The use of osmium in the fixation and staining of tissues

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[Plates 5 to 7]

The use of gallic acid derivatives in the visualization of osmium in tissue sections has been re-investigated. By the use of alkyl esters of gallic acid greatly improved results can be obtained. Fixation with buffered osmium tetroxide followed by ethyl gallate affords a simple and reliable method for staining fat droplets, mitochondria, etc.

According to the hypothesis put forward the distribution of osmium is determined chiefly by the distribution of unsaturated fatty acids; none is bound by nucleic acids and relatively little by protein. The result is claimed to be an histology based primarily on lipids, which is contrasted with the customary histology based on nucleic acids and proteins.

Evidence is given that osmium tetroxide causes polymerization of unsaturated lipids by the cross-linking of ethylenic double bonds. This is particularly liable to occur in layers of oriented lipids. Such layers are widely distributed in living cells; their stabilization by linkage through osmium is considered to play the most important part in cytological fixation by osmium tetroxide.

INTRODUCTION

It has long been recognized that of all histological fixatives osmium tetroxide causes the least disruption of cell structure (Hardy 1899; Monkeberg & Bethe 1899; Strangeways & Canti 1927; Porter 1953; Frederic 1956). This property has become important in recent years in the preparation of thin sections for the electron microscope (Palade 1952a; Sjöstrand 1956), but the nature of the fixative action has remained obscure.

Osmium tetroxide is also employed as a stain for fats. This use is dependent on its rapid reduction by ethylenic linkages in unsaturated fatty acids to give the black hydrate of osmium dioxide. But there are so many uncertainties about this reaction as it occurs in tissues that osmium tetroxide is not now regarded as a useful histochemical reagent (Hoerr 1936; Lison 1953; Pearse 1953).

When tissues are fixed with osmium tetroxide, osmium becomes bound by many structures in the cell. Since osmium strongly scatters electrons this makes osmium tetroxide an excellent ‘stain’ for thin sections studied with the electron microscope. But it is not always clear whether the opacity of a given structure is dependent upon the uptake of osmium or upon natural density (Palade 1952b), though Bahr (1955) has argued that when tissue sections are examined without removal of the plastic embedding material, almost nothing will be visible in the electron microscope but osmium.

If the distribution of osmium in the fixed cell could be made visible in the light microscope this should provide a valuable histological procedure, which would give preparations resembling those seen with the electron microscope, and might be expected to throw light on the mechanism of osmium fixation. Those were the aims of the present work.
Material

As material for this study I have used chiefly the abdominal wall of the fourth-stage larva of the blood-sucking bug *Rhodnius prolixus*. These insects were starved for 4 or 5 months so as to reduce to a low level the stores of fat and other reserves. They were then fed and the tissues examined during the succeeding days when renewed growth and food storage were in progress.

A cut along each margin of the abdomen will separate the dorsal integument (tergites) from the ventral (sternites). These two halves are then detached from the blood-filled alimentary canal; they consist of the cuticle with the underlying single layer of epidermal cells, and below this the lace-like fat body. Other tissues are the oenocytes, the tracheae and tracheoles, the sensory nerves and sense cells, the dermal glands, the heart and pericardial cells, the various types of haemocytes, and the slender intersegmental muscles with their nerve supply. All these tissues are so thinly spread that they can be mounted entire and examined with the highest powers of the light microscope. A single specimen may be divided and comparative tests carried out on the fragments. The most useful cells for study are the large fat body cells.

The results obtained on *Rhodnius* were then confirmed on tissue sections from the mouse.

*The use of polyphenols in the visualization of osmium in droplets of neutral fat*

One of the characteristic reactions of osmium is the intense blue-black colour given with polyphenols. This has been used by botanists as a test for tannins since early in the nineteenth century. It was suggested by Bolles Lee (1887) that this reaction might be used in histology, and Kolossow (1892) developed such a method to some extent; using a mixture of tannic acid and pyrogallic acid he obtained satisfactory results with endothelial cells, but found that other tissues coloured too darkly to give useful preparations. Hoerr (1936) records good staining of the myelin sheath of nerves.

In the present tests the *Rhodnius* tissues at 1 day after feeding were fixed for 1 h in 1% osmium tetroxide buffered to pH 6·8 with citrate-phosphate buffer. After brief washing they were then immersed for 15 min in a mixture similar to that used by Kolossow, consisting of tannic acid 5%, pyrogallol 5%, glycerol 8% and ethanol 15% in water, and then mounted in Farrants's medium.

In such preparations the cells of the fat body show dark staining of the fat droplets. The nucleolus and nuclear membrane stain a faint grey, there are some grey droplets around the nucleus, and the cytoplasm containing large colourless vacuoles stains a diffuse grey with no clearly defined structure (figure 3, plate 5). Figure 4, plate 5 shows for comparison similar cells stained with Ehrlich's haematoxylin after fixation with picric acid.

The use of polyphenols in this way provides an admirable method for staining fats. If the osmium fixed preparations are transferred to 70% alcohol the fat droplets become dark, and for droplets measuring 5 or 10 µ in diameter there is little to choose between this standard procedure and the polyphenol method.
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But with droplets of 1 µ or less the polyphenol method is greatly superior. A drop of olive oil was shaken up in 2% agar which was then spread as a thin film, allowed to harden and treated by the two methods. As seen in figures 1 and 2, plate 5 the staining of the minute droplets is much more intense after treatment with the polyphenol mixture. The difference is still more marked in natural fat droplets when these contain relatively little unsaturated fatty acid.

The visualization of osmium in nuclei, mitochondria, etc.

By the procedure described above the osmium contained in the droplets of neutral fat is readily revealed. When first mounted the preparations showed little else, but when exposed to the bright light (and heat) of the microscope lamp the nucleus and nucleolus gradually became visible and the cytoplasm darkened to some extent. This suggested that more prolonged treatment with the polyphenols might reveal more of the bound osmium.

This proved to be the case. Preparations were kept for several days in the mixture of tannic acid and pyrogallol and then mounted in Farrant's medium to which this mixture had been added. The nuclear membrane and nucleolus were already visible on mounting, and on exposure to the bright light the mitochondria began to appear.

Even in these preparations the mitochondria were rather faint. Much better results were obtained when the water-soluble polyphenols were replaced by oil-soluble esters of gallic acid (ethyl gallate in aqueous solution, nonyl gallate or cetyl gallate in 50% alcohol). The procedure finally adopted was as follows. (i) Fixation in 1% buffered osmium tetroxide (15 min). (ii) Brief washing. (iii) Immersion in a saturated solution of ethyl gallate ('Progallin A' of Nipa Laboratories Ltd) in 0-25% cresol for 24 h or longer. (iv) Mounting in Farrant's medium containing ethyl gallate.

The preparations now showed some staining of the nuclei and mitochondria on mounting, but further darkening took place after keeping on the warm plate for several days and still more during illumination with the microscope lamp. Figure 5, plate 5 shows the mainly globular mitochondria in the fat body cells of the starved insect. Figure 6 shows the elongated and branching forms developing within 24 h after feeding, and figure 7, the slender filaments present in many of the cells at this stage. In such preparations it is possible to see the mitochondria in all the tissues (epidermal cells, oenocytes, haemocytes, muscles, nerves, sense cells, pericardial cells, etc.) in a single mount. Some of these have already been illustrated elsewhere (Wigglesworth 1956, 1957), but in these early preparations the tissues were treated with Hansen's trioxythaematin, and it was thought that this stain was responsible for much of the coloration of the mitochondria. That does not appear to be so; equally good mitochondrial staining is obtained without the haematoxylin.

Demonstration of mitochondria, Golgi apparatus, etc., in tissue sections

For the preparation of sections small pieces of tissue (1 to 2 mm³) were fixed at 0 to 4°C for 3 h in 1% osmium tetroxide buffered at pH 7.25 with veronal acetate buffer in isotonic sodium chloride (Palade 1952a; Sjöstrand 1956). This
may be followed by saturated picric acid in water to complete the hardening of the tissues (say 1 h); and sometimes it is convenient to treat with 2% potassium chlorate for $\frac{1}{2}$ h to remove the black deposits of osmium dioxide (Hoffmann 1912). The object is then transferred to a saturated solution of ethyl gallate in 0.25% of cresol (as a preservative) and stored in this for a day or longer.

After this treatment the tissue resembles a small piece of coal. Several procedures have been used for sectioning.

(i) The pieces are transferred gradually to 25% gelatin, kept at 37°C for 24 h, hardened in formol for 1 day, cut with the freezing microtome at 5μ (or less) and mounted in Farrants’s medium containing ethyl gallate.

(ii) The fragments are dehydrated in alcohol, cleared in cedar-wood oil (the staining is apt to be removed by xylol) embedded in paraffin and cut at 1 to 2μ. The wax is removed by brief immersion in xylol and sections mounted in cedar-wood oil containing nonyl gallate, or taken down to water and mounted in Farrants’s medium containing ethyl gallate.

(iii) If the tissue is sufficiently compact, it may be taken up to 90% alcohol, transferred to equal volumes of alcohol and polyethylene glycol wax, embedded in the pure polyethylene glycol wax, and cut at 0.5 to 1μ. The sections are spread singly on water and mounted in glycerine jelly containing ethyl gallate.

The illustrations show some of the results obtained. Figure 8, plate 5 shows part of a rather thick frozen section cut horizontally through the fused thoracic ganglia of Rhodnius. The giant axons are almost unstained and appear as white tracts containing elongated mitochondria seen in longitudinal and transverse section.

Figure 9, plate 6 (frozen section) shows the familiar palisade of mitochondria in the proximal convoluted tubules of the mouse kidney.

Figure 10, plate 6 is a 1μ paraffin section of the intestine of the mouse showing the mitochondria in epithelial cells cut longitudinally (to the left) and transversely (to the right). It also shows the intense osmium concentration in the intercellular boundary, the thickening of this boundary when it reaches the lumen (better seen in figure 11), and the osmiophil zone immediately below the brush border (cf. Sheldon, Zetterqvist & Brandes 1955).

Figure 11 is a 3μ paraffin section through two adjacent intestinal villi in the mouse. In addition to the points already noted it shows the Golgi apparatus, best seen in the lower row of cells, as a system of colourless canals and vacuoles in an osmiophil matrix just distal to the nucleus (cf. Simpson 1941; Bensley 1951). In the upper row of cells the Golgi apparatus has been cut through obliquely. In figure 12 a villus is seen in oblique section with the cells cut transversely at all levels. Below are the nuclei, in the middle the clear canals of the Golgi apparatus, and above the distal region of the cells with mitochondria.

Figure 13 is a paraffin section of intestinal villi showing fat absorption. In the preparation the blue-black droplets of fat are quite distinct from the grey-brown mitochondria.

Figure 14 is a 2μ section of mouse liver cut in polyethylene glycol wax. The few blue-black fat droplets are again quite distinct from the deep grey mitochondria.
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As an example of the unreliability of osmium tetroxide as a histochemical reagent for lipids, it was pointed out by Hoerr (1936) that during fixation of the adrenal gland the adrenalin precursor in the medulla causes this to blacken in osmium tetroxide more rapidly than the cortex, which is rich in lipids (cf. Cramer 1918). This is so in the adrenal gland of the mouse; but after treatment with ethyl gallate the cortex shows many cells filled with intense blue-staining granules of lipid (figure 15), whereas the cytoplasm in the medulla contains fine grey granules of a totally different appearance, among which the scattered mitochondria can be seen (figure 16). These grey granules are readily removed by brief treatment with potassium chlorate.

Finally, figure 32, plate 7 is a 1 μ section cut in polyethylene glycol wax showing the insertion of the fibrillar flight muscle of the blowfly Calliphora into the cuticle. Between the large striated fibrils are the rows of giant mitochondria or sarcosomes. It is of interest to note also that the fan-like ‘tendons’ in which the fibrils end are also strongly osmiophil.

The distribution of osmium in resting and dividing cells

The general appearance presented by the preparations described above agrees closely with that seen in thin sections with the electron microscope. In the cytoplasm between the mitochondria in the fat body of Rhodnius or in the liver or kidney of the mouse, there is only a faint diffuse staining. Staining is dense in the ergastoplasm at the base of the intestinal epithelium; it is dense throughout the cytoplasm of the pancreas and it is dense around the Golgi apparatus.

In the mitochondria the outer wall is sometimes visible but no further structure. Their staining properties differ somewhat in different tissues. Staining is particularly intense in the kidney.

In the nucleus as a rule only the nuclear membrane and the nucleolus stain, and as in electron microscope preparations after osmium fixation, the nucleolus has a reticular appearance (cf. Bernhard, Bauer, Gropp, Hagnau & Oberling 1955). The uniform staining of the nuclear plasma in the fat body cells of Rhodnius is rather deeper than that of the cytoplasm; in the mouse liver, kidney and pancreas the nuclei show up as pale islands against the dark cytoplasm.

The distribution of osmium has been followed in dividing cells. The epidermis of Rhodnius 6 or 7 days after feeding has been used; at this time mitotic figures are very numerous.

Figures 17 to 21, plate 7 show cells fixed in Carnoy’s fixative and stained with Hansen’s trioxyhaematin. There is little staining in the cytoplasm; the conspicuous objects are always the chromosomes.

Figures 22 to 26, plate 7 show a corresponding succession of stages in mitosis in the same individual insect, stained by the osmium-ethyl gallate method. The numerous dark granules and filaments in the cytoplasm are mitochondria. The chromosomes are completely unstained. The resulting picture resembles a photographic negative of the normal histological appearance. During prophase (figure 22) the nucleus swells and the nucleolus assumes the form of a loose coil of fine granules. Perhaps this is comparable with the ‘nucleolonema’ described by
Bernhard et al. 1955 and Estable & Sotello (1955), and the filamentous structure assumed by the nucleolus at mitosis (Lettré 1955). At metaphase (figure 23) the nucleolar granules have disappeared, the chromosomes appear as a colourless bar through the equator, the spindle is faintly stained, the asters show up as colourless regions at the poles. At telophase (figure 24) the chromosomes are again invisible; the mitochondria are appearing between the dividing cells. Figure 25 shows a late telophase where the nuclear membrane is reforming, but there is no sign of a nucleolus. In figure 26 the daughter nuclei are enlarging and a minute nucleolus has appeared in each.

*The state of osmium in the fat droplets*

If the fat body of *Rhodnius* is fixed in osmium tetroxide for 2 min, 15 min or 1 h and then transferred up the alcohols to a mixture of equal volumes of alcohol and ether for 24 h, the dark grey contents of the fat vacuoles are dissolved out. If the preparation is then taken down to water and placed in the saturated solution of ethyl gallate, each empty vacuole becomes demarcated by a blue-black membrane (figure 33, plate 7). This membrane is exceedingly thin after exposure to osmium tetroxide for 2 min and 15 min; it is more distinct after 1 h. Clearly osmium has been retained in a thin insoluble layer of fat over the surface of the droplet.

We have seen that if they are treated with polyphenol before extraction with alcohol and ether the droplets stain more or less uniformly throughout; osmium is present throughout the droplet, but only in the surface layer is it rendered insoluble.

Now the molecules of fat in the surface of the droplet will be oriented with their hydrophil groups outside and the paraffin chains aligned. These chains will have a more or less constant composition, and therefore the double bonds of corresponding components will lie adjacent to one another. That suggests that in this layer osmium may be binding the chains together and causing the fat to polymerize over the surface in a manner analogous to the hardening of a drying oil on contact with a surface.

The thin black line bounding the empty fat vacuoles in these preparations is clearly much more than a monolayer; but any imperfections in the orientation of molecules in the surface will permit cross-linkage with molecules in the bulk of the droplet and thus lead to a gradual thickening of the insoluble shell. This process will result in a selective deposition in the surface of the droplet of those components most rich in double bonds. If the whole droplet contains only unsaturated fats all the contents will eventually polymerize in this way and the entire droplet will become insoluble.

The next question is the nature of the osmium linkage in these oriented fats. As was shown by Criegee (1936) and Criegee, Marchand & Wannowius (1942) osmium tetroxide readily forms cyclical esters with ethylenic double bonds (I)
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These monoesters are easily hydrolyzed to form diols, which in turn may link up with another molecule of the monoester to form a diester (II)

The diesters are exceedingly stable.

Consideration of the complexes formed by the platinum group with olefines suggests another possibility in place of reaction II: the osmium dioxide may form a double co-ordinate link directly with the carbons of the double bond (III)

It seems reasonable therefore to suggest that the orientation of the fats in the droplet surface may favour the cross-linkage of adjacent chains in one of these ways. Alternatively, it is possible that when two unsaturated bonds are closely opposed in adjacent chains, the osmium tetroxide might form monoester linkages directly with the carbon atoms of adjacent chains and thus bind the chains together without intermediate reactions (IV)

The osmium bound in the surface of the fat droplet reacts with ethyl gallate; and the resulting darkly coloured compound remains insoluble in fat solvents and in water; the osmium is still firmly bound. Perhaps that might be taken to favour a type of linkage in which the monoesters of osmic acid will still be free to form
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diesters with the adjacent hydroxyl groups of the ethyl gallate (V) (or perhaps to oxidize the benzene ring and unite with this).

Whatever may be the precise nature of the osmium linkage it will, of course, cause the fats to polymerize only if there are several unsaturated chains in one molecule (as in triolein for example), or if there are several unsaturated links in one chain (as in linoleic acid).

*Variations in the fat and the binding of osmium*

Residual shells of undissolved fat have often been observed in paraffin sections after osmium fixation. These were attributed by Hoerr (1936) to oxidation of the fat in the surface of the droplets and perhaps to prolonged oxidation rendering the fat soluble again. Hoerr recognized that there was ‘firm adsorption or some kind of combination’ of the osmium with the fat.

It has been possible to show that in *Rhodnius* the reaction of the fat droplets with osmium tetroxide varies with the state of nutrition of the insect. In the fourth-stage larva which has been starved for 5 or 6 months the residual droplets of fat in the fat body do not stain deeply with osmium and ethyl gallate; they colour a rather pale blue with a deeper blue periphery, and only a fine limiting membrane is darkly stained. At this stage the contents are readily dissolved by alcohol and ether, even after exposure to osmium tetroxide for 2 h. But if similar insects are fed, and dissected 8 days later, when new fat droplets are beginning to accumulate in the cells, these droplets stain more deeply, and after extraction with alcohol and ether all have thick black membranes or crescents on the surface and many of them are darkly stained throughout. Presumably the residual fats present after prolonged starvation contain relatively few unsaturated fatty acids, but large amounts of unsaturated acids appear in the newly forming droplets. Polymerization and binding of osmium go hand in hand.

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**Description of Plate 5**

**Figure 1.** Droplets of olive oil in agar treated with osmium tetroxide and 70% alcohol.

**Figure 2.** Droplets of olive oil in agar treated with osmium tetroxide and ethyl gallate. Exposure and printing identical with figure 1.

**Figure 3.** Fat body of *Rhodnius*, fed after 5 months’ starvation, fixed 1 day after feeding. Osmium tetroxide followed by tannic acid and pyrogallol. Whole mount. Fat droplets black, watery vacuoles colourless.

**Figure 4.** As figure 3, fixed with saturated aqueous picric acid, stained with Ehrlich’s acid haematoxylin.

**Figure 5.** Fat body of *Rhodnius* fed after 4 months’ starvation, fixed within an hour after feeding. Osmium tetroxide and ethyl gallate. Whole mount. Mitochondria mostly globular.

**Figure 6.** As figure 5, fixed at 24 h after feeding. Many mitochondria becoming elongated.

**Figure 7.** As figure 6, showing filamentous mitochondria.

**Figure 8.** Fused thoracic ganglia of *Rhodnius*. Osmium tetroxide and ethyl gallate. Horizontal frozen section. The colourless strands are large axons with elongated mitochondria; other axons seen in transverse section.
DESCRIPTION OF PLATE 6

Sections of mouse tissues fixed with buffered osmium tetroxide followed by picric acid and ethyl gallate.

Figure 9. Frozen section of kidney showing columnar mitochondria of proximal convoluted tubule.

Figure 10. Epithelium of intestine; paraffin section at 1 μ. To the right, the epithelial cells are cut longitudinally; elongated mitochondria in apical region of cells. To the left, the apical region of the cells has been cut transversely.

Figure 11. The same; paraffin section at 3 μ. Below, the Golgi apparatus appears in the form of colourless convoluted canals in an osmiophil matrix, just distal to the nuclei.

Figure 12. Tangential section through an intestinal villus. Below, the cells are cut at the level of the nuclei; in the middle, at the level of the Golgi apparatus; and above, through the apical region.

Figure 13. Intestinal villi, showing mitochondria and fat in process of absorption.

Figure 14. Liver. Polyethylene glycol wax section at 1 μ, showing mitochondria and a few droplets of neutral fat.

Figure 15. Adrenal cortex. Polyethylene glycol wax section at 2 μ, showing cells filled with lipid granules.

Figure 16. Adrenal medulla; from the same section as figure 15.
The neutral fats of most insects are liquid at the temperature of the environment. They usually have a high iodine value (up to 160) and contain large amounts of oleic, linoleic and linolenic acids. In the aphids, on the other hand, the fat is liquid because it contains a high proportion of short-chain fatty acids (butyric, caprylic, lauric) and the iodine value ranges only from 1.5 to 22 (Timon-David 1930; Wigglesworth 1953). The fat body of the aphid *Megoura viciae* was fixed for 15 min in osmium tetroxide and then treated with ethyl gallate. The fat body cells in this aphid contain droplets of fat 15 μ or more in diameter. These remained colourless, but they were covered by a uniform dark grey or black skin only a fraction of a micron thick. Where the fat had escaped from the vacuoles in the process of mounting, the dark skin of polymerized fat was much wrinkled.

*Model experiments*

The foregoing conclusions on the polymerization of fats by osmium tetroxide have been verified by means of simple model experiments. A drop of oil was added to hot 2% agar in water, shaken up vigorously, spread as a thin film on a coverglass, allowed to set, and then treated with osmium tetroxide and ethyl gallate. Refined liquid paraffin ('Nujol') was used as the non-reacting vehicle and other oils were added to this, usually at the rate of 1%.

Figure 27, plate 7 shows a droplet of 'Nujol' containing 1% of olive oil, after fixation and staining. There is a well-defined grey skin over the surface, which has been ruptured at one point during mounting, and from this surface membrane interlacing filaments run through the contents of the droplet. The surface skin and the filaments are insoluble in alcohol and ether; they remain intact after the 'Nujol' has been extracted.

**Description of Plate 7**

**Figures 17 to 21.** Epidermal cells in *Rhodnius* fourth-stage larva 7 days after feeding; whole mounts showing stages in mitosis. Carnoy's fixative; Hansen's trioxyhaematin.

**Figures 22 to 26.** Epidermal cells from the same insect, showing similar stages in mitosis. Osmium tetroxide and ethyl gallate.

**Figures 27 to 31.** Droplets of 'Nujol' containing 1% of other lipids, in 2% agar, after treatment with osmium tetroxide and ethyl gallate.

**Figure 27.** Olive oil.

**Figure 28.** Oleic acid.

**Figure 29.** Linoleic acid.

**Figure 30.** Sphingomyelin.

**Figure 31.** Cephalin.

**Figure 32.** Longitudinal section of indirect flight muscles of *Calliphora* showing insertion of fibrils into the cuticle, and sarcosomes between the fibrils. Polyethylene glycol wax section at 1 μ. Osmium tetroxide and ethyl gallate.

**Figure 33.** Fat body of *Rhodnius* at 1 day after feeding; whole mount. Osmium tetroxide 1 h, extraction with alcohol and ether 24 h, followed by ethyl gallate. Fat droplets appear as black rings; watery vacuoles have an indefinite margin; mitochondria stained.
Figure 28 shows a droplet of ‘Nujol’ with 1% oleic acid. There is no robust surface membrane; minute black granules are formed throughout the droplet, but they are not connected to one another and eventually most of them settle on the lower wall. Since oleic acid contains only one chain with a single double bond polymerization would not be expected.

Figure 29 represents a droplet of ‘Nujol’ with 1% linoleic acid. There is a delicate surface skin and from this darkly coloured filaments run inward, as with olive oil. Since there are two double bonds in the single chain, polymerization through osmium can occur.

Figure 30 shows a droplet of ‘Nujol’ with 1% of sphingomyelin, seen in optical section. There is no obvious surface membrane and no filaments in the bulk of the droplet, but only some small lens-like dark deposits in the surface. Sphingomyelin contains one ethylenic double bond in the sphingosine base, but the fatty acid part of the molecule is usually saturated (Lovern 1955); multiple cross-linkage by osmium was therefore not to be expected.

Figure 31 is of a droplet of ‘Nujol’ containing 1% of cephalin. Since cephalin has two fatty chains with a large number of unsaturated links, it was to be expected that it would bind more osmium and would polymerize more effectively than any of the other substances tested. It forms a robust surface membrane from which innumerable trabeculae pass inwards. These anastomozing filaments and membranes are most densely packed at the periphery of the droplet; towards the centre the meshwork is looser and encloses larger spaces. In addition, the meshwork includes a number of intensely black spheres; in all, a large amount of osmium has been taken up.

The state of osmium in mitochondria, etc.

According to the hypothesis here put forward the primary mechanism of osmium fixation is a polymerization of lipids through the osmium atoms. Films of oriented lipids are now regarded as one of the fundamental building materials of living tissues, not only in the surface membranes of the cells and in the myelin sheath of nerve, but in the walls and transverse membranes of the mitochondria and in the laminated or tubular structures of the cytoplasm (ergastoplasm, ‘endoplasmic reticulum’) (Palade 1953; Porter 1954; Sjöstrand & Hanson 1954; Sjöstrand 1955; Dalton & Felix 1956). Such membranes are ideally constituted for cross-linkage by osmium tetroxide.

It was to be expected, therefore, that osmium would be taken up very actively by the mitochondria. The preparations already described show that that is so. A striking example was obtained as follows. The fat body cells of the blowfly Calliphora contain large amounts of fat, and stain so intensely with osmium and ethyl gallate that it is impossible to see the mitochondria in whole mounts. But if the tissues are left in the osmium tetroxide solution for 2 min only, whereas the more superficial cells are completely blackened, the deeper cells may be coloured only a pale grey. In these the fat is colourless, but the mitochondria are strongly stained throughout the cell; the traces of osmium which have penetrated to these cells have been taken up preferentially by the mitochondria.
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It has long been recognized that mitochondria are rich in lipids. The mitochondrial fraction of mouse liver contains 62% of the total lipid of the cell, and 85% of the phospholipid; 20% of the fatty acids in the mitochondria have four double bonds (Kretchmer & Barnum 1951). In the mitochondria of rat liver the dry matter contains 63% of protein and 29% of lipids, of which phospholipids form 79%, neutral fat 17% and cholesterol 4.4%. The phosphatides consist of 45% lecithin, 47% cephalin and 8% sphingomyelin (Swanson & Artom 1950). The model experiments described above show that such substances must bind very large amounts of osmium.

The lipid-protein membranes which have been most studied in recent years are those which make up the myelin sheath of nerve. Here the lamination is clearly seen with the electron microscope, particularly after osmium fixation (Sjöstrand 1953; Fernández-Moran 1954). The laminae are so accurately aligned that they give a reproducible X-ray diffraction pattern, the intensity of which is increased by osmium (Finean 1953), and similar diffraction patterns are obtained after osmium tetroxide fixation in retinal rods and chloroplasts (Finean, Sjöstrand & Steinmann 1953). From such observations Finean (1954) infers that there must be a deposition of osmium ‘at specific points which repeat in a regular fashion in the radial direction’ and he points out that the unsaturated olefinic linkages are the most likely sites. He confirms that lipids become insoluble after osmium fixation, and suggests as one possible explanation a lipid-to-lipid bonding (cf. Fernández-Moran 1954). The binding of osmium by protein was not ruled out by Finean (1954), and in the membranes of mitochondria as seen with the electron microscope, Sjöstrand & Rhodin (1953) and Sjöstrand & Hanson (1954) regard the two outer dark lines as layers of protein with which osmium has combined, and the clear intermediate zone as a layer of lipid.

In the course of the present work I have made many experiments in which osmium tetroxide was mixed with or preceded by other fixatives. In none of these was there a clear-cut staining of the mitochondria. Altmann’s fixative (osmium tetroxide and potassium bichromate) was followed by very poor staining of the mitochondria. Schaudinn, Zenker without acetic, or potassium dichromate, preceding the osmium tetroxide were quite unsatisfactory. After neutral formal the mitochondria will stain by the osmium-ethyl gallate method, but only faintly. It was thought that this might perhaps be due to denaturation of the proteins upsetting the alinement of the fatty acid chains and thus preventing the orderly cross-linkage by osmium. This idea is supported by the effect of formalin fixation on the myelin sheath as seen with the electron microscope (Fernández-Moran 1954), and by the observation of Finean (1954) that osmium tetroxide failed to produce a diffraction pattern in myelin which had been frozen or fixed in formalin—a result which he likewise attributed to a disarray of the reactive double bonds. But the striking reduction in the binding of osmium by the lipid-rich mitochondria which follows formalin fixation is puzzling and is not satisfactorily explained.
Exposure of tissues to osmium tetroxide has three results: (i) the deposition of lower oxides of osmium, (ii) the binding of osmium by organic compounds, and (iii) the fixation of the tissues. Osmium tetroxide will oxidize many organic substances and radicals (Bahr 1954). In the process it is reduced to lower oxides, and it has been argued in the past that if these are deposited at the site of the reaction, osmium should serve as a histochemical reagent for the substances in question. That has not proved to be the case; the blackening of the tissues has been shown to be capricious and unreliable (Hoerr 1936). On the other hand, the binding of osmium by the tissues, as revealed by ethyl gallate, gives consistent and reproducible results.

As pointed out by Bahr (1954), osmium tetroxide does not react with nucleic acids or carbohydrates; it reacts with sulphhydryl and disulphide groups, with polyphenols, including ascorbic acid, adrenalin, etc., and with the nitrogen of tertiary bases such as tryptophane, proline, etc., and therefore reacts to some extent with proteins. But the predominant reactive group is undoubtedly the ethylenic linkage as it exists in fatty acid chains.

If the abdominal wall of Rhodnius is immersed for 5 min in Ringer’s solution containing bromine, in order to saturate the ethylenic double bonds before fixation in osmium tetroxide, no osmium is taken up by the droplets of neutral fat and very little by the nuclei and mitochondria. But this treatment causes no reduction at all in the amount of iron taken up after fixation, by the proteins and nucleic acids.

It is therefore probably safe to neglect the other reactants and, as a first approximation, to regard the binding of osmium during brief fixation as a histochemical test for unsaturated fatty acids. For in order to be visible in sections, massive amounts of osmium must be retained. A small amount almost certainly unites with proteins, but with comparatively rare exceptions (such as the medulla of the adrenal gland) unsaturated lipids are probably the main substances present in the requisite quantities.

This same argument was applied to the reaction of fixed tissues with iron. Iron will form complexes with a great variety of tissue constituents; but acidic proteins and nucleic acids preponderate to such an extent that iron becomes in effect a histochemical reagent for these two components (Wigglesworth 1952).

It is also suggested in the present paper that polymerization of the lipids in the cell forms the basis of fixation by osmium tetroxide. That raises the question of the effect of osmium tetroxide on proteins. Monkeberg & Bethe (1899) noted that osmium tetroxide applied to a solution of egg albumen changes the protein so that it can no longer be coagulated by heat or by the usual protein precipitants. They ascribed both this change and the difficulty in staining protein after osmium fixation, to the oxidation of reactive groups in the protein molecule.

It was observed by Porter & Kallman (1953) that osmium tetroxide causes instant cessation of Brownian movement in the cytoplasm, an apparent gelation. It also causes gelation of albumen, globulin and fibrinogen in vitro. This is presumably an effect on the protein itself; Porter & Kallman suggest that osmium
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may be acting on the tryptophane in the protein. But such gelation is temporary only; on further treatment with osmium tetroxide the gels become soluble again; and all observers are agreed that on prolonged treatment with osmium tetroxide much of the cell substance dissolves out (Bahr 1955). As Porter & Kallman write, 'what remains of the cell may be thought of as a membrane skeleton'. It is the hardening of this 'membrane skeleton' by the polymerization of lipids through osmium that is here regarded as the essential feature of osmium fixation. Whether the reaction with protein is also of importance remains to be determined.

To what extent can all the sites of osmium binding as illustrated in this paper be regarded as concentrations of unsaturated lipids? The droplets of neutral fat, the mitochondria, the nuclear membrane, the intercellular boundary membrane, are probably all sites of lipid accumulation. Phospholipids have long been known to occur in the nucleolus (Baker 1946). The fact that cells most rich in 'ergastoplasm' stain most deeply is likewise to be expected; for the electron microscope has shown ergastoplasm to be made up of multiple membranes, presumably composed largely of lipids (Porter 1954; Sjöstrand 1955). The deep staining of the Z line of the muscle fibril (figure 32, plate 7) remains to be considered.

In the earlier paper on the uptake of iron by fixed tissues (Wigglesworth 1952) it was shown that certain structures will reduce ferric to ferrous iron—notably the nuclear membrane and nucleoli, the Z line of striated muscle, the keratohyalin granules and Henle's layer of the root-sheath of hairs. A number of substances likely to occur in tissues were tested and it was shown that di- and polyphenols including ascorbic acid, and compounds with sulphydryl groups would reduce ferric iron in vitro. It was suggested that sulphydryl groups were probably the reducing substances present in the root-sheath of hairs; the nature of the reducing substance in the nuclei and in the Z line was not known.

Subsequently, a more extensive series of compounds was tested by Lillie & Burtner (1953) and among the substances capable of reducing ferric iron in vitro they include poly-unsaturated fatty acids. It is therefore interesting to speculate whether the reduction of ferric iron in the nuclear membrane and nucleoli and in the Z line of muscle, and the binding of osmium at these sites, may not both be due to unsaturated lipids, presumably combined with protein.

Conclusions

Gallic acid derivatives greatly intensify the coloration of neutral fat after fixation with osmium tetroxide. Except in the rare instances where the fat is almost devoid of unsaturated fatty acids this provides a useful method for staining minute fat droplets in tissues. The osmium taken up by mitochondria, the nuclear membrane and nucleoli, etc., is largely bound in invisible form. It can be made visible by prolonged treatment of the osmium-fixed tissues with oil-soluble esters of gallic acid, notably ethyl gallate. This provides a simple method for the demonstration of mitochondria, which is invariably reproducible and requires no particular skill or experience. It is suggested that this bound osmium is held chiefly by unsaturated lipids, particularly phosphatides, and that the whole procedure thus constitutes
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an histology based on lipids, which forms a useful counterpart to the normal histology which is based on proteins and nucleic acids.

Under the action of osmium tetroxide unsaturated fatty acid chains become linked together. When there are several unsaturated chains in one molecule, or several unsaturated links in one chain, this will result in polymerization of the lipid. An insoluble complex of lipid and osmium is thus formed. This process is particularly liable to occur in layers of oriented lipids. Such layers form one of the chief structural components of living cells; their stabilization by linkage through osmium is considered to be the basis of cytological fixation by osmium tetroxide.

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