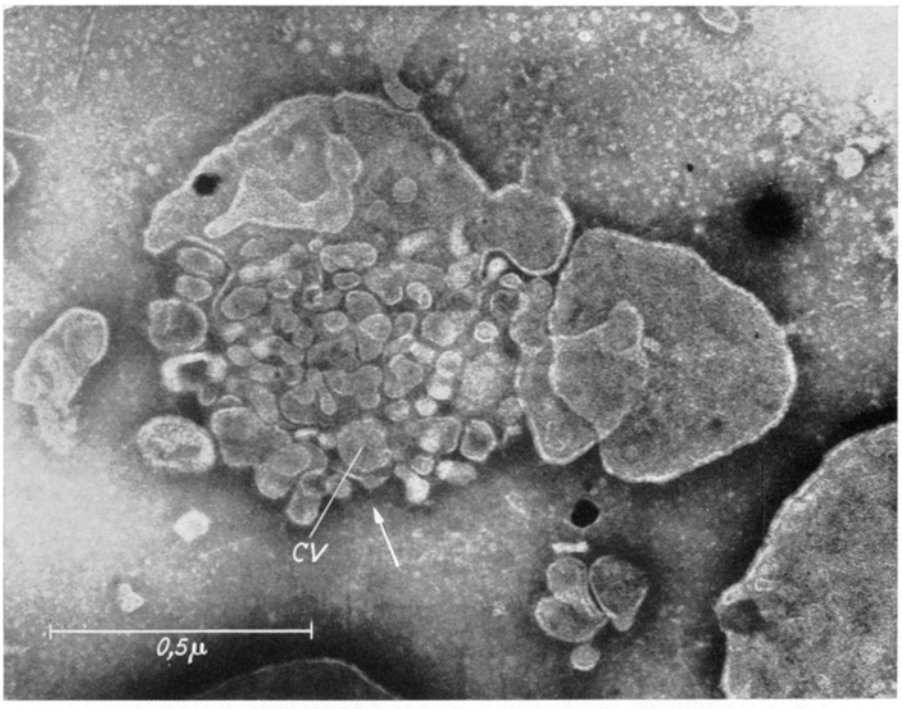

Fig. 9

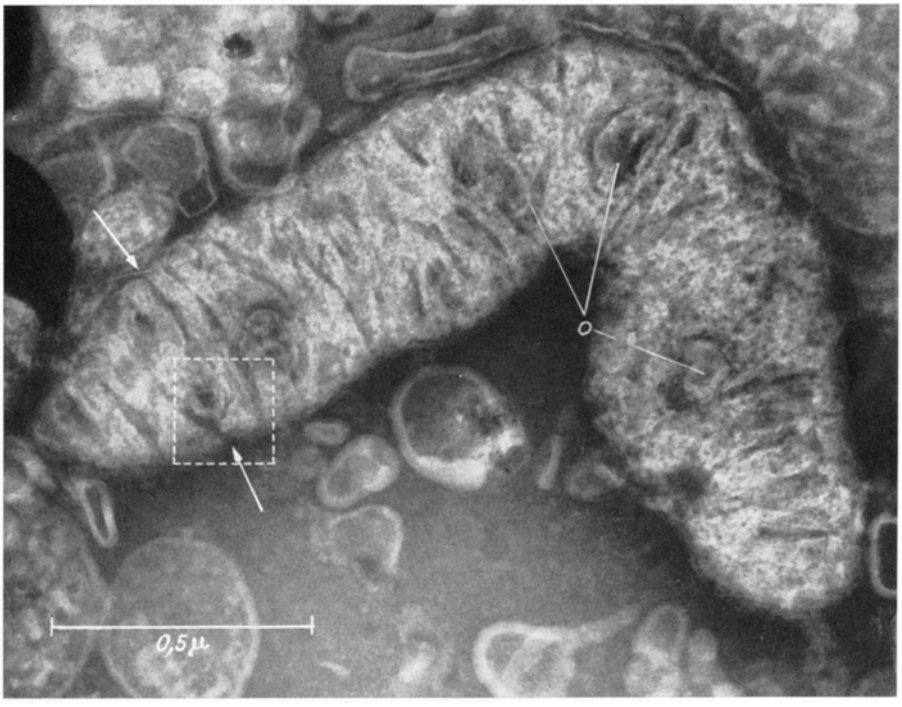

Fig. 10

Fig. 9. Nerve ending particle of P_2 fraction negatively stained by method I. Note outer membrane absent on side indicated by arrow, compound vesicle (?) at (*CV*) and mass of vesicles remaining adherent

Fig. 10. Long mitochondrion, fixed with 0.01 % osmium tetroxide, negatively stained by method II and sprayed onto grid. Note orifices of cristae at *o* and (in side view) at arrows

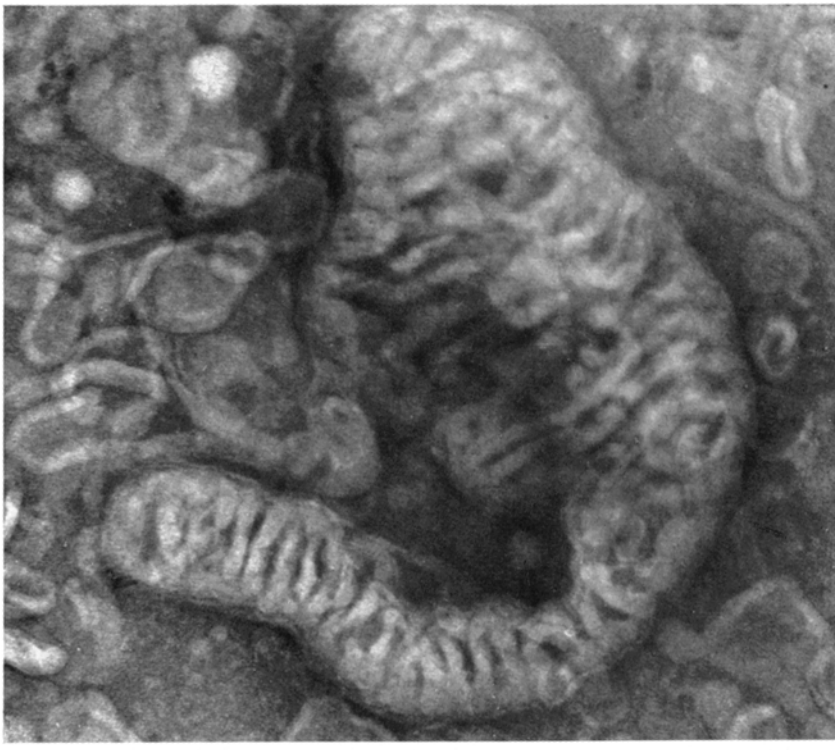

Fig. 11

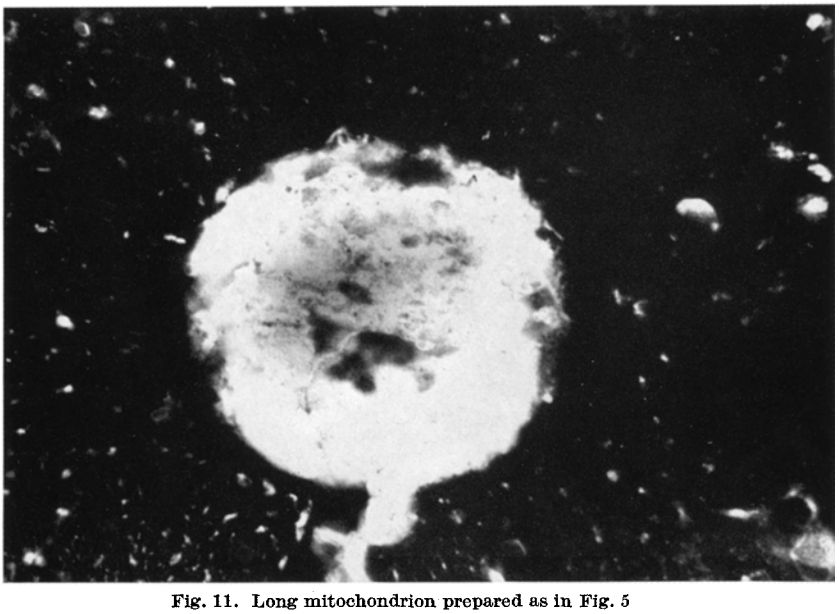

Fig. 12

Fig. 11. Long mitochondrion prepared as in Fig. 5

Fig. 12. Nucleus in P_1 fraction, negatively stained by method II and pipetted onto grid

about 500 Å in diameter and one or more small mitochondria. In addition a length of post-synaptic membrane may be adherent (GRAY and WHITTAKER 1962), though this is not seen in the examples shown in Figs. 3 and 6.

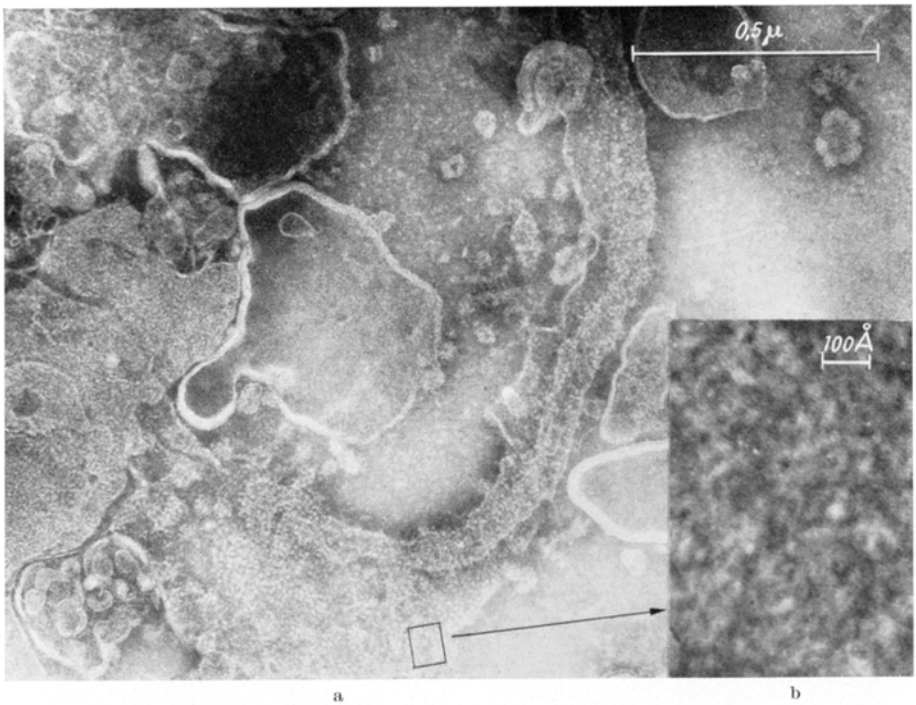

Fig. 13 a b

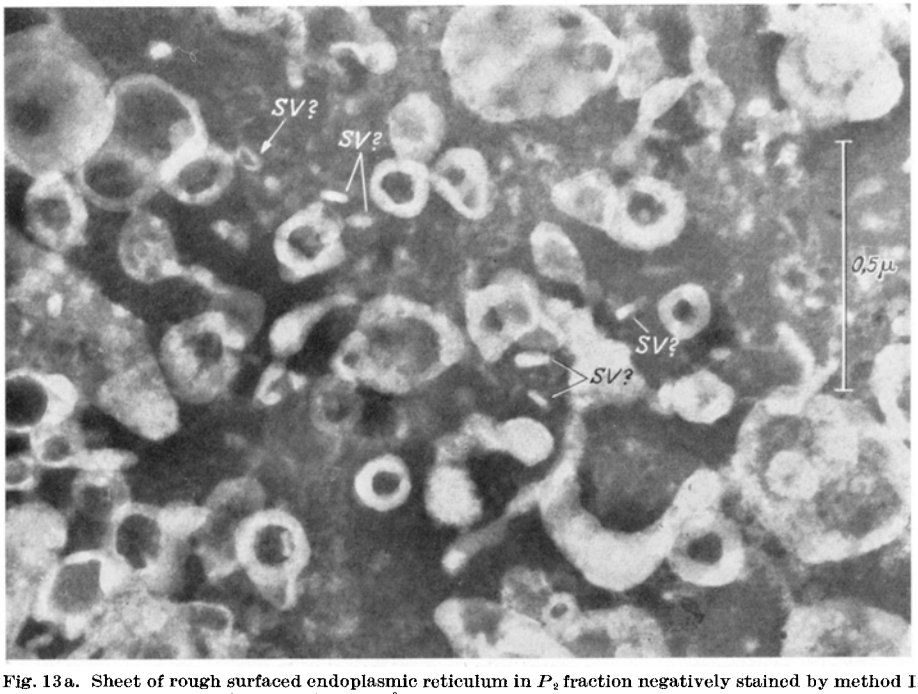

Fig. 14

Fig. 13a. Sheet of rough surfaced endoplasmic reticulum in P_2 fraction negatively stained by method I. Doughnut structure of ribosomes 80—100 Å in diameter is seen in high magnification view in Fig. 13 b

Fig. 14. P_3 fraction, negatively stained by method I. Note profusion of small oval or circular profiles, some of which may be isolated synaptic vesicles (*SV*?)

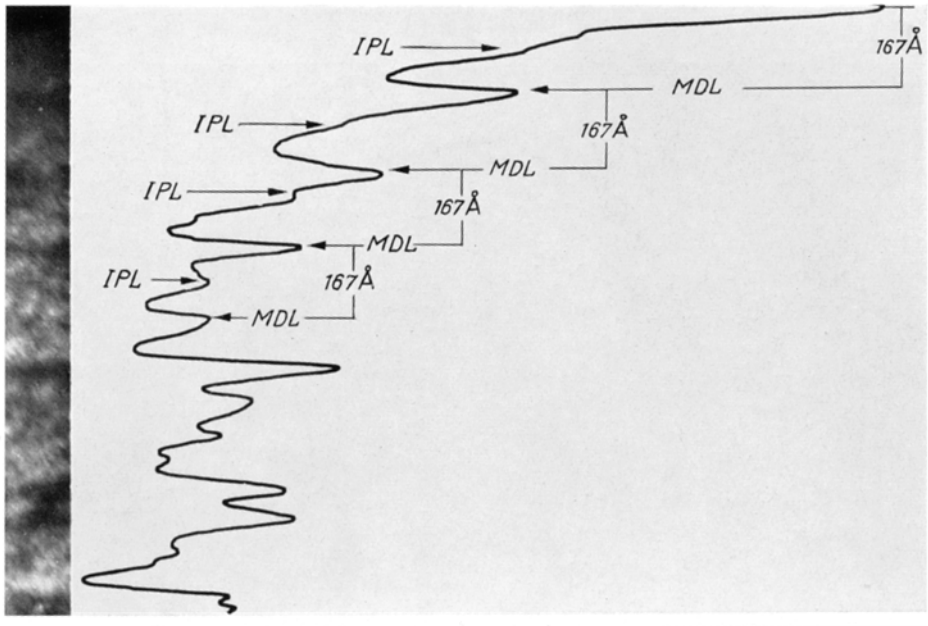

Fig. 15

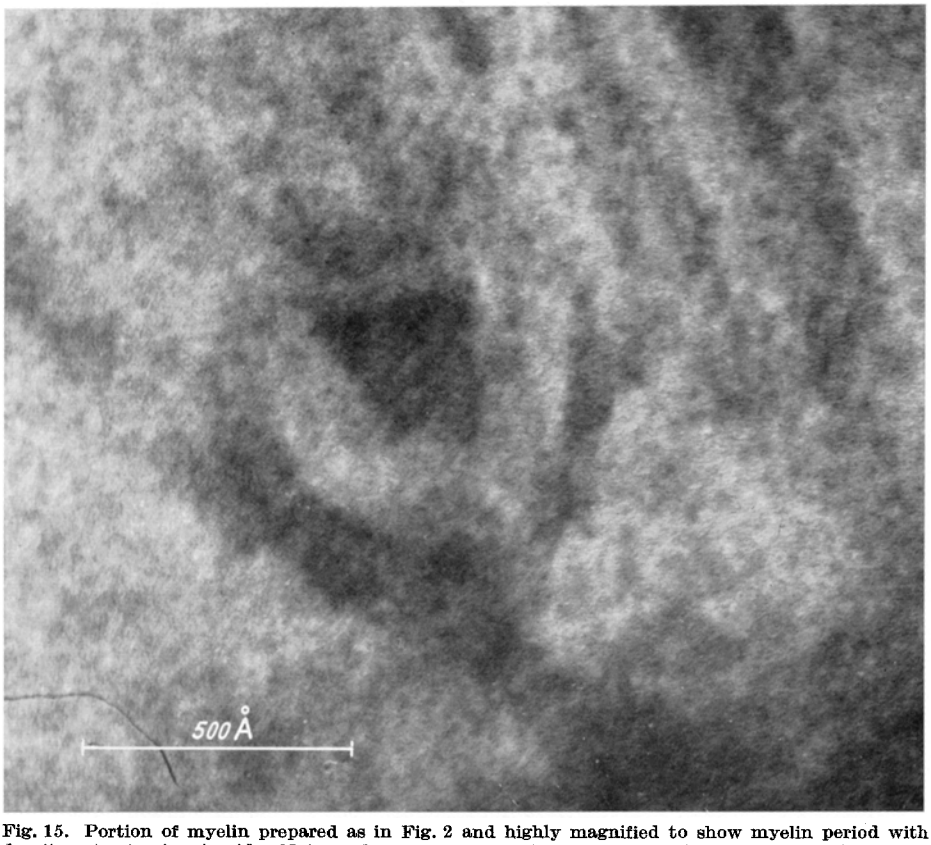

Fig. 16

Fig. 15. Portion of myelin prepared as in Fig. 2 and highly magnified to show myelin period with densitometer tracing to side. Note peaks corresponding to major dense lines (*MDL*) with spacing between centres of 167 Å and minor peaks or shoulders representing interperiod lines (*IPL*)

Fig. 16. Enlargement of cristal orifice (dotted rectangle in Fig. 10)

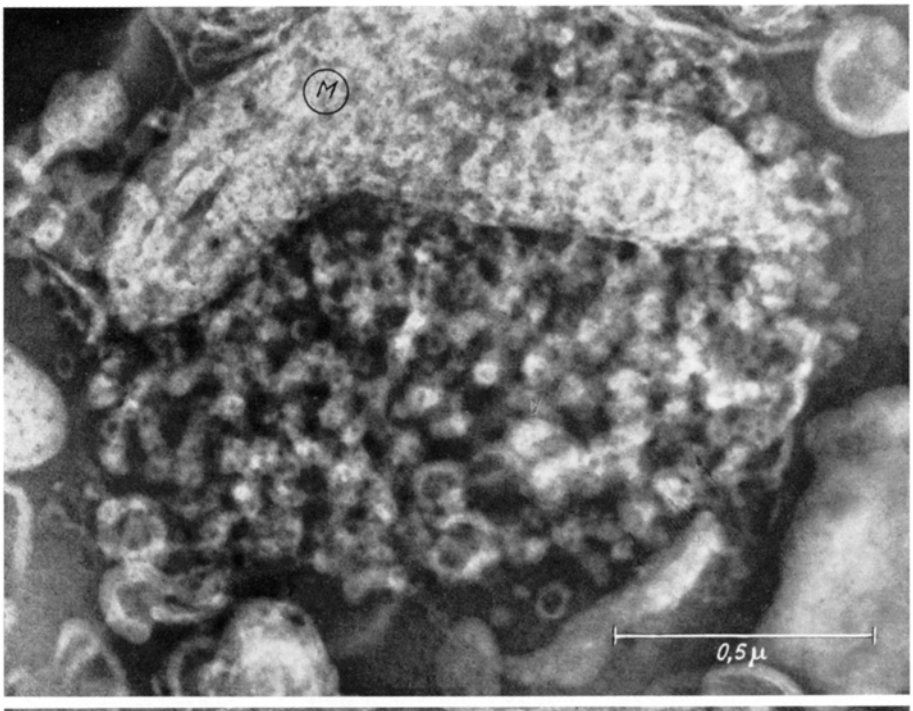

Fig. 17

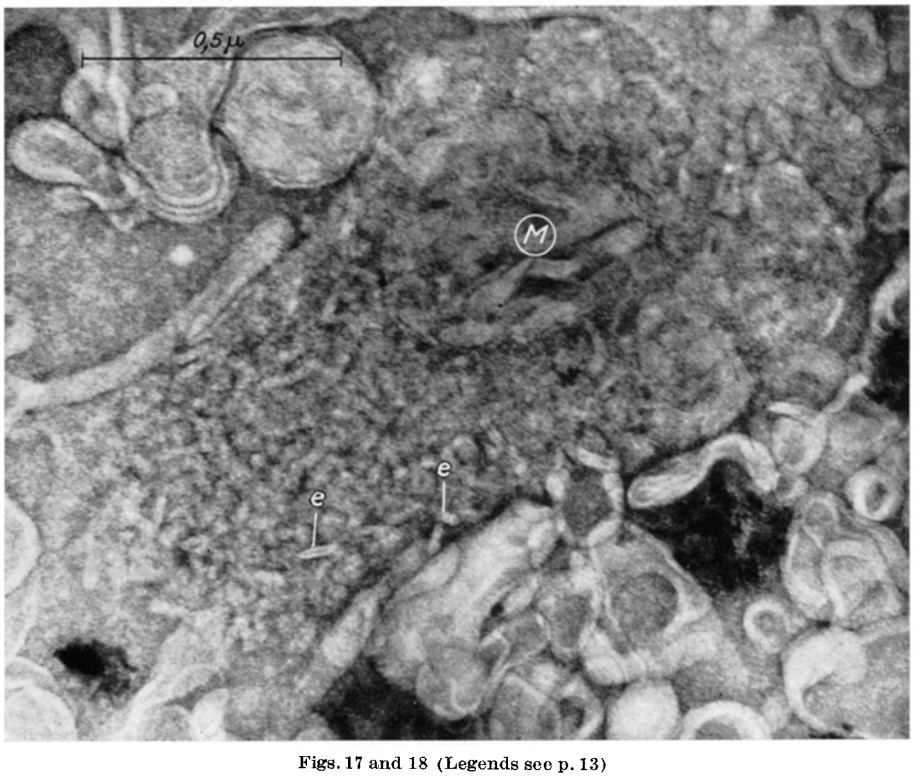

Fig. 18

Figs. 17 and 18 (Legends see p. 13)

In negative staining, the appearance of the nerve ending particles depends on the extent to which phosphotungstate has been able to penetrate into the particle. In the preparation shown in Fig. 4, the disruption of the outer membrane which occurred when the nerve ending was resuspended in sucrose-free, hypotonic phosphotungstate enabled the latter to penetrate into the cytoplasm and reveal the synaptic vesicles, swollen and distorted by the hypotonic conditions, and a fairly large mitochondrion. In Fig. 5, the hypotonic shock was lessened by the the presence of sucrose; two mitochondria and occasional synaptic vesicles are dimly perceived through the envelope of the particle. This preparation was fixed in 0.01% osmium tetroxide, which is insufficient to break down the permeability of the particle envelope; appearances similar to Fig. 5 were obtained with formaldehyde and without fixative in the presence of sucrose, but when 0.5% osmium tetroxide was used, the internal structure became more clearly visible.

Results with negative staining have provided the following information about synaptic vesicles. In shape, they often appear to be flattened discs rather than spheres and are frequently seen tilted or edge-on (Fig. 18); the less regular and more spherical shape seen in Figs. 4 and 9 is probably due to swelling in hypotonic media. The flattened shape may be due to drying but this seems unlikely. The vesicles appear to be embedded in a jelly-like cytoplasm; this is well shown in Figs. 9 and 17 in which the outer membrane has completely disappeared all down one side, yet the vesicles remain clustered together. This conslusion is supported by much evidence from thin sections of isolated nerve endings and whole tissue preparations and explains why it has not so far been possible to isolate intact mono-dispersed synaptic vesicles from nerve endings by disruptive techniques (WHITTAKER 1962).

Post-synaptic membranes have not been positively identified as it has proved difficult to exclude the possibility that the apposed membranes sometimes seen are not chance juxtapositions of separate particles.

Mitochondria. The best results were obtained with preparations fixed in osmium tetroxide (Figs. 8, 10, 11) or formaldehyde. In such preparations the spaces between the walls of the cristae and between the matrix and the external enveloping membrane are beautifully revealed by the penetration of phosphotungstate. These spaces are therefore most probably hydrophilic. The cristae are frequently seen opening out into the space between the matrix and the external membrane (arrow, Fig. 10) and orifices of vertically orientated cristae are seen from above at *o* in Figs. 8 and 10. These observations are consistent with the Palade-Robertson model of the mitochondrion (see ROBERTSON 1961). In this, the cristae are assumed to be invaginations of a unit membrane surrounding the matrix. The external membrane is also assumed to be a unit membrane. Since the surface layers of a unit membrane are assumed to consist of protein, the space between two unit membranes (the 'outer compartment') must be hydrophilic and readily penetrable by phosphotungstate once the semipermeable character

Fig. 17. Nerve ending particle showing Y-shaped mitochondrion (*M*) and vesicles remaining adherent in spite of lack of external membrane. Fixed with 0.5 % osmium tetroxide in 0.32 M sucrose, negatively stained by method II and sprayed onto grid

Fig. 18. Nerve ending particles showing mitochondrion (*M*) and closely packed vesicles, some with 'edge-on' profile (*e*). Prepared as in Fig. 8

of the external membrane is destroyed by fixation. The same considerations would not apply to the SJÖSTRAND (1956) model in which the space between the double membranes is assumed to be filled with lipid.

An interesting further finding was the prevalence of long thin mitochondria as seen in Figs. 10 and 11. These were often curved or horseshoe-shaped and waisted, as though budding off. Y-shaped mitochondria were also seen (Fig. 17). Thin sections would have shown these long or branched mitochondria as multiple oval profiles, confirming the deductions of WESSEL (1960).

Molecular subunits in membranes. In sharply focussed pictures a granular appearance was often seen in myelin, nerve ending and mitochondrial membranes (Figs. 4, 5 and 9) which could not be accounted for by background grain and is probably due to molecular subunits in the membranes. Similar subunits have been seen recently (HORNE and BANGHAM, to be published) in artificial lipid membranes using negative staining.

Other structures. Many unidentified smooth walled circular profiles are seen in negatively stained P_2 preparations as in thin sections. In addition, structures (Figs. 13, a, b) not previously noted in thin sections are not infrequently seen; these appear to be sheets of rough-surfaced endoplasmic reticulum studded with ribosomes. Plaques or sheets of such material have been observed in the cytoplasm of oligodendrogliocytes by PALAY (personal communication).

Other brain fractions

Little work has as yet been carried out on the nuclear (P_1) and microsomal (P_3) fractions but results so far obtained indicate that the negative staining method is applicable to the study of the material in these fractions also. The particles of the P_1 fraction were too large to be sprayed and had to be applied with a micropipette; large myelin fragments and nuclei (Fig. 12) have been identified, the latter sometimes with strands of material, perhaps deoxyribose-nuclei acid, coming out of them. In the P_3 fraction, large numbers of small circular doughnut shaped particles were seen (Fig. 14); some of the smallest of these might be isolated synaptic vesicles, which had broken away from the nerve ending cytoplasm.

Other tissues

Preliminary studies indicate the applicability of the negative staining method to rat liver and muscle preparations.

Discussion

The results presented in this paper demonstrate the applicability of the negative staining method to the identification and study of subcellular fractions from animal tissues. The method should prove useful in several ways, in particular: (1) for the rapid evaluation of subcellular fractions obtained by differential centrifugation, density gradient centrifugation or density gradient electrophoresis; (2) as a means of visualizing extremely small structures such as synaptic vesicles or ribosomes, which are difficult to see clearly in thin sections when isolated; (3) for visualizing complex structures which are difficult to reconstruct from thin

sections; (4) for providing information regarding the molecular structure of animal cell membranes and organelles. The survival, following homogenization, of tissue or cell fragments containing organized cytoplasm provides a means whereby the high resolution of the method can be applied in cytological studies.

Summary

1. The negative staining (negative contrast) method for investigating the structure of particulate biological material (viruses, bacteria) in the electron microscope has been found to be applicable to subcellular fractions of guinea pig brain and other animal tissue preparations.

2. The method involves the transfer to specimen grids of fine droplets of the particulate material mixed with potassium phosphotungstate at pH 7.4. Under suitable conditions the droplets leave, on drying, a glass of electron-dense phosphotungstate in which the subcellular particles show up as areas of relatively low electron-density. The phosphotungstate appears to protect the particles from excessive distortion; it has the property of penetrating into minute pores and clefts and shows up fine structure with a potentially much higher resolution than is obtainable in thin sections.

3. The potentialities of the method have been illustrated by work with myelin fragments, mitochondria and particles derived from nerve endings.

4. The myelin period has been estimated to be 140—170 Å, the mean value of which is somewhat below the diffraction results of FINEAN.

5. The results with mitochondria are consistent with the Robertson rather than the Sjöstrand model. Long sausage-shaped mitochondria are frequently seen which would appear in thin sections as multiple oval profiles, thus confirming the deductions of WESSEL.

6. The results with nerve ending particles clearly demonstrate the existence, as discrete cytoplasmic organelles, of synaptic vesicles. They are seen to have a flattened, disc-like shape rather than a spherical, and remain firmly stuck together even when the outer wall of the particle is broken.

7. Other structures seen in brain fractions include nuclei, microsomes and sheets of endoplasmic reticulum studded with ribosomes.

8. The method appear to be highly suitable for work with subcellular fractions and to be capable of giving valuable information complementary to that obtainable with thin sections.

Literature

BRENNER, S., and R. W. HORNE: A negative staining technique for high resolution of viruses. Biochim. biophys. Acta (Amst.) **34**, 103—110 (1959).

FINEAN, J. B.: X-ray diffraction studies of the myelin sheath in peripheral and central nerve fibres. Exp. Cell Res., Suppl. **5**, 18—32 (1958).

GRAY, E. G., and V. P. WHITTAKER: The isolation of synaptic vesicles from the central nervous system. J. Physiol. (Lond.) **153**, 35—37 P (1960).

— — The isolation of nerve endings from brain: an electron-microscopic study of cell fragments derived by homogenization and centrifugation. J. Anat. (Lond.) **96**, 79—88 (1962).

HALL, C. E.: Electron densitometry of stained virus particles. J. biophys. biochem. Cytol. **1**, 1—12 (1955).

HORNE, R. W.: In techniques for electron microscopy (ed. D. D. KAY). Oxford: Blackwell's Scientific Publications Ltd. 1961.

HUXLEY, H. E.: Some observations on the structure of tobacco mosaic virus. 1st Regional Conference on Electron Microscopy. Stockholm: Almquist & Wiksell 1956.

ROBERTSON, D. J.: In: Regional Neurochemistry: the regional chemistry, physiology and pharmacology of the nervous system (ed. S. S. KETY and J. ELKES). Oxford: Pergamon Press 1961.

SJÖSTRAND, S. F.: The ultrastructure of cells as revealed by the electron microscope. Int. Rev. Cytol. **5**, 455—533 (1956).

VALENTINE, R. C., and R. W. HORNE: An assessment of negative staining techniques for revealing ultrastructure. J. Ultrastruct. Res. (Suppl.), in press (1962).

WESSEL, W.: Über die Form der Mitochondrien in elektronenmikroskopischen Bildern. Z. Zellforsch. **52**, 712—714 (1960).

WHITTAKER, V. P.: The isolation and characterization of acetylcholine-containing particles from brain. Biochem. J. **72**, 694—706 (1959).

— The separation of subcellular structures from brain tissue. Biochem. Soc. Symp. in press (1962).

R. W. HORNE,
Agricultural Research Council, Institute of Animal Physiology
Babraham, Cambridge, England