

LIPID STAINING FOR THE ELECTRON MICROSCOPE: A NEW METHOD

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SUMMARY

Tissues fixed in osmium tetroxide or in combined osmium and glutaraldehyde (Hinde), embedded in Spurr's medium, cut at 0.5-1 μ m and mounted in Farrants' gum medium containing ethyl gallate, show good staining of lipid-containing structures (droplets of triglyceride, membranes, mitochondria, etc.) in the light microscope.

Such preparations show moderate contrast in the electron microscope without further staining. But a specific increase in contrast in lipid-rich structures is obtained by partition of the tissues, before embedding, in 70% ethanol saturated with the monoterpene hydrocarbon myrcene, with or without the addition of 0.1% ethyl gallate, followed by osmium tetroxide.

This method will visualize both saturated and unsaturated lipids, including waxes.

INTRODUCTION

Tissues fixed with osmium tetroxide and then treated with ethyl gallate* before double embedding in agar and esterwax, show intense staining of membranes, mitochondria, nucleoli, Golgi bodies and lipid droplets. Since undenatured proteins take up relatively little osmium, and nucleic acids and carbohydrates are completely unreactive, this procedure was described as a method for visualizing lipids (Wigglesworth, 1957). It was confirmed by Hayes, Lindgren & Gofman (1963) that virtually all the osmium taken up by fresh tissues is in fact bound by unsaturated lipids. In tissues treated with acid fixatives (Bouin, Carnoy, etc.) the denatured proteins react strongly with osmium and ethyl gallate, which then becomes a general protein stain (Wigglesworth, 1964).

The early preparations for the electron microscope, fixed in osmium tetroxide alone, cut in methacrylate and examined without further contrast staining, likewise revealed largely lipid which had reacted with osmium tetroxide. But dense packing of any organic material will give some electron scattering; such intrinsic electron opacity could be seen in material fixed in formaldehyde alone. In tissues treated by methods which are now standard (fixed with glutaraldehyde before brief treatment with osmium tetroxide, embedded in Araldite by way of propylene oxide, and treated with metallic stains on the grid) it is impossible to tell how much, if any, of the electron density is due to lipid.

Furthermore, osmium tetroxide will react only with unsaturated lipids. In dealing

* Progallin A of Nipa Laboratories, Trading Estate, Cardiff.

with insect tissues in particular it is desirable to have a method which will also reveal saturated lipids, as well as solid waxes with a very low iodine number. This paper describes the development of such a method.

RESULTS

Incorporation of unsaturated lipid

The principle of the method is to incorporate additional unsaturated lipid into existing lipids in order to increase the amount of osmium they will bind. There are doubtless many suitable materials which could be employed for this purpose. I have used the monoterpene hydrocarbons D-limonene (puriss., Koch-Light) and myrcene (technical, said to be 95%, Aldrich Chemical Co.). These substances have the same empirical formula ($C_{10}H_{16}$), but limonene has two double bonds whereas myrcene has three. They are completely miscible with lipids, but will dissolve up to 0.5–1% in 70% ethanol or 60% pyridine.

Representative lipids, dissolved at 0.2% in chloroform, were pipetted in 1- μ l samples at the centre of small disks of lens paper, which were then cut into radial segments for comparative trials. The segments were embedded in 2.5% agar below a coverglass and the embedded slips were cut out and immersed in the medium for partition.

Ten microlitres of limonene or myrcene per ml of 60% pyridine, shaken gently and filtered after saturation was complete, gave satisfactory results. Control slips, and slips exposed to partition for 1–24 h, were then treated for 1 h with osmium tetroxide (1%); the bound osmium was visualized by immersion for several hours in 1% aqueous ethyl gallate, and the slips mounted in Farrants' gum medium containing ethyl gallate.

Results on isolated lipids

Fig. 1 shows a preparation of 'Nujol' (pure medicinal paraffin) treated as described. The droplets of oil have rounded up on the surface of the lens paper fibres and show intense osmium staining down to the limits of resolution. Nujol preparations not exposed to partition show no staining at all.

Results with other materials are predictable. *Olive oil* forms irregular adherent droplets; it takes up much osmium without partition (Fig. 2); after partition with limonene, staining is perhaps a little darker, but the difference is not striking.

Cephalin forms irregular films and flakes on the surface of the fibres and shows deep black staining without partition (Fig. 3). After partition the flakes have mostly rounded up into small deeply stained droplets (Fig. 4).

Cholesterol, which has one ethylenic double bond, forms irregular flakes which stain without partition, but not very deeply. After partition much of the cholesterol has rounded up and now stains strongly.

Stearic acid, which is fully saturated, is totally unstained in the absence of partition. After partition intense staining of droplets of all sizes occurs.

Sphingomyelin forms irregular films and flakes. Treated with osmium directly it

shows very weak staining with some black deposits and minute droplets (perhaps due in part to other lipid impurities). After partition it is mostly rounded up into deeply staining droplets.

Beeswax forms small flakes on the surface, which stain rather weakly with osmium/ethyl gallate without partition. After partition at room temperature the flakes persist, but now stain intensely (Fig. 5). If partition is carried out at 65 °C black-staining droplets are everywhere (Fig. 6).

Figs. 7, 8 show electron micrographs of droplets of Nujol. Nujol, 1:50 in 2.5% agar, was heated to 60 °C and emulsified by vigorous shaking. Small blocks of the cooled agar were then exposed to partition in myrcene in 60% pyridine (10 µl/ml) for 18 h; treated with osmium tetroxide (1% for 1 h) and embedded in Spurr (1969) medium.

It will be noted in Figs. 7 and 8 that, except in very minute droplets, the electron-dense layer forms a sheath around the droplet. This phenomenon is familiar in the fixation of lipid droplets in tissues with osmium tetroxide. The osmium induces a polymerization of the lipid in which the osmium is incorporated (Wigglesworth, 1957). After a brief exposure of 2 min, an almost invisibly thin film is formed over the surface of the droplets. As exposure is prolonged the sheath thickens; but with droplets 5–10 µm in diameter there always remains a central core which escapes polymerization and is thus extractable with ethanol and ether (Fig. 9).

The probable chemical nature of the cross-linking of double bonds by osmium was discussed at some length (Wigglesworth, 1957). The most obvious process would be diester formation as described by Criegee (1936) and Criegee, Marchand & Wannowius (1942); but there are other possibilities, and the actual process is not known.

In any case polymerization will result in a selective deposition in the surface of the droplet of those components most rich in double bonds. In extreme starvation in the blood-sucking bug *Rhodnius* the unsaturated lipids in the fat body are preferentially consumed and the droplets of triglycerides become progressively less reactive with osmium tetroxide. Under these conditions a thin shell of polymerized lipid containing osmium is confined to the periphery of the droplets (cf. Fig. 9).

It was also pointed out (Wigglesworth, 1957) that polymerization through osmium will occur only if there are several unsaturated chains in one molecule (as in triolein), or if there are several unsaturated links in one chain (as in linoleic acid).

Visualization of lipid in tissues

The tissues of the body wall of *Rhodnius* have been used as test material. By cutting along the margins of the abdomen the dorsal and ventral halves are detached and the tissues adhering to the body wall are readily exposed and fixed; they lend themselves well to comparison between areas differently treated.

Two tissues have been utilized: the fat-body cells, with droplets of triglyceride, large mitochondria, plasma membranes and stacks of laminated ER; and the epidermal cells responsible for secretion of the cuticle, during the formation of which they provide good material for nucleus, nucleolus and nuclear sap, mitochondria,

laminar and vesicular ER; numerous Golgi bodies liberating droplets of lipoprotein, microtubules running from the Golgi bodies to the cuticle, microvilli and plasma membranes.

Initial experiments with osmium-fixed tissues were disappointing in the small amounts of additional osmium-binding which followed partition in myrcene solutions. Indeed, if the exposure to myrcene was prolonged or repeated there might be an actual reduction in osmium uptake. This was traced to the extraction of osmium from the tissues by the myrcene, and perhaps myrcene oxidation. The defect was finally corrected by exposing the tissues to 70% ethanol for 24 h before partition in the myrcene solution, and by addition of a small amount of ethyl gallate to the partition mixture to serve as an antioxidant and to bind to the osmium in the tissues. Myrcene proved definitely superior to limonene in the partition experiments; and 70% ethanol as a solvent had a less deleterious effect on tissue structure than 60% pyridine.

The final procedure adopted consisted in the following. (i) Rapid dissection in ice-cold 2.5% glutaraldehyde in cacodylate buffer containing 7% sucrose. (ii) Fixation in ice-cold osmium tetroxide: 1% in cacodylate buffer containing 6% sucrose (2 h) followed by 2.5% glutaraldehyde (as above) at 4 °C for 24 h. Alternatively, equally good results, and sometimes better preservation of structure, were obtained by fixation in combined osmium tetroxide and glutaraldehyde (Hinde, 1971) for 1 h at 0 °C. (iii) Partition in 1% myrcene in 70% ethanol containing 0.1% ethyl gallate, gently shaken until saturated and then filtered. Partition for 1–18 h with gentle agitation. (iv) Removal of excess myrcene with 50% ethanol followed by renewed treatment with osmium tetroxide (1 h). (v) Embedding in Spurr's medium by way of ethanol only, omitting propylene oxide.

Results on tissue sections

Fig. 18 shows a 0.5- μ m section of the fat body of a 4th-stage larva of *Rhodnius* at 9 days after feeding; fixed in osmium tetroxide and embedded without further treatment in Spurr's medium. There is only moderate contrast of lipid droplets, mitochondria, etc. Fig. 19 is from the same material but including partition in myrcene (1 h) and renewed osmium treatment. There is increased contrast in triglyceride droplets, nucleoli, mitochondria and plasma membranes. It is this *increase* in osmium binding after myrcene partition which affords evidence of lipid content.

Extraction of tissues with propylene oxide (18 h at 22 °C) after osmium tetroxide fixation, followed by direct embedding in Spurr's medium, caused very little decrease in contrast as compared with Fig. 18.

Fig. 17 shows the typical appearance in the electron microscope of a section of the epidermis of a 4th-stage larva of *Rhodnius* at 9 days after feeding, prepared by the method: osmium tetroxide fixation, partition in 70% ethanol with 0.1% ethyl gallate and saturated with myrcene, for 16 h followed by osmium tetroxide (1 h). The sections lack the crisp outlines of membranes and of ribosomes given by uranyl acetate and lead nitrate staining after glutaraldehyde fixation. Characteristic are the deep lipid staining of the nucleolus and of inclusions passing through the nuclear sap presumably in process of liberation from the nucleus; nuclear membranes well stained;

mitochondria and Golgi droplets deeply stained; walls of saccules of ER showing diffuse lipid staining.

Fig. 13 shows the surface of part of an epidermal cell at 7 days after feeding, where the cells have become recently detached from the cuticle and are developing long slender microvilli. Fixation in osmium tetroxide; direct embedding in Spurr. Differentiation rather weak. Fig. 14 shows the same material after myrcene partition. There is much stronger contrast in the plasma membranes, in mitochondria, and in cytoplasmic inclusions (rough and smooth ER).

Figs. 15 and 16 show a similar comparison at 8 days after feeding, when the outer epicuticle is forming as a uniform lipid-staining layer with the convoluted plasma membrane and microvilli below. Abundant mitochondria collect near the cell apex and small lipid or lipoprotein vesicles are in process of incorporation into the cuticle. In this distal region of the cell, microtubules are abundant at this stage of development (see Wigglesworth, 1973: figs. 16, 17); but they appear not to contain lipid and are almost invisible in the present micrographs. Fig. 16, in which fixation was followed by 16-h partition in myrcene, shows increased contrast in all lipid-containing structures, as compared with Fig. 15, in which myrcene partition was omitted.

Fig. 22 is comparable with Fig. 17 and was prepared by the standard myrcene partition procedure after simultaneous osmium/glutaraldehyde fixation. Fig. 23 was prepared from the same material (an epidermal cell at 9 days after feeding) without myrcene partition but stained with lead citrate. The chief differences lie in the conspicuous black ribosomes but relatively pale mitochondria after lead staining, and the more darkly stained mitochondria and other lipid inclusions in the nucleus and cytoplasm after myrcene partition.

Fig. 20 shows part of a fat-body cell after osmium fixation and myrcene partition, with the margin of a triglyceride droplet (below right), together with laminated ER and darkly staining plasma membrane and mitochondria. Fig. 21 shows the same material without myrcene partition but after lead staining. The triglyceride droplet (to right) is only weakly stained and the plasma membranes and mitochondria are much paler. Ribosomes are darkly stained but do not show up well at this magnification.

The amount of osmium taken up at the osmium-binding sites before or after myrcene partition can be increased and the contrast therefore enhanced by means of the OTO procedure described by Seligman, Wasserkrug & Hanker (1966). When using Spurr's medium it is possible to apply this method to the tissues before embedding. After osmium treatment they are exposed for 30 min in 0.2% thiosemicarbazide in 20% acetic acid for 1 h at room temperature; and after washing well in 10% acetic acid they are again treated with 1% osmium tetroxide for 1 h. Fig. 24 shows part of an epidermal cell at 11 days after feeding, fixed in Hinde osmium/glutaraldehyde mixture and embedded in Spurr's medium. Fig. 25 shows part of the same preparation exposed to the OTO procedure as described above before embedding. It illustrates the general increase in the intensity of staining as compared with Fig. 24. The relative distribution of staining is unchanged.

Figs. 11 and 12 are examples of 0.5- μ m sections for the light microscope, of the fat

body after Hinde fixation, myrcene partition and OTO treatment, showing good staining of nucleoli, nuclear membrane, mitochondria, plasma membranes and triglyceride droplets.

DISCUSSION

Accessibility of lipid to partition

The accessibility of lipid in the tissues to the partition of lipid-soluble dyes varies with the method of fixation. Direct fixation in osmium tetroxide results in the binding of osmium to the lipid of mitochondria and other membranous organelles, and such lipid becomes readily stainable. Whereas fixation in formaldehyde or glutaraldehyde fixes much of the lipid in an inaccessible state (Wigglesworth, 1957), presumably being masked by protein, like unsaturated fatty acids incorporated in the channels of urea crystals to form inclusion compounds.

In the present experiments, the accessibility of lipid to partition in myrcene is ensured by fixation in osmium tetroxide alone or in mixed osmium tetroxide and glutaraldehyde (Hinde, 1971). The latter mixture combines the advantages of initial osmium fixation and of glutaraldehyde fixation (for example, in the preservation of microtubules).

But the question remains whether there may not still be lipid present in the tissues which is not accessible to partition in myrcene after this fixation procedure. Much of the lipid in insect cuticle is inaccessible to partition; but it can be revealed by treatment with nitric acid saturated with potassium chlorate (van Wisselingh's method) (Wigglesworth, 1933). It can be unmasked in a more controlled fashion with sodium hypochlorite, the concentration and timing of exposure to which can be easily regulated (Wigglesworth, 1970). It was later shown that sodium hypochlorite, which leads to oxidative breakdown of associated protein, was effective in the unmasking of lipid in tissue sections; so that lipid-containing structures were rendered stainable by Sudan black B for the light microscope (Wigglesworth, 1971, 1973). This procedure has been used for the visualization in the electron microscope of lipid in the insect cuticle (Wigglesworth, 1975*a, b*); but so far it has not proved possible to apply these methods to soft tissues without unacceptable damage to the fine structure.

Enhancement of optical staining and electron density

The union of ethyl gallate with osmium is highly effective for the visualization of osmium in the light microscope (Wigglesworth, 1957); but it does not increase the electron density. On the other hand, a large increase in osmium uptake, and therefore in electron density, can be obtained by the OTO procedure of Seligman *et al.* (1966) in which thiosemicarbazide applied after osmium fixation serves as a 'multidendate ligand' to combine with osmium in the tissues and then to bind further osmium at the same sites. Seligman *et al.* (1966) noted that osmium and thiosemicarbazide are removed during embedding in Epon or Araldite; this OTO treatment was therefore applied by these authors to the sections after mounting on gold or platinum grids. It would be more convenient to be able to apply this treatment to the whole preparation

before embedding. This has been made possible by the use of Spurr's medium with dehydration in ethanol, and omitting propylene oxide (see Fig. 25).

It must, however, be pointed out that the OTO procedure merely enhances the uptake of osmium at sites where it is already bound. It does not increase the specificity of lipid staining. The virtue of the method described in the present paper is that any increase in osmium uptake which follows partition in myrcene in 70% ethanol is confined to sites of lipid in the tissues. Since most of the lipids in tissues contain ethylenic double bonds the distribution of osmium before and after treatment with myrcene is the same – but the increased density after myrcene treatment affords evidence that this osmium is indeed localized in lipid-rich sites. In practice, it has been found that the myrcene procedure as described (1–18 h partition) gives adequate electron density in the sections, without further treatment with thiosemicarbazide and osmium.

A specific objective of the present work was to devise a method for the electron-microscopic demonstration of insect waxes. If the surface of the cuticle of the *Rhodnius* larva is very gently rubbed with fine abrasive dust (Linde B) the peaks and crests of the epicuticular folds are deprived of their waterproofing wax; and during the next few days new wax is secreted to form a faint 'bloom' on the surface (Wigglesworth, 1945). Fig. 10 shows a section through this amorphous deposit of wax as seen in the electron microscope after myrcene partition and osmium staining.

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Figs. 1-6. Free lipids deposited on lens paper: whole mounts. $\times 750$.

Fig. 1. Nujol: limonene partition, osmium tetroxide, ethyl gallate.

Fig. 2. Olive oil: osmium tetroxide, ethyl gallate.

Fig. 3. Cephalin: osmium tetroxide, ethyl gallate.

Fig. 4. Cephalin: limonene partition, osmium tetroxide, ethyl gallate.

Fig. 5. Beeswax: limonene partition at 20 °C, osmium tetroxide, ethyl gallate.

Fig. 6. Beeswax: limonene partition at 65 °C, osmium tetroxide, ethyl gallate.

Fig. 7. Nujol emulsified in agar, partition in 60% pyridine with myrcene, osmium tetroxide. $\times 8000$.

Fig. 8. As Fig. 7. $\times 17000$.

Fig. 9. Whole mount of fat body showing: *a*, lipid droplets with osmium bound only in the wall; *b*, watery vacuoles; and mitochondria (Wigglesworth, 1957). $\times 1000$.

Fig. 10. Wax deposit on *Rhodnius* cuticle some days after abrasion. The membrane on which the wax rests has separated from the cuticle; hence the clear space below. $\times 20000$.

Fig. 11. Fat body: 1- μ m section. Osmium tetroxide, myrcene, OTO. $\times 720$.

Fig. 12. Fat body: 0.5- μ m section. Hinde, myrcene, OTO. $\times 1000$.

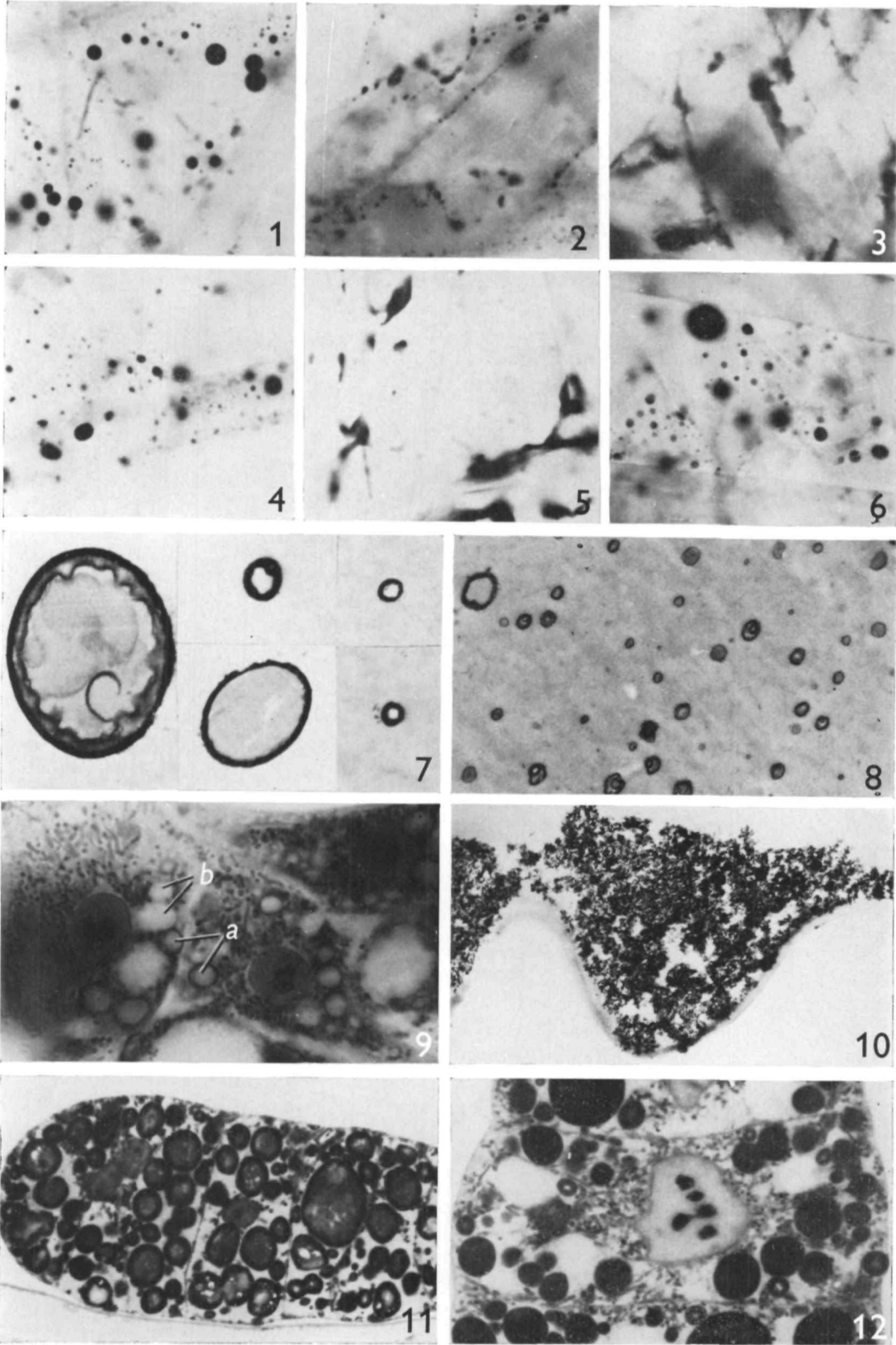


Fig. 13. Surface of epidermis of *Rhodnius* 4th-stage larva, at 7 days after feeding. Osmium tetroxide, Spurr's medium. $\times 20\,000$.

Fig. 14. As Fig. 13. Myrcene partition, osmium, Spurr. $\times 20\,000$.

Fig. 15. Epidermal cell at 8 days after feeding; outer epicuticle formed. Osmium tetroxide, Spurr. $\times 20\,000$.

Fig. 16. As Fig. 15. Myrcene partition, osmium, Spurr. $\times 20\,000$.

Fig. 17. Nucleus and cytoplasm of epidermal cell at 9 days after feeding. Osmium tetroxide, myrcene, osmium, Spurr. *er*, endoplasmic reticulum; *g*, Golgi bodies; *m*, mitochondria; *n*, nucleus; *nu*, nucleolus. $\times 20\,000$.

Fig. 18. Fat body, $1\text{-}\mu\text{m}$ section. Osmium tetroxide, Spurr. $\times 1000$.

Fig. 19. As Fig. 18. Myrcene partition, osmium, Spurr. $\times 1000$.

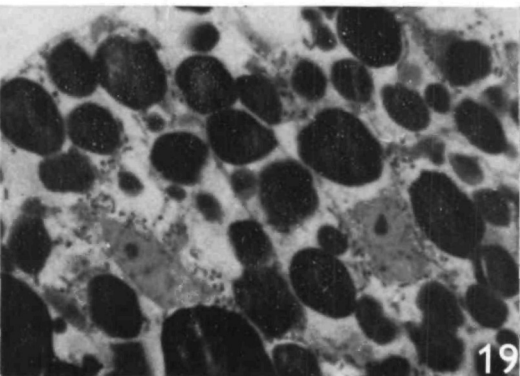
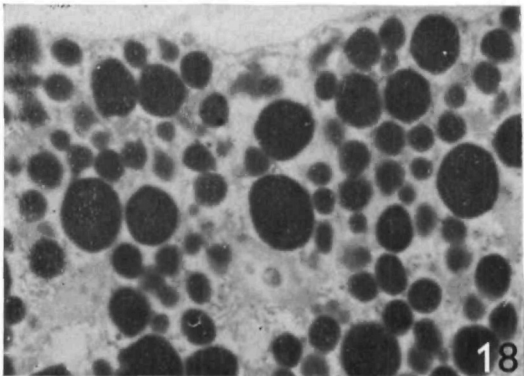
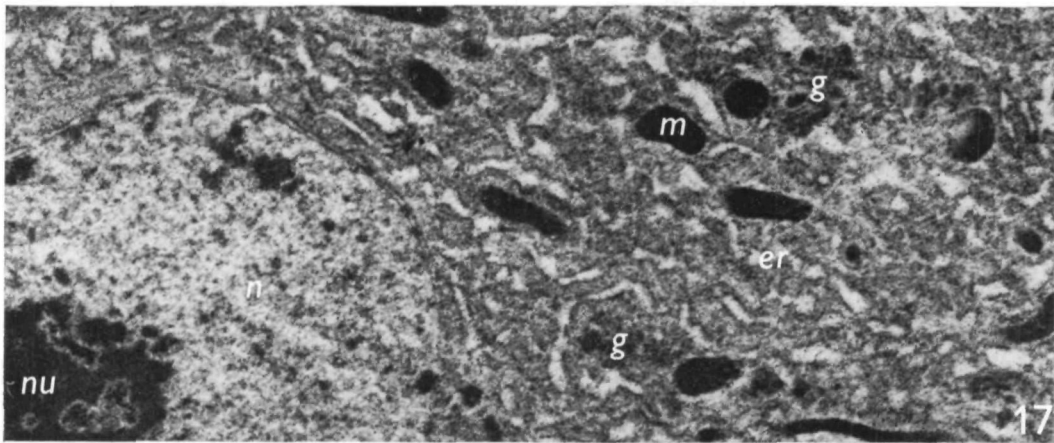
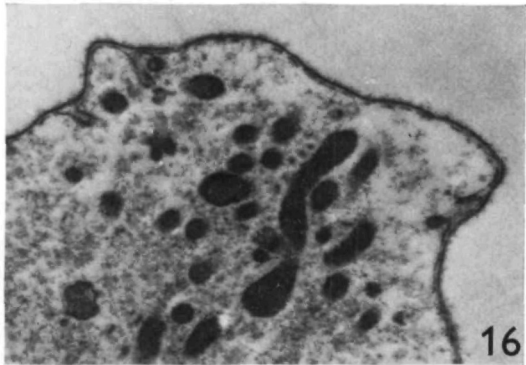
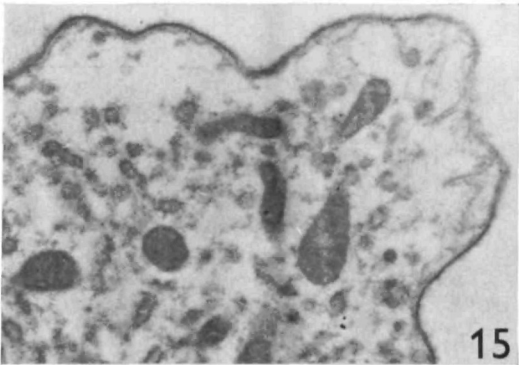
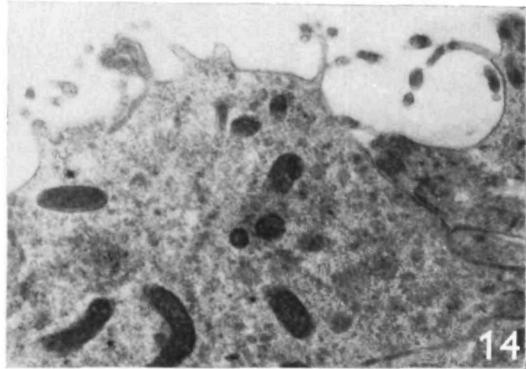
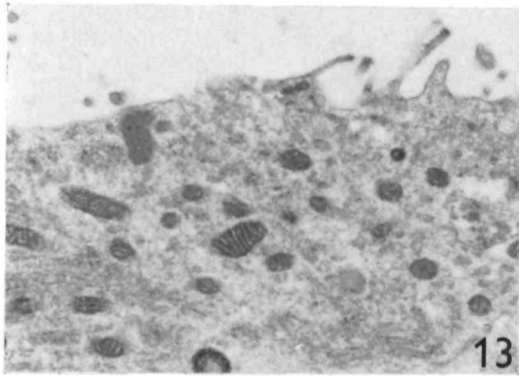


Fig. 20. Part of fat-body cell, showing mitochondria, laminated ER, plasma membrane (above left) and margin of lipid droplet (below right). Osmium fixation, myrcene partition, Spurr. $\times 20000$.

Fig. 21. As Fig. 20, without myrcene partition but with lead citrate staining. Plasma membrane (above) and margin of lipid droplet (at right). $\times 20000$.

Fig. 22. Part of epidermal cell with mitochondria and vesicular ER. Osmium/glutaraldehyde fixation and myrcene partition. $\times 20000$.

Fig. 23. As Fig. 22, without myrcene partition but stained with lead citrate, showing conspicuous microsomes on the ER. $\times 20000$.

Fig. 24. Part of nucleus and cytoplasm of epidermal cell at 11 days after feeding. Osmium/glutaraldehyde fixation, Spurr's medium. *er*, vesicular endoplasmic reticulum; *m*, mitochondria; *n*, nucleus; *nu*, nucleolus; *p*, plasma membrane. $\times 26000$.

Fig. 25. As for Fig. 24, treated with thiosemicarbazide and osmium tetroxide before embedding. $\times 26000$.

