BOUND LIPID IN THE TIessions OF MAMMAL
AND INSECT: A NEW HISTOCHEMICAL
METHOD

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SUMMARY
'Bound' lipids persist in ester wax sections of tissues fixed in glutaraldehyde. They do not
take up Sudan black B. They are unmasked by dilute sodium hypochlorite and then stain readily
with the Sudan dye and are extractable in warm chloroform and methanol.

Bound lipids have been studied in tissues from the mouse and from the insects Rhodnius,
Periplaneta and Calliphora. They are so widely distributed that Sudan staining alone gives an
informative histological picture.

They occur in nucleoli and the nuclear envelope, in the nuclear matrix and in the cytoplasm
(especially when rich in endoplasmic reticulum), in plasma membranes and their invaginations
and evaginations (microvilli), in septate desmosomes, and particularly in mitochondria, dictyos-
somes and myelin.

In erythrocytes they are not only concentrated in the surface membrane but are distributed
throughout the interior of the cell. In muscle fibrils (papillary muscle of the ventricle and fibri-
lar flight muscle of Calliphora) bound lipid occurs in small amounts in the dark regions of the
unstained muscle ('A'-bands) but is normally absent from the 'Z'-line and from the 'I'-
bands. In highly contracted muscle this relation is reversed: lipid is concentrated around the
'Z'-line. The elastic layers and fibrils in arteries are rich in bound lipid.

Bound lipids are presumably lipoproteins. Purified lipoprotein from egg yolk examined
histochemically has the same properties.

INTRODUCTION
There have been many unsuccessful attempts to demonstrate bound lipids in tissues
by histochemical means (Lison, 1953). According to Gabe (1968) this still lies beyond
the range of present day histochemistry. For the unmasking of lipid in plant and insect
cuticle the method of van Wisselingh (warming in nitric acid with potassium chlorate)
has been used (Kühnelt, 1928; Wigglesworth, 1933). In a recent paper (Wigglesworth,
1970) I described a more gentle method involving the oxidative breakdown of protein
with dilute hypochlorite.

The action of sodium hypochlorite on tissues has been extensively studied since the
introduction of 'Dakin's solution' for the treatment of infected wounds in 1916.
Engfeldt (1922) showed that proteins are readily attacked, whereas lipids and carbo-
hydrates are relatively inert. Under alkaline conditions the chief mode of protein
breakdown is by oxidative fission. Terminal amino acids are detached and rupture
may occur at any point in the peptide chain; but the detailed chemistry of the process
The present work arose from an attempt to apply sodium hypochlorite for the unmasking of lipid in tissue sections. The greater part of the bound lipid in cells consists of phospholipids, which in the bound state do not take up Sudan dyes. Phospholipids can be revealed by Weigert's method of staining with haematoxylin after prolonged treatment with chromium salts, as modified by Dietrich; but mitochondria, which contain most of the phospholipid in the cell, are not stained by this method. They are sometimes but not always stained by J. R. Baker's (1946) modification.

Most of the bound lipids in cells contain ethylenic double bonds which react with osmium tetroxide. The osmium is bound in colourless form, invisible in the light microscope; but it is readily visualized by treatment with ethyl gallate to give an intense blue-black coloration. This procedure was described as a 'histology based on lipids' (Wigglesworth, 1957); it provides excellent staining of mitochondria, Golgi complex, nucleoli, nuclear envelope and plasma membrane, as well as droplets of triglyceride (Wigglesworth, 1957, 1959b) (Fig. 2). Under some conditions, however, an appreciable amount of osmium is bound by protein (Wigglesworth, 1964). The results of Sudan staining of lipid unmasked by hypochlorite have now been compared with those given by the osmium/ethyl gallate method.

**MATERIAL AND METHODS**

Representative tissues from the mouse and from the insects *Periplaneta, Rhodnius* and *Calliphora* have served as test material. Two fixatives have been used: (i) glutaraldehyde, 2.5 or 5%, in cacodylate buffer at pH 7.4, with 2% sucrose, for 2-3 h; or (ii) osmium tetroxide, 1%, in isotonic Ringer solution, for 2-3 h. The tissues are embedded in agar (2.5% for 1 h at 60 °C); the specimen is oriented during cooling and the block trimmed. The trimmed blocks are embedded in ester wax and rapidly cooled (Wigglesworth, 1959b). (Ester wax of Steedman, 1947 formula (British Drug Houses Ltd) must be used; ester wax of Steedman, 1960 formula, like paraffin wax, will not impregnate the agar block.)

Sections are cut with a steel knife on the Cambridge rocking microtome as earlier described (Wigglesworth, 1959b) or with a glass knife on the Huxley microtome. They are cut at 0.5-2 μm; for most tissues 1 μm is the optimal thickness. The sections are cut on to the surface of water. With the Huxley microtome they are shepherded with a bristle into the middle of the
trough and enclosed within a barrier as shown in Fig. 1A. This barrier is cut out of black ‘weighing paper’, which is matt and wettable below, glazed and hydrophobe above. The floating barrier is then guided with a dissecting needle on to the surface of a half coverslip (11 x 22 mm) and lifted out of the bath. Serial sections can be obtained by shepherding the sections into a suitable passage cut in a black paper slip before enclosing the whole in the barrier as before (Fig. 1B).

If the sections are dried and dewaxed before treatment with hypochlorite they are apt to come away from the coverslip before sufficient oxidation has occurred. They are therefore treated with hypochlorite while floating at the water surface, before dewaxing. This may be done by placing the coverslip, with the sections still enclosed within the barrier, on a waxed surface, floating the sections in a few drops of hypochlorite for the required time, drawing off the excess fluid with filter paper, removing the barrier and drying the sections on the warm plate.

Sodium hypochlorite, 10% (i.e. containing 10 g available chlorine per 100 ml) has been used as stock solution. This is freshly diluted 1:100 in distilled water to give a solution of pH about 11.5. Since one is observing transient phases in the unmasking and subsequent breakdown of lipid it is desirable to treat groups of sections for periods increasing from 15 s to 5-10 min in order to get a complete ‘spectrum’. The time, however, is not critical; for a good overall picture I have found 3-4 min the optimum time after glutaraldehyde fixation; after osmium tetroxide fixation 2 min is generally about right.

The dried sections are passed rapidly through xylene, down to 70% ethanol and stained in Sudan black B of about 0.15% in 50% ethanol (that is, about 0.2 ml of 2% stock solution in acetone freshly added to 3 ml of 50% ethanol). Stain 10 min at room temperature. Differentiate for 30 s with agitation in 70% ethanol. Mount in Farrants’ gum medium.

Results may often be improved by taking the sections down to water after dewaxing, and dipping the coverslip into the hypochlorite solution again for 1 min before taking up to 50% ethanol and staining. This procedure ensures that the sections are exposed to hypochlorite from both surfaces.

Tissue sections, treated or untreated, are diffusely coloured by Sudan black in 50% ethanol. After rinsing 1-μm sections for 20-30 s in 70% ethanol the dye is retained only by the unmasked lipids; sections not treated with hypochlorite become completely colourless.

**RESULTS**

**Tests on isolated lipoprotein**

A low-density lipoprotein fraction from egg yolk was put at my disposal by Dr V. B. Kamat and Professor D. Chapman. This was made up of 86-89% lipid and 11-14% protein on a dry weight basis; the lipid fraction being approximately 75% neutral lipid and 25% phospholipid. On contact with water this material emulsified slowly without giving myelin forms. The resulting droplets, for the most part, stained very faintly with Sudan black B.

Smears of this lipoprotein on coverslips were treated as the tissue sections. When fixed in buffered glutaraldehyde, washed and dried and then extracted with xylene (1 min) they left residual membranes which remained colourless after Sudan black staining. If these residual membranes, after extraction with xylene, were exposed to hypochlorite (1:100) for 4 min before placing in Sudan black, they showed deep purple staining, appearing black in optical section, and in places were giving rise to minute black-staining droplets.

These results show that large amounts of lipid can be ‘masked’ by association with protein, in both the fresh and ‘fixed’ state; and that the lipid becomes stainable after exposure to dilute hypochlorite.
Tests on tissue sections: mouse liver

Liver sections, fixed in glutaraldehyde, exposed to hypochlorite for 10–15 s only, show a diffuse pale staining of cytoplasm and nuclei with Sudan black, with the mitochondria scarcely detectable. As hypochlorite treatment is prolonged the mitochondria stain more deeply, with a still darker bounding membrane. The nuclear envelope appears as a fine uniform black line, the nucleoli darken and the nuclear matrix develops a uniform coloration, distinctly darker than the cytoplasm. The uptake of Sudan dye reaches a peak at about 3–4 min exposure in 1:100 hypochlorite (Fig. 3). After treatment for 10 min (Fig. 4) the mitochondria have become pale again and are often seen with difficulty. The nuclear matrix is pale, the nucleoli still dark and conspicuous, the nuclear membrane tending to break up into minute black granules.

The liver represented in these figures was from a mouse starved for 16 h, and the cells contained many droplets of triglyceride. These appear as round colourless vacuoles in Figs. 3 and 4. Fig. 5 is from the liver of the same mouse fixed with osmium tetroxide and the section stained with Sudan black. The lipid droplets are deeply stained; nuclear envelope, nucleoli and mitochondria are faintly coloured.

Fig. 2 is a 0.5-μm section from the liver of a young mouse, fixed with osmium and ethyl gallate, embedded in Araldite, treated with hot Sudan black, and retreated with ethyl gallate, after sectioning, for comparison.

Mouse kidney sections

Fig. 6 is a 1-μm section of kidney (glutaraldehyde-fixed) showing the distal tubule with perpendicular mitochondria at the base of the low columnar cells and round mitochondria at the apex. The minute microvilli are not visible at this magnification. Fig. 7 shows the proximal convoluted tubule from the same preparation with the thick brush border well preserved and the walls of the individual microvilli showing Sudan staining.

Erythrocytes

The erythrocytes colour deeply with Sudan black after glutaraldehyde fixation and hypochlorite treatment. Fig. 3 shows a single red cell in a capillary; likewise Fig. 6. Fig. 8 shows the margin of Bowman’s capsule in the kidney with many erythrocytes in the capillaries, and Fig. 9 the outer sheath of the kidney with subjacent tubules and capillaries. In all these sections, although the surface membrane of the erythrocyte stains most deeply, the dye is also taken up by the cell contents.

The only evidence that this is indeed lipid material is its retention of Sudan black after agitation of 1-μm sections for 0.5 min in 70% ethanol, and its extractability. Sections left in xylene for 2 h at room temperature after hypochlorite treatment show some reduction in the subsequent uptake of Sudan black. Fig. 10 shows a section comparable with that shown in Fig. 9 which was kept in chloroform/methanol (2:1) for 30 min at 60 °C before staining in Sudan black. A little of the dye is still taken up by the erythrocytes; only traces appear in the mitochondria, nuclei etc. Extraction at 60 °C for 2 h removes virtually all traces of stainable material.
In the preparation illustrated in Fig. 4, which had been treated for 10 min in hypochlorite, the erythrocytes were becoming pale and some showed minute Sudan-staining droplets attached to the residue of the membrane.

Pancreas sections

After hypochlorite and Sudan black, nuclei and erythrocytes appear as in liver and kidney. The elongated mitochondria stain darkly against the blue-staining endoplasmic reticulum, which shows occasional clefts that indicate the general orientation of its constituent membranes. The spherical zymogen granules show quite strong lipid staining which is absent from treated sections immersed in warm chloroform and methanol for 1 h.

Intestine

In the epithelium of the small intestine the osmium/ethyl gallate method stains the nuclear envelope, mitochondria, microvilli, the 'terminal web' lying close to the apex of the cell below the brush border, the convoluted intercellular plasma membranes and particularly the dense 'terminal bar' where these membranes reach the striated border. The Golgi complex forms colourless convoluted canals just distal to the nuclei (Wigglesworth, 1957). After glutaraldehyde fixation and hypochlorite treatment all those structures which stain with osmium and ethyl gallate show strong Sudan black staining.

Mesenteric artery

A small artery, 200 μm in diameter, was studied in transverse section after fixation in glutaraldehyde. When stained in Sudan black without previous treatment the walls were completely unstained, the erythrocytes in the lumen virtually unstained. Fig. 11 shows a section after hypochlorite treatment and Sudan black staining: the erythrocytes stain quite strongly as usual; the convoluted inner elastic layer shows intense black staining; the media is coloured a pale blue with some fine convoluted black-staining elastin fibrils; the outer elastic layer likewise stains black; the collagenous adventitia is pale staining but contains black-staining elastin fibres of varying thickness.

Fig. 12 shows a section extracted with chloroform/methanol (2:1) for 1 h at 60 °C after hypochlorite treatment and then stained with Sudan black as before. The walls are now colourless; the elastic layers are refractile but completely unstained; the erythrocytes still take up a trace of the dye.

Native elastin clearly contains a large amount of bound lipid.

Sciatic nerve

A segment of sciatic nerve after fixation in glutaraldehyde and staining directly in Sudan black without sectioning showed uptake of the dye by the myelin sheaths, particularly at the exposed ends of the axons; but this staining was not very intense: blue rather than black. Transverse sections of sciatic nerve that had been fixed in glutaraldehyde (and, of course, extracted in cellosolve during embedding and in xylene and ethanol before staining) showed no staining in Sudan black. After treatment
with hypochlorite the myelin stained a blackish purple. By far the greater part of the lipid in myelin is in the bound state.

Heart muscle

The papillary muscle of the ventricle of the mouse was fixed in glutaraldehyde and cut in longitudinal section. The large mitochondria (sarcosomes) arranged in rows between the fibrils stain conspicuously. The staining of the fibrils agrees closely with that seen in the flight muscles of Calliphora as described below. Over the greater part of the muscle (Fig. 13) the fibrils shows lipid staining of the A-band with a pale or colourless transverse zone in the I-band and with no staining of the Z-line between sarcomeres. The A-bands often show a narrow pale band in the middle (H-band region) and in some fibres this band may widen. At the cut end, where excessive contraction appears to have taken place, many of the fibres show a uniform pale staining with a narrow, darkly stained band at the position of the Z-line. Other fibres, apparently at a still more advanced stage of contraction, show these dark bands much thickened and closer together (Fig. 14).

Flight muscles of Calliphora

The longitudinal indirect flight muscles of the blowfly Calliphora were exposed by slitting the thorax down the mid-line and fixing the muscles without detaching them from their insertions. In life the flight muscles probably do not shorten by more than 1–2 % of their length; but sometimes fibres are cut through during dissection and this gives them an opportunity for excessive contraction.

Muscles fixed in either osmium tetroxide or glutaraldehyde, followed by hypochlorite and Sudan black, show a narrow pale band in the region of the Z-line. Each sarcomere has a broad lipid-staining A-band which darkens at the 2 ends and is interrupted by a second, less-distinct pale zone in the region of the H-band (Figs. 15, 17). Rows of deeply staining sarcosomes lie between the fibrils. In preparations treated with osmium and ethyl gallate the same appearance is seen except that the pale cross-bands are usually less conspicuous.

Where the muscle has been cut and excessive contraction has occurred one can see the other type of banding (as observed in the heart muscle of the mouse) in which the fibrils, now relatively pale, are crossed by narrow dark bands at the level of the Z-line. It is sometimes possible to see the transition between the 2 types of banding. Fig. 16 illustrates such a case: below are the narrow resting fibrils as just described; above are the highly contracted fibrils.

In his classic description of the visible changes in contracting and relaxing muscle Engelmann (1873) describes how the dark appearance of the 2 dark bands ('Querscheiben'), which in the resting state correspond with the anisotropic region ('A'), spread progressively into the isotropic region during contraction; and the original dark bands become paler, until the whole sarcomere has a uniform appearance. In the most extreme contraction the dark appearance becomes concentrated on the 2 surfaces of the Z-line and the rest of the sarcomere is uniformly pale, with the exception of a
Unmasking of bound lipid

A faint irregular dark band which appears in the middle (Hensen’s line, ‘H’, in the ‘Mittelscheibe’). There is in fact a reversal of the banding: the dark band of the resting muscle becomes the pale band of the contracted muscle and vice versa.

Englemann attributed these changes to imbibition of water causing greater transparency, and coagulation or precipitation causing increased darkening. He showed that there was no reversal of birefringence between the zones. It is interesting to observe, however, that the changes in the distribution of lipid in the contracting muscle reproduce precisely the changes in opacity seen by Englemann in living fibres observed by transmitted light. That is seen in Fig. 16; and Fig. 20 is a drawing showing the changes more clearly, alongside a copy of Engelmamn’s well-known figure.

It may be that bound lipid (lipoprotein) is responsible for the regional differences in the opacity of the fibre (for example, by the partial reflection of transmitted light by lipid-rich fibrils) and that there is a change in the distribution of lipid during contraction. We know that the myosin fibrils of the A-band slide towards the Z-line during contraction (Huxley, 1969). It may be that lipids play some role in these movements. Precisely the same changes can be seen in contracted fibrils stained with osmium and ethyl gallate.

In order to make sure that it is really lipid that is taking up the Sudan dye the same tests have been applied as for the erythrocytes and for elastin: the muscle fibres take up no trace of Sudan black before treatment with hypochlorite. After treatment there is again no staining with Sudan black if the sections are first placed in chloroform/methanol (2:1) at 60 °C for 1 h. The quantity of lipid involved is probably very small.

**Rhodnius Malpighian tubules**

The Malpighian tubules of the blood-sucking bug *Rhodnius* consist of 2 segments; an upper two-thirds which is secretory and a lower one-third which is reabsorptive (Wigglesworth, 1931). They have been studied with the electron microscope (Wigglesworth & Salpeter, 1962). Fig. 18 shows a 1-μm section (glutaraldehyde, hypochlorite, Sudan black) through the upper segment, showing the brush border in which elongated mitochondria extend from the body of the cell into individual microvilli. Other mitochondria appear as dark filaments wholly within the microvilli. The cytoplasm contains numerous granules whose contents have been dissolved out to leave small vacuoles; the plasma membrane at the base of the cell is thrown into deep folds with mitochondria between.

Fig. 19 shows a section through the lower segment in which the brush border is made up of more widely separated filaments (microvilli), many of which again contain elongated mitochondria. These filaments may extend to the middle of the lumen at the height of excretory activity (Wigglesworth, 1931). In Fig. 19 they are contracted down. The clear areas at the base represent the sites of uric acid granules (now dissolved) which crystallize out in this position, displacing the adjacent microvilli. Septate desmosomes in the cell boundaries of both upper and lower segments also stain with Sudan black, but are not visible in the photographs. These sections also show small tracheae with lipid-staining taenidia, and tracheoles with lipid-staining walls.
Rhodnius testis

Testes from 5th-instar larvae of Rhodnius at 12 days after feeding showed all stages from meiosis of spermatocytes to the development of spermatids into spermatozoa. No attempt is made to describe the stages in this process which has been fully dealt with in several species of Hemiptera (Moriber, 1956; Gupta, Bedi & Nath, 1960). A few sections only are reproduced to illustrate the potentiality of the method.

Fig. 21 shows dividing spermatocytes at metaphase. It seems to be only at this stage that the chromosomes take up the Sudan dye, rather weakly. The spindle, which is unstained, is enclosed in very fine, intensely staining mitochondria, arranged longitudinally like a cage. Fig. 22 shows meiosis at telophase. The mitochondrial cage is closely applied to the straight-sided spindle, which is divided in the middle by a deeply staining transverse bar (not yet formed in this specimen). Soon after this stage the mitochondria fuse to form the round ‘Nebenkern’. Fig. 23 shows young spermatids with darkly staining globular ‘Nebenkern’, pale nucleus, and deeply stained annular or horseshoe-shaped dictyosome (acroblast).

In Fig. 24 the ‘Nebenkern’ has divided into 2 halves which are extending along the unstained tail filament and beginning to twist around it. The dark staining acroblast is seen and the unstained acrosome is beginning to form. In the follicle above, the tail filament is seen in transverse section between the divided ‘Nebenkern’.

The distribution of lipid as indicated by this method agrees with that described by Moriber (1956) and Gupta et al. (1960).

Periplaneta ganglion

In Figs. 25 and 26 the third thoracic ganglion of the cockroach Periplaneta is shown in transverse section after glutaraldehyde, hypochlorite and Sudan black. Fig. 25 shows a recognized group of motor axons which are unusual in being medullated or ‘tunicaled’. After osmium/ethyl gallate this ‘myelin’ sheath appears uniformly dark, with still darker concentric membranes (Wigglesworth, 1959a). As seen here the diffuse staining is absent but the concentric membranes stain deeply. Fig. 26 shows a section at the boundary between the ganglion cell layer and the neuropile. Above are medium-sized ganglion cells, with invaginated plasma membranes, invested by the dark staining processes of glial cells. Below them are 2 nuclei of the glial cells that surround the neuropile. Lower still is the neuropile with abundant mitochondria within the axons and in the glial cytoplasm between them.

It is noteworthy that the glial cells which have deeply staining cytoplasm containing still more deeply staining membranes have nuclei with exceptionally high lipid content.

DISCUSSION

Specificity of the test for bound lipid

Do the positive results described indicate the presence of lipid? Sudan black is notorious for its tendency to non-specific adsorption. Applied to tissues in high con-
centration in acetone (Berenbaum, 1958) it becomes firmly bound to protein and other constituents; it does not afford evidence of lipid (Locke, 1959). Even in solution in 55% ethanol, followed by ‘rinsing in 50% ethanol’, Sudan black will stain fat-free heat-coagulated protein on paper chromatograms (Kutt, Lockwood & McDowell, 1959). In order to avoid non-specific adsorption it is necessary to differentiate thoroughly in 70% ethanol, as has been done in the present work.

It is conceivable that certain proteins may take up Sudan dye by reason of their high concentration of non-polar side chains. There is no proof of this, but it has been suggested as the reason for the Sudan staining of elastin (Partridge, 1962). In connexion with the present work this hypothesis must be considered also in relation to haemoglobin (or other proteins of the erythrocyte) and the proteins of muscle fibres.

In the absence of treatment with hypochlorite, elastin in the arteries, after glutaraldehyde fixation, is completely uncoloured by the present staining procedure; and so are the muscle fibrils. The erythrocytes are just detectably coloured. Treatment with hypochlorite leads to intense black staining of elastin; intense staining of the erythrocyte membrane and quite strong staining of the contents; moderate to intense staining of certain bands in the muscle fibril.

Oxidative fission of protein under the action of sodium hypochlorite leads to the formation of hydrophilic breakdown products (R. W. R. Baker, 1947). It is therefore difficult to explain the development of affinity for Sudan black in the treated tissues except by the unmasking of lipid. This conclusion is supported by the fact that although the affinity of elastin for Sudan black, after hypochlorite treatment, is somewhat resistant to extraction by xylene at room temperature, it is readily abolished completely by chloroform/methanol (2:1) at 60 °C. In erythrocytes and muscle fibrils Sudan staining is eliminated, or reduced to very low levels, after similar extraction for 1 h. Even if the residual staining material were of a non-lipid nature it would be of negligible significance in the results described.

**Osimum binding and Sudan staining**

The distribution of material staining with Sudan black after treatment with hypochlorite agrees in general with the staining given by the osmium/ethyl gallate method: nucleolus, nuclear envelope, mitochondria, plasma membrane and desmosomes are strongly coloured. This supports the view that the binding of osmium by tissues at neutral pH is due chiefly to lipids and that the osmium/ethyl gallate method reveals unsaturated lipids (cf. Hayes, Lindgren & Gofman, 1963).

But denatured protein readily reacts with osmium tetroxide, chiefly through its amino and imino groups. In whatever form the osmium is held it again reacts with ethyl gallate. Thus in tissues preserved with acid fixatives, and freed from lipids by extraction, the osmium/ethyl gallate method becomes a general stain for protein (Wigglesworth, 1964). Proteins rich in free amino groups, such as the basic histones, are particularly reactive (Bahr, 1954). In the chromosomes of the living cell these reactive amino groups are masked by the formation of salt linkages with the phosphoric acid residues of the nucleic acids. If these amino groups are protonated by acid fixatives, or even by exposure of the living cell to carbon dioxide (pH 3.9–4.1) before
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fixation in neutral osmium tetroxide, the chromosomal and cytoplasmic nucleoprotein will stain intensely with osmium and ethyl gallate (Wigglesworth, 1964). This exposure of charged amino groups was shown to be reversible within the living cell. The staining of erythrocytes with osmium/ethyl gallate was attributed to reaction between osmium tetroxide and the histone haemoglobin (Wigglesworth, 1964). That may well be true; but the present work shows that there is also a substantial amount of bound lipid in the erythrocyte.

There may be other proteins in the living cell which will reduce osmium tetroxide at neutral pH, but they are certainly not conspicuous. Most structures which react with ethyl gallate after fixation in neutral osmium tetroxide have now been shown to be rich in lipids.

‘Free’ and ‘bound’ lipid

Osmium tetroxide causes the polymerization of unsaturated lipids by forming diester bridges between the ethylenic double bonds of neighbouring fatty chains (Wigglesworth, 1957; and see Korn, 1966). These insoluble droplets can then be stained with ethyl gallate or with Sudan black (Wigglesworth, 1967). There is a transition between such readily extractable ‘free’ lipids and the so-called ‘bound’ lipids in cell membranes etc. Thus the lipid in the ‘externum’ of the dictyosomes in the ganglion cells of Periplaneta, which is conspicuous in osmium-fixed material (Wigglesworth, 1960) is completely absent after fixation in glutaraldehyde, and only the lipid of the ‘internum’ persists. Likewise in the myelin sheath: a substantial part of the lipid will take up Sudan black when newly fixed in glutaraldehyde; after embedding and sectioning, this lipid has been removed but the myelin still shows intense Sudan staining after hypochlorite treatment. A similar gradation is to be seen in the testis.

In order that the lipid in the cytomembranes, etc., may remain firmly bound, fixation with glutaraldehyde or osmium tetroxide must be rapid. In solid tissues such as liver or muscle, although the deeper cells may appear well fixed, the amount of lipid retained in the mitochondria etc. falls off rather steeply from the exposed surface. Maximum retention of lipid is confined to a layer several cells deep.

Lipid in ‘membranes’

It is generally believed that the dielectric properties and the high degree of impermeability of some cell membranes are controlled by films of oriented lipid molecules (monolayers or ‘bilipid’ layers). On the other hand there is increasing evidence that in the structurally visible ‘membranes’ of cells (erythrocyte ghosts, membranes of mitochondria, etc.) the bulk of the lipid is in the form of lipoprotein in which the ‘protein would form a tightly bound complex with the lipid, stabilized by multi-point and multi-type bonding’, including salt linkages between NH₃⁺ groups on the protein and exposed anionic groups on the lipid surface, and between carboxyl groups and the basic groups of zwitterionic phospholipids; and possibly also hydrogen bonding and hydrophobic bonding of side chains of the protein penetrating into the spaces opened up between the lipid molecules (Dawson, 1968).

It is evident from the present work that the amount of lipid, that is, the apparent
thickness of the membrane, differs widely in different membranes in the cell. One
kind of nucleus differs from another; plasma membranes also vary. The surface
membrane of the erythrocyte appears exceptionally thick. The description of the
erthrocyte by Ponder (1961) indicates a low content of bound lipid in the interior of
the cell rising steeply towards the surface. The observations described in this paper fit
in well with that description. The observations of Chapman (1968) on the nuclear
magnetic resonance spectrum of the erythrocyte membrane likewise suggest some
intimate interaction between lipid and protein which restrains the mobility of the lipid
chains.

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On all micrographs and on Fig. 20, the scale indicates 10 μm.

Fig. 2. Liver of young mouse; 0.5-μm section in Araldite; osmium tetroxide/ethyl gallate; section stained with renewed ethyl gallate and Sudan black at 60 °C. ×1600.

Fig. 3. Liver of mouse, glutaraldehyde-fixed; 1-μm section in agar and ester wax; Sudan black after hypochlorite for 4 min. Clear vacuoles mark site of droplets of triglyceride; single erythrocyte in capillary, top left. ×1000.

Fig. 4. As Fig. 3, after hypochlorite for 10 min. Mitochondria show pale staining. ×1000.

Fig. 5. Same liver as Fig. 3; osmium tetroxide-fixed; stained with Sudan black direct. Triglyceride droplets black; nuclei and mitochondria very faint. ×1000.

Fig. 6. Kidney of mouse; glutaraldehyde-fixed; 1-μm section; Sudan black after hypochlorite 4 min. Distal convoluted tubule; single erythrocyte in capillary, bottom left. ×1000.

Fig. 7. As Fig. 6. Proximal convoluted tubule, glancing section showing brush border. ×1000.

Fig. 8. As Fig. 6. Margin of Bowman’s capsule showing erythrocytes in capillaries of glomerulus. ×1000.

Fig. 9. As Fig. 6. Margin of kidney showing erythrocytes in blood vessels. ×1000.

Fig. 10. As Fig. 9, but section treated with chloroform/methanol for 0.5 h at 60 °C after hypochlorite but before Sudan black. Erythrocytes faintly stained; mitochondria etc. almost colourless. ×1000.
Unmasking of bound lipid

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Fig. 11. Mesenteric artery of mouse (200 μm diameter) glutaraldehyde-fixed; 2-μm section; Sudan black after hypochlorite. Elastin fibres in media and adventitia and inner and outer elastic layers stain black; erythrocytes dark blue. × 1000.

Fig. 12. As Fig. 11, but section treated with chloroform/methanol for 1 h at 60 °C after hypochlorite but before Sudan black. Erythrocytes very pale staining; arterial wall and elastin completely colourless. × 1000.

Fig. 13. Papillary muscle of ventricle of mouse, longitudinal section. Glutaraldehyde-fixed; 1-μm section; hypochlorite, Sudan black. Normal fibres staining in region of A-bands, with rows of sarcosomes between. × 1200.

Fig. 14. The same; at the cut end of the muscle, showing fibres in excessive contraction with reversed striation as seen after lipid staining, and a single nucleus with invaginated membrane. × 1200.

Fig. 15. Indirect flight muscle of Calliphora, longitudinal section. Glutaraldehyde-fixed; 1-μm section; hypochlorite, Sudan black. Individual fibrils with pale I-bands and dark A-bands interrupted by a narrower pale zone, and with deeply staining sarcosomes between. × 1200.

Fig. 16. The same; fixed in osmium tetroxide; otherwise as Fig. 15. Normal fibrils below, becoming excessively contracted above (near the point of transection) showing progressive reversal of striation. × 1200.

Fig. 17. As Fig. 15, showing whole unsectioned fibrils. × 1200.

Fig. 18. Malpighian tubules of Rhodnius. Glutaraldehyde-fixed; 1-μm section; hypochlorite/Sudan black. Upper segment showing closely packed microvilli containing mitochondria; mitochondria within invaginated plasma membrane at base of cells; tracheoles with deeply staining walls are seen in cross-section outside the tubule above and below. × 1000.

Fig. 19. As Fig. 18. Lower segment with widely spaced filamentous microvilli, displaced at the base of sphaerocrystals of uric acid which are now dissolved. × 1000.
Fig. 20. A, drawing of muscle fibril of Calliphora flight muscle showing progressive reversal of lipid-stained banding during excessive contraction. Based on detail of Fig. 16. B, Engelmann's (1873) drawing of contraction wave in unstained abdominal muscle of Telephorus showing reversal of banding. The vertical lines indicate the middle of each sarcomere.

Fig. 21. Testis of Rhodnius. Glutaraldehyde, hypochlorite, Sudan black, at 1-μm. Spermatocytes in metaphase meiosis, showing mitochondria as fine dark filaments around unstained spindle; chromosomes weakly stained. ×1200.

Fig. 22. As Fig. 21. Telophase meiosis showing deeply stained mitochondrial filaments around the straight-sided spindle. ×1200.

Fig. 23. As Fig. 21. Early spermatids showing pale nucleus, deeply staining rounded Nebenkern and crescentic acrobeast (dictyosome). ×1200.

Fig. 24. As Fig. 21. Developing spermatids showing pale nucleus, darkly staining paired Nebenkern on either side of the unstained axial filament (seen in transverse section in the follicle above) and dark acrobeast with colourless acrosome vacuole. ×1200.

Fig. 25. Third thoracic ganglion of Periplaneta in transverse section at 1 μm. Glutaraldehyde, hypochlorite, Sudan black; showing glial nuclei and transverse sections of axons with 'medullated' sheaths made up of concentric lipid-staining membranes. ×1000.

Fig. 26. As Fig. 25. Ganglion cells with dictyosomes and mitochondria above, 2 darkly staining glial cell nuclei below, and neuropile with axons containing mitochondria at the foot. ×1000.
Unmasking of bound lipid