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Imidazole-buffered osmium tetroxide: an excellent stain for visualization of lipids in transmission electron microscopy

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Summary

The usefulness of imidazole-buffered osmium tetroxide as a stain for lipids in transmission electron microscopy has been investigated. Rat liver and other tissues were fixed by perfusion with glutaraldehyde and post-fixed with osmium–imidazole and the appearance of lipid droplets was compared with that after post-fixation in unbuffered aqueous osmium tetroxide or an osmium solution buffered otherwise. Prominent electron-opaque staining of lipid droplets and of lipoprotein particles was noted after post-fixation with 2% osmium–imidazole, pH 7.5, for 30 min. The lipid droplets appeared well circumscribed with no evidence of diffusion. In contrast, the intensity of staining was much less and there was some diffusion around lipid droplets in material post-fixed in aqueous or cacodylate-buffered osmium tetroxide. Spot tests on filter paper revealed that unsaturated fatty acids, especially linolenic and linoleic acids reacted more intensely with osmium–imidazole than with aqueous osmium tetroxide. These findings demonstrate that osmium–imidazole provides an excellent stain for lipids in transmission electron microscopy and that most probably it stains lipids with unsaturated fatty acids.

Introduction

The study of the ultrastructure of biological materials by electron microscopy depends to a large extent on the deposition of osmium compounds in the tissue. These deposits are formed during fixation with buffered osmium tetroxide, which is also a routine procedure for material fixed primarily by glutaraldehyde. Altmann, in 1894, found that osmium tetroxide blackens oleic acid and olein, both of which are unsaturated, but does not react with palmitic and stearic acids, which are saturated and it is by now generally accepted that osmium tetroxide reacts with unsaturated lipids in tissues. In addition, however, the type of buffer used as a vehicle for osmium tetroxide is also important for the quality of fixation (Wood & Luft, 1965; Ericsson *et al.*, 1965). Imidazole is a potent substance, which stimulates the activity of cyclic nucleotide phosphodiesterase (Butcher & Sutherland, 1962) and inhibits selectively thromboxane synthetase in platelets

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(Needleman *et al.*, 1977). Tu *et al.* (1968) reported that imidazole increased significantly the peroxidatic activity of haem-peptides and Simionescu *et al.* (1975) and Straus (1980) used imidazole in cytochemical studies to enhance the intensity of staining of haem-proteins. Imidazole is also used as a buffer for biochemical and cell biological studies (Gibbons & Fronk, 1979; Gibbons & Gibbons, 1979).

In the present study, we have used imidazole-buffered osmium tetroxide for post-fixation of tissues fixed primarily by glutaraldehyde perfusion and compared their ultrastructural appearance with material post-fixed in osmium tetroxide buffered in another way. Furthermore, the reaction of osmium–imidazole with saturated and unsaturated fatty acids has been studied in a spot test on filter paper. The results indicate that osmium–imidazole reacts more intensely with unsaturated fatty acids than aqueous solutions of osmium tetroxide, and that osmium–imidazole provides an excellent stain for tissue lipids.

Materials and methods

Normal male Sprague–Dawley rats, weighing 150–200 g were used. In one experiment, animals received 30 000 IU of vitamin A (Vogan-Aquat, Bayer). The liver was fixed by perfusion with 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 4% polyvinylpyrrolidone and 0.05% $CaCl_2$. Other tissues were fixed with the same fixative by retrograde perfusion through the abdominal aorta. 50 μm-thick sections were prepared with a Smith–Farquhar TC_2-chopper and placed in 0.2 M imidazole to which 4% aqueous osmium tetroxide was added. We tested the effect of varying the pH of the imidazole solution between 6 and 10, and the post-fixation time from 5 to 60 min.

For comparison, tissues were post-fixed in osmium tetroxide in the following buffers at pH 7.2: cacodylate, veronal–acetate (Caulfield, 1957), *s*-collidine (Bennett & Luft, 1959), and unbuffered aqueous osmium tetroxide (Claude, 1970), as well as osmium tetroxide reduced with potassium ferrocyanide (Karnovsky, 1971). All material was dehydrated with ethanol and embedded in Epon 812. Ultra-thin sections were contrasted with lead citrate for 1 min and examined in a Zeiss EM 10 electron microscope.

The fatty acids oleic, linolenic, linoleic, palmitic and stearic acids were purchased from Sigma Chemie GmbH Munich, West Germany. Palmitic and stearic acids were dissolved in chloroform. All five acids were spotted out onto filter paper and stained either with 2% osmium–imidazole, pH 7.5, or 2% aqueous osmium tetroxide.

Results

The figures presented here illustrate the staining of lipids in rat liver and brown fat, but similar observations have been made in other tissues such as kidney, adrenal and peripheral nerves. In material post-fixed with osmium tetroxide prepared in conventional buffers at pH 7.2, the lipid droplets showed moderate contrast, with a pale central region surrounded by a thin rim of electron-dense material (Figs. 1a–d). The lipid droplets appeared well circumscribed after post-fixation in veronal–acetate buffer (Fig. 1a) and reduced osmium tetroxide (Fig. 1d) with no evidence of diffusion. In cacodylate-buffered (Fig. 1b) and aqueous osmium tetroxide (Fig. 1c), however, diffuse

electron-opaque precipitates around some lipid-droplets were noted. The strongest contrast of cytomembranes with the abovementioned buffers was obtained after post-fixation with reduced osmium, which also stained glycogen deposits (Fig. 1d). Prominent electron-opaque staining of lipid droplets was obtained after post-fixation with 2% osmium–imidazole, pH 7.5, for 30 min. Whereas larger lipid droplets (>3 μm) showed a lighter central core, the smaller ones (which could also be sections through the peripheries of larger droplets) were completely blackened (Fig. 2a). These were all well circumscribed and there was no evidence of diffusion or precipitation around them. The very low density lipoprotein particles also stained quite strongly with osmium–imidazole (Fig. 2b).

The injection of vitamin A resulted in a marked increase in size and number of lipid droplets in perisinusoidal fat-storing cells, causing focal indentation of the nucleus (Wake, 1971; Kobayashi *et al.*, 1973). The contrast between the appearance of these lipid-droplets in material treated with osmium–imidazole on the one hand and with osmium–cacodylate on the other is illustrated in Figs. 3a and b, respectively. By shortening the time of treatment with osmium–imidazole to 5 min only, we noted differences in the intensity of staining of lipid droplets in hepatocytes and in fat-storing cells (Fig. 4a). It should be noted that osmium–imidazole penetrated 50 μm chopper sections within 5 min and required only 30 min for penetration of 1 mm^3 tissue blocks.

At an alkaline pH, some evidence of lipid extraction was observed. The lipid droplets lost part of their contents (Fig. 4b), and cytomembranes, especially of sinus lining cells, were interrupted. A brown precipitate was noted in vials containing the tissue. In such material, the lipoprotein particles showed a translucent core surrounded by an electron-dense coat (Fig. 4c).

The lipid droplets in brown adipose tissue from the interscapular region were similar

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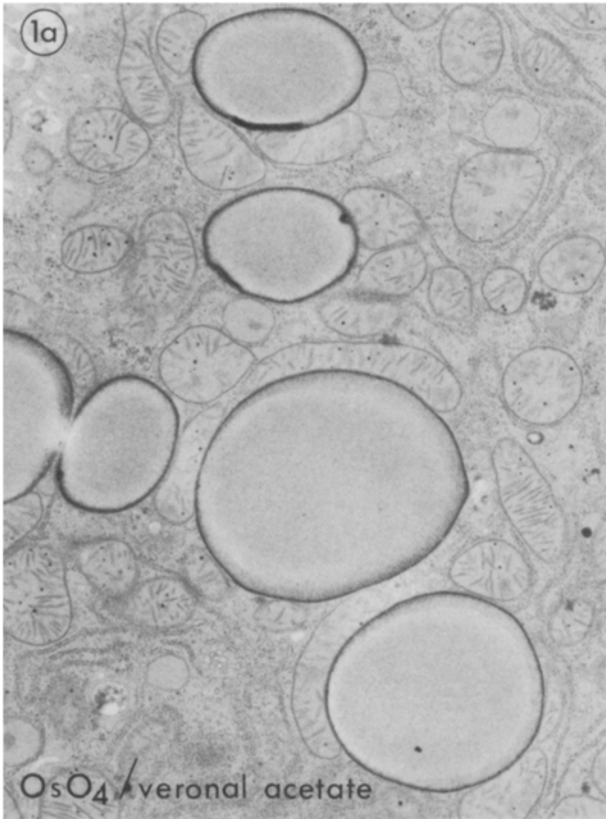
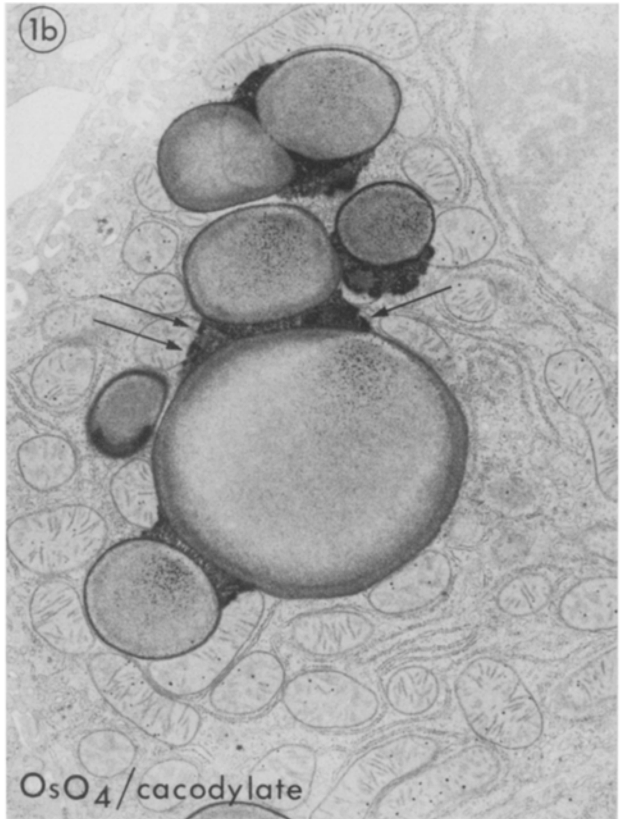
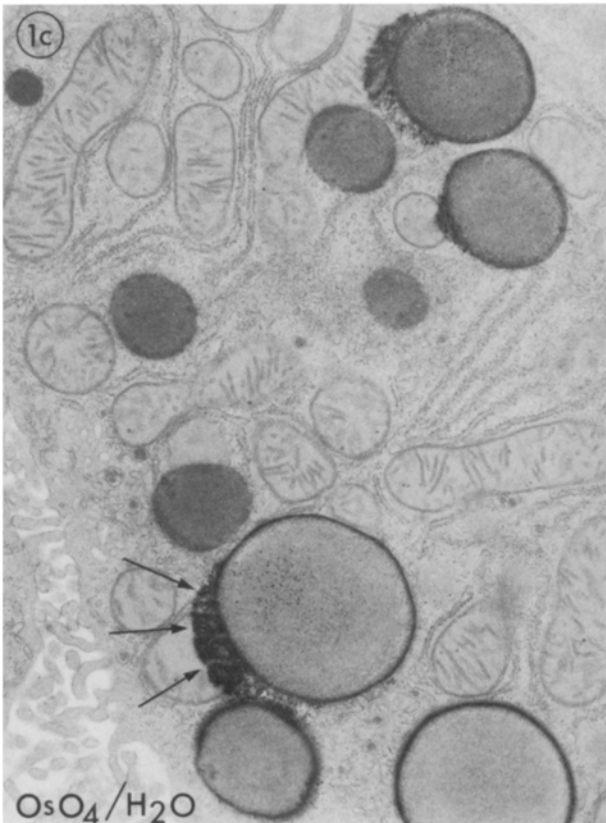
Fig. 1. Rat liver post-fixed with osmium tetroxide prepared in conventional buffers at pH 7.2. Note the appearance of lipid droplets with a pale centre and a thin electron-opaque rim. The droplets are well circumscribed in veronal–acetate buffered (a) and in reduced (d) osmium, but show some diffusion (arrows) after post-fixation in cacodylate-buffered osmium (b) or in aqueous osmium tetroxide (c). The glycogen particles stain positively in reduced osmium (glycogen). (a) × 12 500; (b) × 10 500; (c) × 14 500; (d) × 14 000.

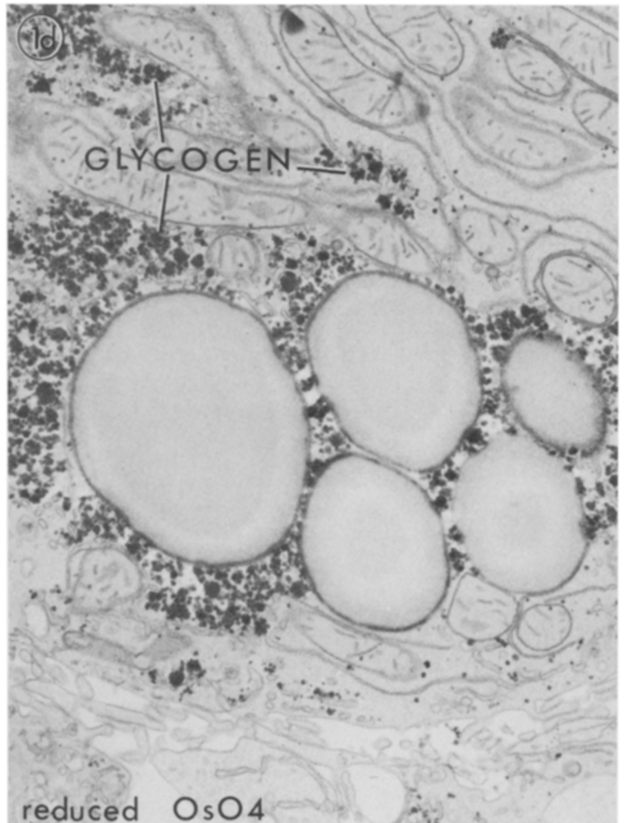
Fig. 2. Rat liver post-fixed with 2% osmium–imidazole, pH 7.5, for 30 min. (a) Note the prominent electron-opaque staining of lipid droplets (L). In larger droplets (> 3 μm), a lighter core is noted (L), but smaller droplets stain diffusely black. BC, bile canaliculi. × 6300. (b) Lipoprotein particles in the Golgi region of a hepatocyte. Note the electron dense appearance of the very low density lipoprotein particles (VLDL). × 34 000.

Fig. 3. Fat storing cells from the liver of a rat treated with vitamin A. Post-fixation with osmium imidazole (a); or with cacodylate-buffered osmium, pH 7.2 (b). Note the large number of lipid droplets (L) in fat storing cells (FSC), which stain much more intensely after osmium–imidazole treatment. (a) × 14 300; (b) × 8400.


1a
OsO4/veronal acetate


1b
OsO4/cacodylate


1c
OsO4/H2O


1d
GLYCOGEN
reduced OsO4

2a

L

L

L

L

L

L

L

L

BC

OsO_4 / imidazole

2b

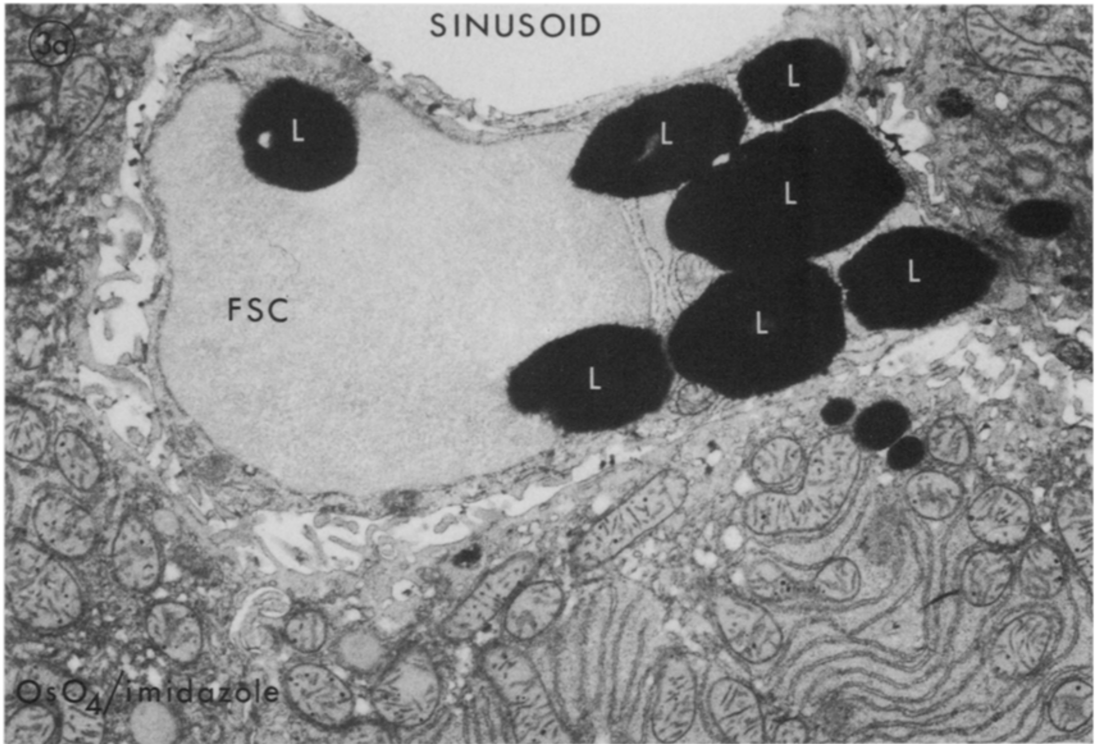
BC

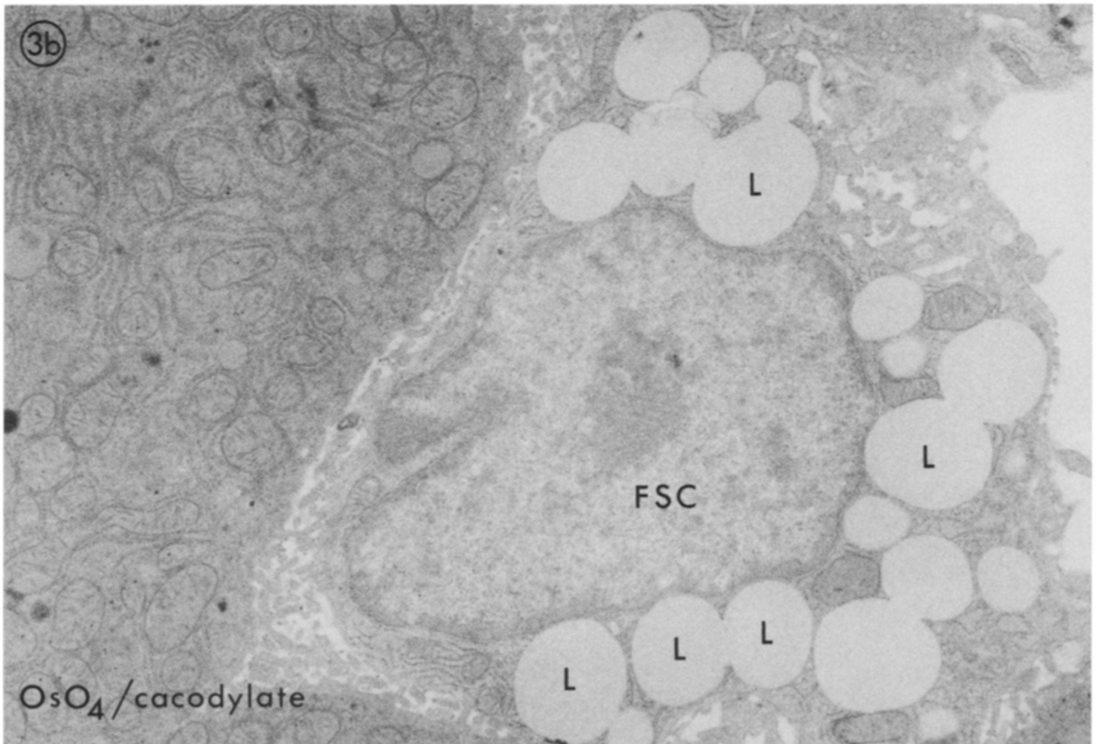
VLDL

VLDL

VLDL

OsO_4 / imidazole


3a
SINUSOID
L
L
L
L
L
L
L
FSC
OsO4/imidazole


3b
L
L
FSC
L
L
L
OsO4/cacodylate

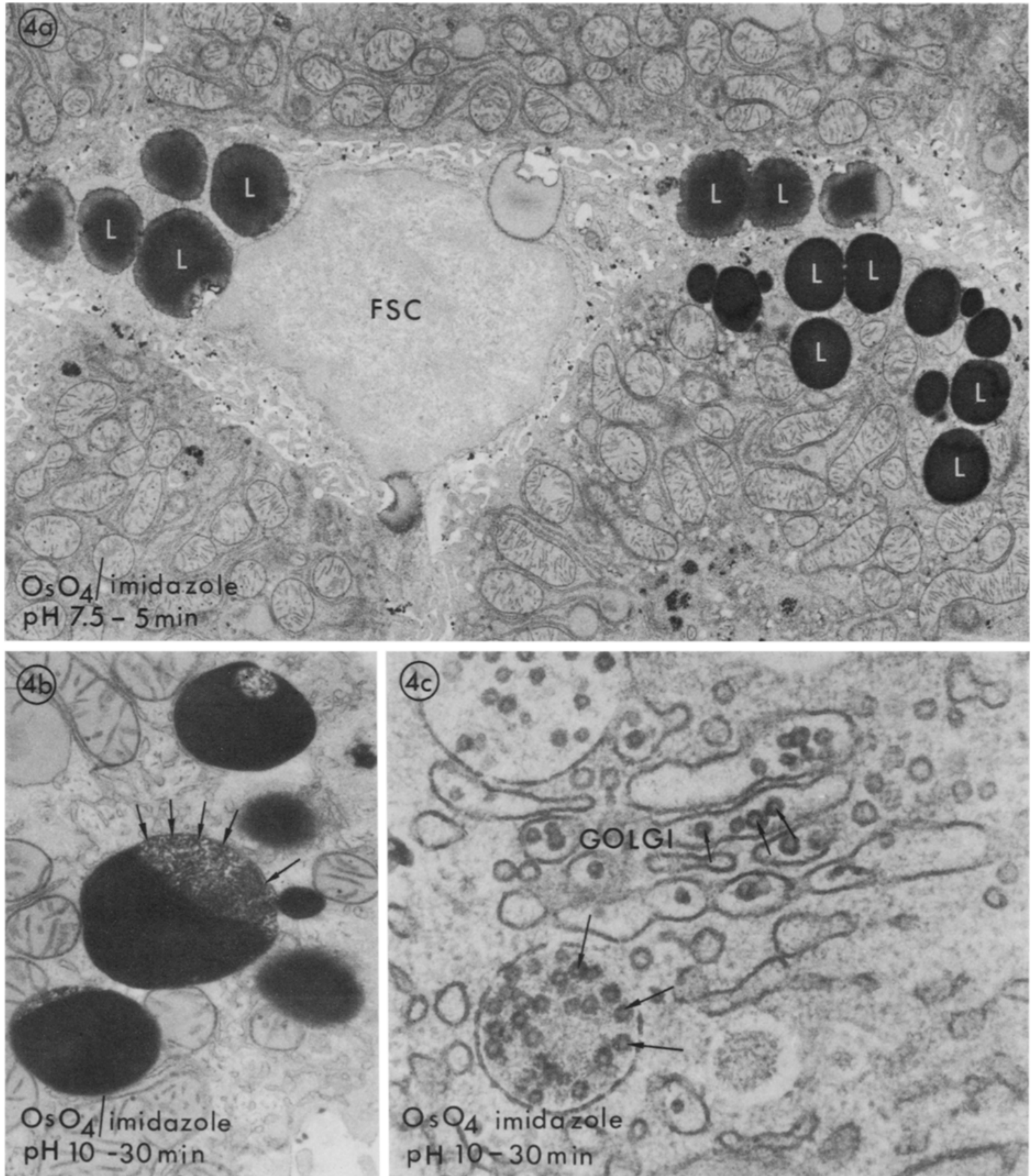
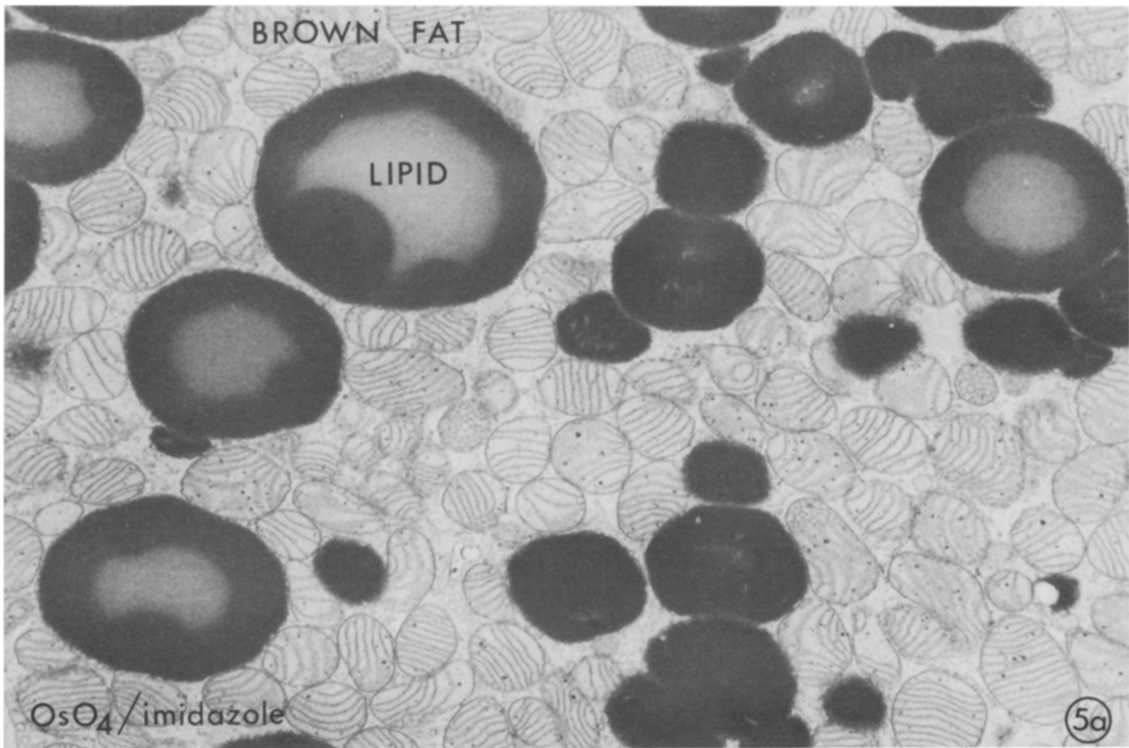


Fig. 4. (a) Vitamin A-treated animal, post-fixation in osmium–imidazole for only 5 min. Note the difference in the intensity of staining of lipid droplets in the hepatocyte and the fat storing cell (FSC). The latter have denser cores and lighter peripheries. × 7000. (b) After the post-fixation in osmium–imidazole at an alkaline pH, evidence of lipid extraction is noted. Lipid droplets show some loss of their contents (arrows). × 19 500. (c) In the same material, VLDL show a translucent core, surrounded by an electron-dense coat (arrows). × 62 000.



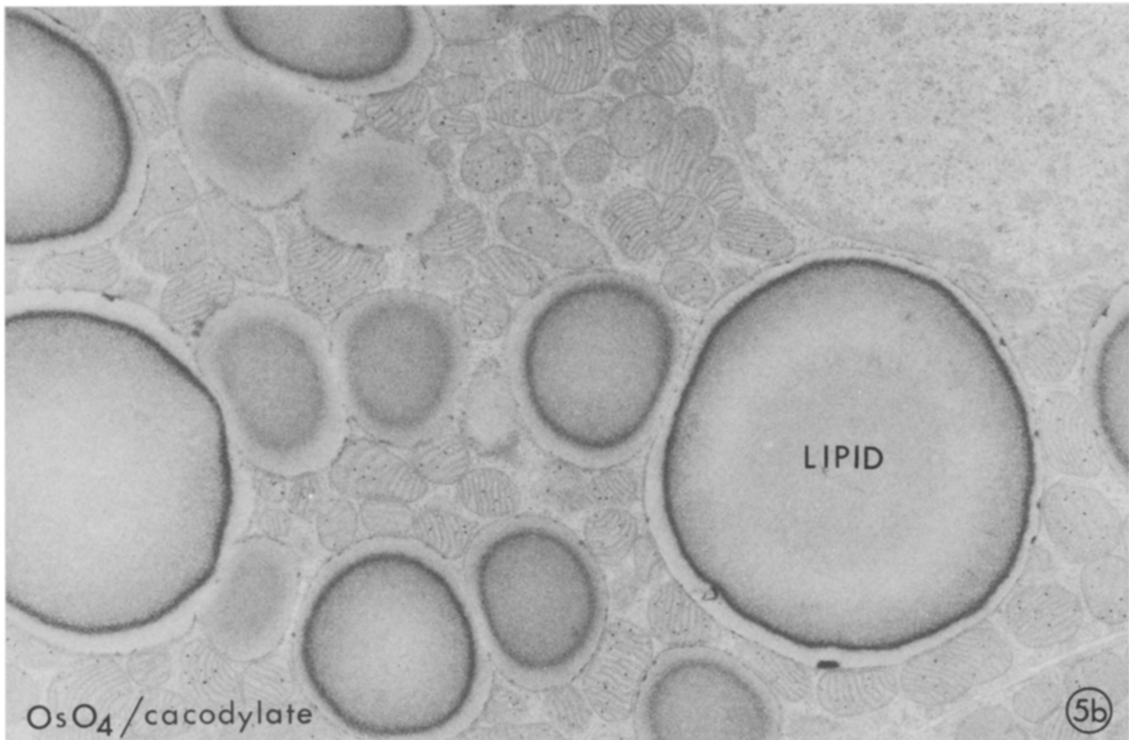


Fig. 5. Brown adipose tissue from the interscapular region of rat, post-fixed either with osmium–imidazole (a) or with cacodylate-buffered osmium (b). The lipid droplets show stronger contrast after osmium–imidazole treatment. Note also the increased contrast of mitochondrial membranes. (a) × 14 000; (b) × 11 500.

to our observations of lipid droplets in the liver in that they also stained more intensely with osmium–imidazole, pH 7.5 (Fig. 5a) than with osmium–cacodylate, pH 7.2 (Fig. 5b).

The spot test on filter paper revealed that linolenic and linoleic acids stained darker

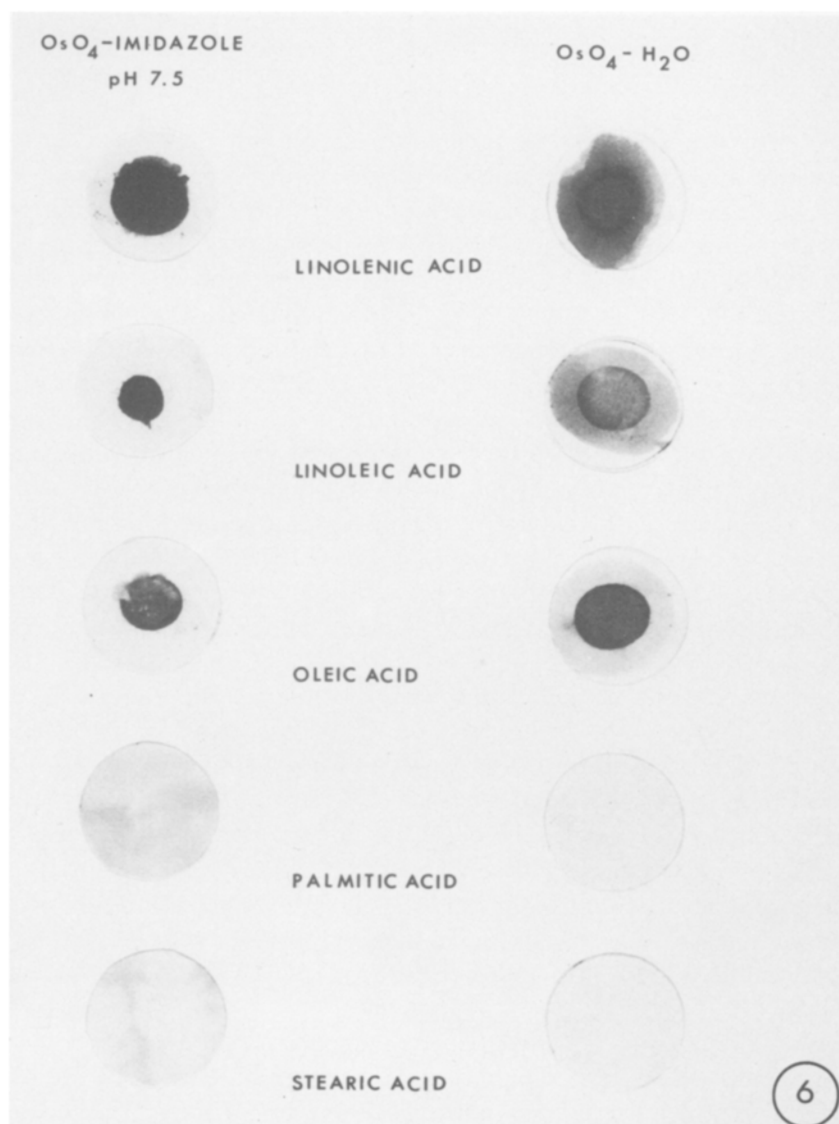


Fig. 6. Spot test on filter paper with fatty acids. Linolenic, linoleic and oleic acids were used directly and palmitic and stearic acids after dissolution in chloroform. All acids were spotted out onto filter paper and stained either with 2% osmium–imidazole, pH 7.5, or with 2% aqueous osmium tetroxide. The linolenic and linoleic acid stain black with osmium–imidazole, but show diffuse greyish reaction with aqueous osmium tetroxide. Spots of osmium–imidazole with unsaturated fatty acids are well circumscribed, but show diffusion with aqueous osmium tetroxide. Saturated fatty acids remain without any significant reaction with either solution.

with osmium–imidazole, but oleic acid reacted more intensely with aqueous osmium tetroxide (Fig. 6). Whereas the spots of aqueous osmium with unsaturated fatty acids showed diffusion, the spots of osmium–imidazole remained well circumscribed. The saturated fatty acids palmitic and stearic acids did not show any significant reaction with either solution (Fig. 6).

Discussion

The present study has revealed that osmium–imidazole provides an excellent stain for lipids containing unsaturated fatty acids in transmission electron microscopy. It penetrates the tissue quite rapidly and stains lipid droplets more profoundly than either aqueous osmium tetroxide or osmium buffered in another way. The stronger reaction of osmium–imidazole with unsaturated fatty acids, linolenic and linoleic acids, was also demonstrated in spot tests on filter paper. These findings suggest that imidazole facilitates the interaction of osmium tetroxide with unsaturated lipids in the tissue.

The rapid penetration of osmium–imidazole and its improved staining of unsaturated lipids can be explained on the following grounds.

(1) As a quasi-aromatic molecule with a strong capacity to bind a number of metals (Barnard & Stein, 1958), imidazole could form a co-ordination compound with osmium (Fig. 7). The formation of similar co-ordination compounds of osmium with aromatic molecules such as pyridine has been well established since the pioneering studies of Criegee and his associates (1936, 1942). These co-ordination compounds have a strong affinity towards double bonds and exhibit superior penetrating characteristics (Zeiss, 1960).

(2) The presence of the tertiary amino group in the imidazole molecule could also explain the rapid penetration of this compound. It is known that the tertiary amino group in local anaesthetics forms a free base, which penetrates easily through tissues and cell membranes (Kuschinsky *et al.*, 1981). The formation of the free base is facilitated at an alkaline pH, which could explain our finding of the more intense reaction of alkaline osmium–imidazole with partial extraction of tissue lipids. The strong affinity of osmium–imidazole to unsaturated lipids is also in agreement with recent observations which indicate that several imidazole derivatives react quite intensely with unsaturated lipids in artificial membranes or certain micro-organisms (Yamagushi & Iwata, 1979; Sud & Feingold, 1981).

As mentioned above, some evidence of lipid extraction is noted when the post-fixation with osmium–imidazole is carried out at an alkaline pH. In such preparations, lipoprotein particles appear as electron-dense rings surrounding a translucent core (Fig. 4c). This is consistent with the lipid core model of lipoprotein particles indicating that these consist of a triglyceride core, surrounded by a coat of protein, phospholipids and cholesterol (Schumaker & Adams, 1969; Jackson *et al.*, 1976). The lipid extraction becomes more pronounced with prolongation of exposure of tissue to osmium–imidazole in alkaline media (pH 9–10). In such material, the plasma membranes of sinus-lining cells

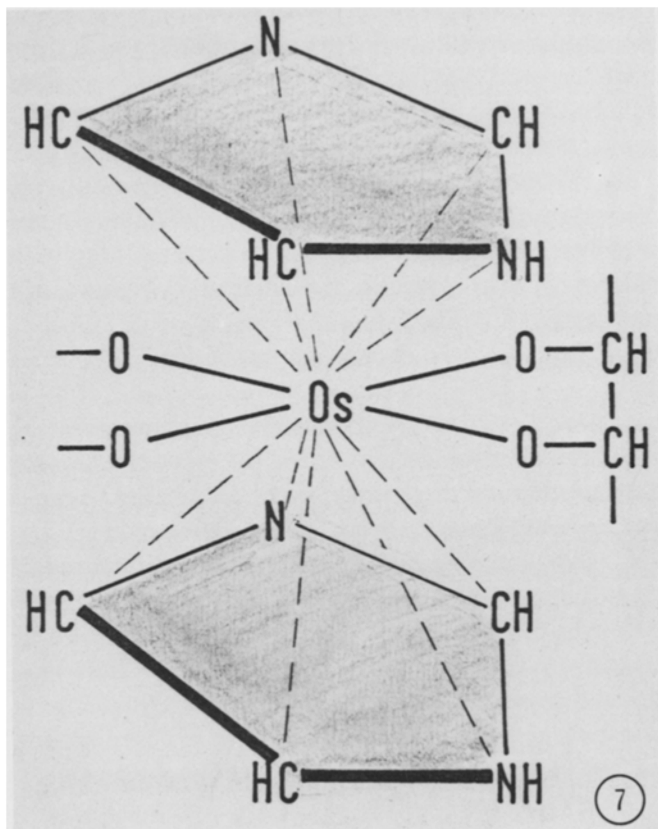


Fig. 7. Proposed model of the co-ordination compound of two imidazole molecules with osmium tetroxide. Interrupted lines symbolize the connection of *p*-electrons between osmium and imidazole.

are disrupted and numerous lipid spheres with a diameter of 1500–2000 Å are found in sinusoidal and other vascular spaces (Angermüller, unpublished observation). These particles exhibit a dark brown colour and have an oily consistency on precipitating during the post-fixation to the bottom of vials containing the tissue. Further analysis of these particles could identify the exact types of lipids that interact with osmium imidazole.

In tests on filter paper, the black spots of osmium–imidazole with unsaturated fatty acids remained well circumscribed and showed little or no diffusion, whereas the spots of aqueous osmium with the same fatty acids exhibited moderate to severe diffusion (Fig. 6). This corresponds to the electron microscopical observations revealing that in hepatocytes post-fixed in osmium–imidazole, lipid droplets exhibited sharp contours (Fig. 2a), while evidence of diffusion was noted in material post-fixed in aqueous osmium tetroxide or cacodylate-buffered osmium (Figs. 1c, b). These findings emphas-

ize the superior quality of osmium–imidazole for demonstration of lipids in electron microscopy.

Feeding of vitamin A causes enlargement of perisinusoidal fat storing cells (Ito, 1973; Wake, 1971; Kobayashi *et al.*, 1973). The large lipid droplets of these cells exhibit the typical green flourescence for vitamin A and are the intracellular sites of storage of this vitamin (Wake, 1980). We found that in tissues exposed briefly (5 min) to osmium–imidazole, the lipid droplets in hepatocytes stained darker than those in fat-storing cells (Fig. 5a). This is consistent with the notion that these two cell types contain different types of lipids. This observation suggests that osmium–imidazole could provide helpful information in differentiating between the various types of lipids in the tissue. Further studies using differential lipid extraction in conjunction with osmium–imidazole could pave the road for the development of more specific cytochemical stains for different types of lipids.

Note added in proof. It is important to adhere to the procedure outlined in Materials and methods by placing the tissue first in imidazole buffer and adding the aqueous osmium solution to it afterwards. Prior addition of osmium to imidazole gives rise to a brown solution and interferes with reproducibility of the method.

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References

ALTMANN, R. (1894) *Die Elementarorganismen und ihre Beziehung zu den Zellen.* 2nd edn. Leipzig.

BARNARD, E. A. & STEIN, W. D. (1958) The roles of imidazole in biological systems. *Adv. Enzym.* **20**, 51–110.

BENNETT, H. S. & LUFT, H. J. (1959) s-Collidine as a basis for buffering fixatives. *J. Biophys. Biochem. Cytol.* **6**, 113–4.

BUTCHER, R. W. & SUTHERLAND, E. W. (1962) Adenosine 3′,5′-phosphatase in biological materials. *J. biol. Chem.* **237**, 1244–50.

CAULFIELD, J. B. (1957) Effects of varying the vehicle for OsO_4 in tissue fixation. *J. Biophys. Biochem. Cytol.* **3**, 827–33.

CLAUDE, A. (1970) Growth and differentiation of cytoplasmic membranes in the course of lipoprotein granule synthesis in the hepatic cell. *J. Cell Biol.* **47**, 745–66.

CRIEGEE, R. (1936) Osmiumsäureester als Zwischenprodukte bei Oxydationen. *Justus Liebigs Ann. Chem.* **522**, 75–96.

CRIEGEE, R., MARCHAND, B. & WANNOWIUS, H. (1942) Zur Kenntnis der organischen Osmium-Verbindungen. *Justus Liebigs Ann. Chem.* **550**, 99–133.

ERICSSON, J. L. E., SALADINO, J. & TRUMP, F. (1965) Electron microscopic observations of the influence of different fixatives on the appearance of cellular ultrastructure. *Z. Zellforsch.* **66**, 161–81.

GIBBONS, I. R. & FRONK, E. (1979) A latent triphosphatase form of dynein 1 from sea urchin sperm flagella. *J. biol. Chem.* **254**, 187–96.

GIBBONS, B. H. & GIBBONS, I. R. (1979) Relationship between the latent adenosine triphosphatase state of dynein 1 and its ability to recombine functionally with KCl–extracted sea urchin sperm flagella. *J. Cell Biol.* **254**, 197–201.

ITO, T. (1973) Recent advances in the study on the fine structure of the hepatic sinusoidal wall: a review. *Gunma Rep. Med. Sci.* **6**, 119–63.

JACKSON, R. L., MORRISETT, J. D. & GOTTO, A. M., JR (1976) Lipoprotein structure and metabolism. *Physiol. Rev.* **56**, 259–316.

KARNOVSKY, M. J. (1971) Use of ferrocyanide reduced osmium tetroxide in electron microscopy. *11th Ann. Meet. Am. Soc. Cell Biol.*, Abstract 284.

KOBAYASHI, K., TAKAHASHI, Y. & SHIBASAKI, S. (1973) Cytological studies of fat-storing cells in the liver of rats given large doses of vitamin A. *Nature New Biol.* **243**, 186–8.

KUSCHINSKY, G., LÜLLMANN, H. & PETERS, T. (1981) *Kurzes Lehrbuch der Pharmakologie und Toxikologie.* pp. 195–197. Stuttgart, New York: Georg Thieme Verlag.

NEEDLEMAN, P., RAZ, A., FERRENDELLI, J. A. & MINKES, M. (1977) Application of imidazole as a selective inhibitor of thromboxane synthetase in human platelets. *Proc. natn. Acad. Sci., U.S.A.* **74**, 1716–20.

SCHUMAKER, V. N. & ADAMS, G. H. (1969) Circulating lipoproteins. *Ann. Rev. Biochem.* **38**, 113–36.

SIMIONESCU, N., SIMIONESCU, M. & PALADE, G. E. (1975) Permeability of muscle capillaries to small heme-peptides. *J. Cell Biol.* 586–607.

STRAUS, W. (1980) Factors affecting the sensitivity and specificity of the cytochemical reaction for the anti-horseradish peroxidase antibody in lymph tissue sections. *J. Histochem. Cytochem.* **28**, 645–52.

SUD, J. & FEINGOLD, D. S. (1981) Mechanisms of action of the antimycotic imidazoles. *J. Invest. Derm.* **76**, 438–41.

TU, A. T., REINOSA, J. A. & HSIAO, Y. Y. (1968) Peroxidatic activity of heme-peptides from horse heart cytochrome *c*. *Experientia* **24**, 219–21.

WAKE, K. (1971) 'Sternzellen' in the liver: perisinusoidal cells with special reference to storage of vitamin A. *Am. J. Anat.* **132**, 429–62.

WAKE, K. (1980) Perisinusoidal stellate cells (flat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A storing cells in extrahepatic organs. *Int. Rev. Cytol.* **66**, 303–57.

WOOD, L. & LUFT, J. H. (1965) The influence of buffer systems on fixation with osmium tetroxide. *J. Ultrastruct. Res.* **12**, 22–45.

YAMAGUSHI, H. & IWATA, K. (1979) Effect of fatty acyl group and sterol composition on sensitivity of lecithin liposomes to imidazole antimycotics. *Antimicrob. Agents Chemother.* **15**, 706–11.

ZEISS, H. (1960) Arene complexes of the transition metals. In *Organo-metallic Chemistry* (edited by ZEISS, H.), pp. 380–425. New York, London: Reinhold.