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Tissue Fixation and Osmium Black Formation with Nonvolatile Octavalent Osmium Compounds*

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Summary. Several compounds of osmium^{VIII}, including potassium osmiamate and coordination complexes of OsO_4 with ammonia and various heterocyclic nitrogen compounds, have been synthesized and characterized. They have also been evaluated as substitutes for OsO_4 in postfixation of biological specimens and in light and electron microscopic cytochemical methods resulting in osmium black formation.

The most useful of these osmic compounds, a molecular addition complex of hexamethylenetetramine (methenamine) with OsO_4, has a negligible vapor pressure of OsO_4. It has the molecular formula $C_6H_{12}N_4.2OsO_4$ and has been designated osmeth. Although it has only limited solubility, aqueous solutions of the compound (or of OsO_4) can be rapidly prepared by dissolution in a minimal amount of dimethylformamide and subsequent dilution with distilled water or buffer. Although stable in the solid state, the complex in solution undergoes partial dissociation releasing OsO_4, and the odor of OsO_4 becomes apparent.

Such solutions of osmeth are ($\sim$0.25%) considerably less concentrated with respect to OsO_4 than solutions (1–2%) ordinarily employed for ultrastructural preservation or in cytochemical studies. Osmeth has limited value for postosmication after glutaraldehyde fixation because the generation (release) of OsO_4 appears to be slow. Adequate osmication of tissue blocks exists only at the surface, but effective osmication can be achieved throughout tissue sections. In cytochemical reactions resulting in the formation of osmium blacks, the osmeth solutions are as effective as OsO_4 solutions of equivalent concentrations. Our findings indicate that OsO_4 solutions of less than 1% may be satisfactorily utilized in many cytochemical studies.

Osmeth is safer and more convenient to handle than OsO_4 because small amounts may be solubilized as needed. It should be considered as a substitute for OsO_4 in ultrastructural cytochemistry.

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These results suggest that the effectiveness of OsO_4 as a fixative may, in part, be related to its nonpolarity. The infrared spectra indicate that the OsO_4 molecule is tetrahedral, perfectly symmetrical and, therefore, as a whole nonpolar. As a consequence, it could be expected to readily penetrate charged surfaces of tissues, cells, and organelles. The spectral studies show that osmeth is much less symmetrical and, to that extent, polar; thus, it penetrates biomembranes less readily.

Introduction

Osmium tetroxide, OsO_4, has many favorable properties as a reagent that preserves biologic ultrastructure while it increases the contrast of specimens. Furthermore, it has recently been applied to the formation of osmium blackend products for light and electron microscopic cytochemistry (Hanker et al., 1964). It is, however, one of the most dangerous osmium compounds to handle on account of its toxicity and vapor pressure; the solid has a vapor pressure of 11 mm at 25° C (Griffith, 1965a). To avoid severe damage to the respiratory tract and eyes, handling with extreme care is required in well ventilated fume hoods. Another problem is the slow solubility of OsO_4 in water. The latter problem has been relieved to some extent, by the commercial availability[1] of OsO_4 solutions. Thus, there is a distinct need for the preparation of less volatile, more soluble compounds of osmium with an oxidation potential equivalent to OsO_4.

The principal aims of this investigation were: (1) the preparation and characterization of nonvolatile soluble octavalent compounds of osmium; (2) comparison of their properties to those of OsO_4 as used in the double fixation of biological specimens; (3) determination of their effectiveness as substitutes for OsO_4 in cytochemical reactions resulting in the formation of osmium blackend products.

Osmium has no less than nine oxidation states (VIII to 0, Griffith, 1965a). The tendency of certain nitrogen ligands which are good π donors, particularly those of the nitride type, to stabilize octavalent osmium is well known (Bailar, 1956). This is also apparent in the case of certain amine donors which react with OsO_4 to form organic osmiamates (Milas and Iliopulos, 1959). It was, therefore, appropriate to study these osmiamates and complexes of OsO_4 which are rather easily prepared with ammonia and various heterocyclic amines (Fig. 1) as possible nonvolatile substitutes for OsO_4 in tissue fixation and in ultrastructural cytochemistry.

Materials and Methods

I. Synthesis and Characterization of Octavalent Osmium Compounds

Osmium tetroxide-methenamine addition compound ("osmeth")[1], $C_6H_{12}N_4 \cdot 2OsO_4$ was prepared by the dropwise addition of 20 ml of 2% OsO_4 with continuous stirring (under a fume hood)

[1] Available from Polysciences, Inc., Warrington, PA 18976

at room temperature to 3 g of hexamethylenetetramine (methenamine) dissolved in a minimum amount of water. Before the addition was completed, gold-orange crystals corresponding to the addition compound previously described by Tschugajeff and Tschernjajeff (1928) appeared. After cooling in an ice bath, the orange crystals were collected on a fluted filter, washed thoroughly with water, and dried on a unglazed porcelain plate under a fume hood. The dried compound lightened during melting, turned white at 130–160°, darkened considerably at 160–170°, and melted at 168–185°. Attempts to recrystallize the products from dimethylformamide-petroleum ether resulted in its decomposition. Elemental analysis (Table 1) confirmed the structure (Fig. 2) proposed earlier by Tschugajeff and Tschernjajeff (1928) even though the value for osmium was low. Osmium determinations on organic compounds give consistently low values (Milas and Iliopulos, 1959), however. The purified compound has practically no odor of OsO_4. It has very limited solubility in water and it is soluble in water only upon stirring over a long period of time. It was found in these studies, however, that instantaneous solution of either OsO_4 or of its addition complex with hexamethylenetetramine could be achieved in water or in aqueous buffer solutions by first dissolving the compound in a minimum amount of dimethylformamide (DMF). Specific direction for the instantaneous preparation of typical OsO_4, or OsO_4-methenamine complex (osmeth) solutions for use in fixation or in cytochemical studies are given below.

Upon dissolution in water the osmium tetroxide-methenamine complex partially dissociates, as shown in Figure 2. It forms a solution with a high vapor pressure similar to OsO_4 solutions and thus must be handled with appropriate precautions. The octavalency of osmium in the complex was suggested by the pink to red-violet coloration given by its solutions with acidic thiourea reagent (Fig. 3) and confirmed by potentiometric titration with hydrazine sulfate and iodometric titration (Fig. 4) performed by modifications of the methods of Crowell and Kirschman (1929) as described below.

Instantaneous Preparation of Aqueous OsO_4 or Osmeth Solutions. These solutions could be readily prepared by dissolution of the appropriate quantity of either OsO_4 (up to 0.4 g) or osmeth (up to 25 mg) in 1 ml dimethylformamide[1] with agitation on a vortex stirrer. The solution was then brought to a total volume of 10 ml by the very rapid addition of 9 ml of water or of an appropriate buffer solution with continuous stirring. Solutions prepared in this manner appeared to have stabilities similar to OsO_4 solutions prepared in the usual manner (Hayat, 1970). After storage for several weeks at room temperature, unprotected from light, a precipitate that could be identified by infrared spectroscopy as methenamine (Fig. 1) had separated from some osmeth solutions.

Osmium tetroxide-methenamine mandelate addition compound, $C_6H_5CHOHCOOH.(CH_2)_6N_4.OsO_4$. When methenamine mandelate[2] was treated with OsO_4 in chloroform solution, as shown in Figure 2, the desired complex was obtained. It began decomposing at 110° and was completely decomposed at 160°. Its solutions behaved toward acidic thiourea and thiocarbohydrazide reagents like osmeth. Elemental analysis of the osmium tetroxide-methenamine mandelate complex (Table 2) suggested the structure shown in Figure 2. It was appreciably more soluble in DMF/water or DMF/buffer combinations than osmeth. It could not be prepared in aqueous solution; if water was present, only the osmium tetroxide-methenamine complex was obtained as shown in Figure 2. Since methenamine mandelate is derived from a weak acid and a weak base, it undergoes instantaneous hydrolysis in aqueous solution.

Potassium osmiamate, $KOsO_3N$, was preapred by a procedure patterned after the general directions in Sidgwick (1950): One gram of osmium tetroxide[1] was dissolved in 25 ml of saturated potassium carbonate solution with magnetic stirring and occasional gentle heating on a water bath. After cooling in an ice bath, 5 ml of NH_4OH (28.5% NH_3) were added dropwise with continuous stirring, whereupon a yellow solid separated. The mixture was gently heated on the water bath for a brief period with continued stirring. After chilling in an ice bath, the precipitate was collected on a Buchner funnel; a second crop of product was obtained from the mother liquors on standing. The bright yellow solid was stable at room temperature and had no odor. It was quite soluble

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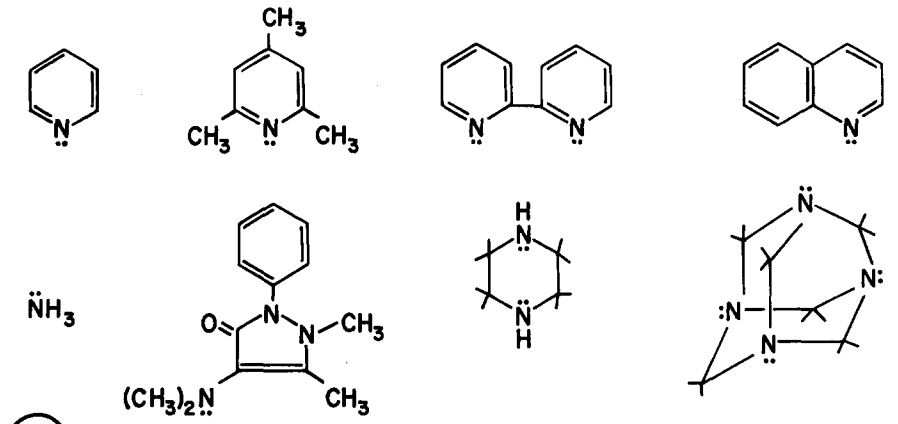
[2] *Methenamine mandelate,* $(CH_2)_6N_4.C_6H_5CHOHCOOH$, was prepared by adding 14.0 g (0.1 mole) of methenamine dissolved in 200 ml ethanol to 15.2 g (0.1 mole) mandelic acid dissolved in 20 ml ethanol. The complex was isolated by precipitation with ether and melted at 128–130°

AMINES STUDIED AS LIGANDS FOR COMPLEX FORMATION WITH OsO_4



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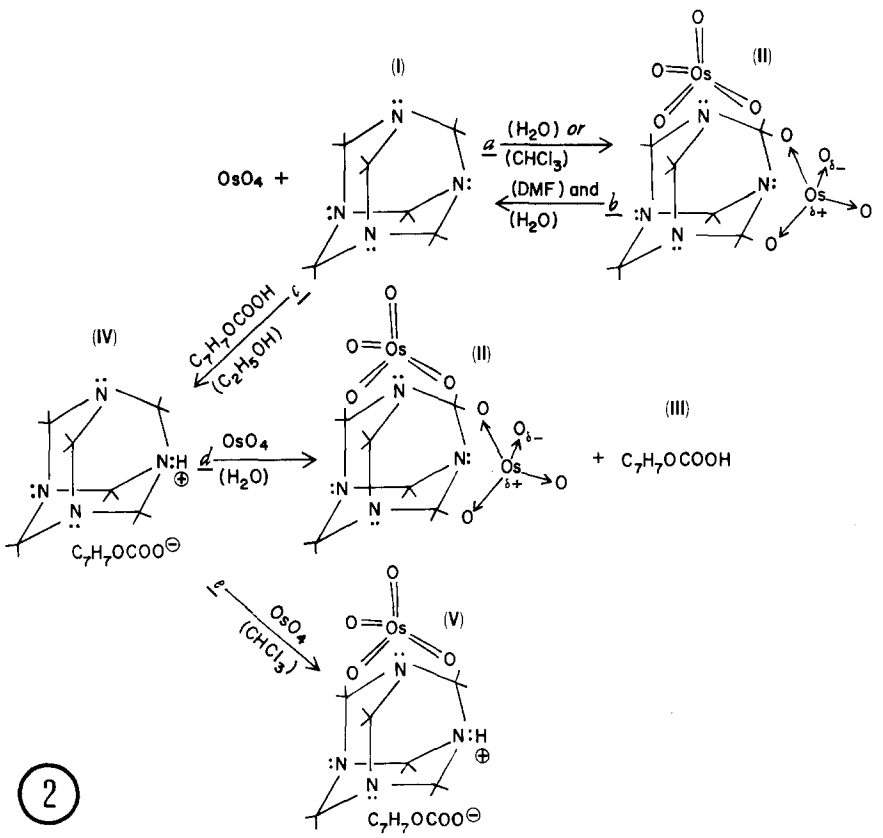

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Table 1. Elemental analysis[a] of osmeth

	For $C_6H_{12}N_4 \cdot Os_2O_8$			
	C	H	N	Os
Calcd.	11.11%	1.90%	8.64%	58.65%
Found	11.31%	2.29%	9.1%	57.13%

[a] Determinations by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y., USA

in water. The aqueous solutions had no odor and appeared relatively stable. The solutions gave an immediate coloration with thiourea in acid solution (Fig. 3) indicative of octavalent osmium (Sauerbrunn and Sandell, 1953) although they react only relatively slowly with thiocarbohydrazide to form osmium blacks (Hanker et al., 1966). The compound darkened at 140° and was completely decomposed by 190° C.

Osmium tetroxide-ammonium hydroxide addition compound, $OsO_4 \cdot 2NH_4OH$, was prepared by the procedure of Krauss and Wilken (1925). The orange crystals had a melting range of 33–37° and were very soluble in water. The deep yellow aqueous solution gave an immediate red-violet coloration with acidic thiourea solution and also gave an immediate osmium black precipitate with thiocarbohydrazide. The solid as well as the solutions had pungent irritating odors of either osmium tetroxide or ammonia. It apparently lost ammonia on standing a short while at room temperature leaving a light yellow residue of OsO_4, which sublimed shortly thereafter. Attempts to produce a potassium bromide disc for infrared spectroscopy resulted in the loss of ammonia. By the time the preparation of a mull in liquid petrolatum was attempted, the stored sample had lost most of its ammonia.

Osmium tetroxide-pyridine addition compound. This bright yellow compound separated out immediately upon the dropwise addition of an equivalent of pyridine to a stirred 10% solution of OsO_4 chilled by an ice bath. The solid started to contract at 65° and melted at 68–70°. The most prominent odor of the compound was that of pyridine. It was insoluble in water and sublimed at room temperature. It did not appear to breakdown in the solid state, however. Elemental analyses were inconclusive as to its structure.

Complexes of OsO_4 with Other Heterocyclic Amines. The other amines shown in Figure 1, *sym.* collidine, α,α'-dipyridyl, quinoline, aminopyrine and piperazine formed brown or black products

Fig. 1. Structures of amines reacted with OsO_4 in attempt to form stable, soluble, nonvolatile octavalent osmium compounds, Upper row, left to right: pyridine, *sym.* collidine, α,α'-dipyridyl, quinoline. Lower row, left to right: ammonia, aminopyrine, piperazine, methenamine

Fig. 2. Reactions involved in the preparation of osmeth (*II*) and the osmium tetroxide-methenamine mandelate addition compound (*V*). The upper row depicts the formation (*a*) of osmeth in water or in chloroform. OsO_4 is a symmetrical nonpolar molecule. The Os–O bonds however are polarized as shown. The partial positive charge on the osmium atom accounts for its ability to complex with the σ electrons of methenamine nitrogen atoms. The hydrolysis (*b*) of osmeth yielding methenamine and OsO_4 is also shown as is the formation (*c*) of methenamine mandelate (*IV*) from methenamine and mandelic acid in alcoholic solution. The center row shows that hydrolysis (*d*) of the salt occurs and osmeth (*II*) is also formed when methenamine mandelate (*IV*) is treated with OsO_4 in water. When treated (*e*) with OsO_4 in chloroform, however, the OsO_4 addition compound of methenamine mandelate (*V*) results

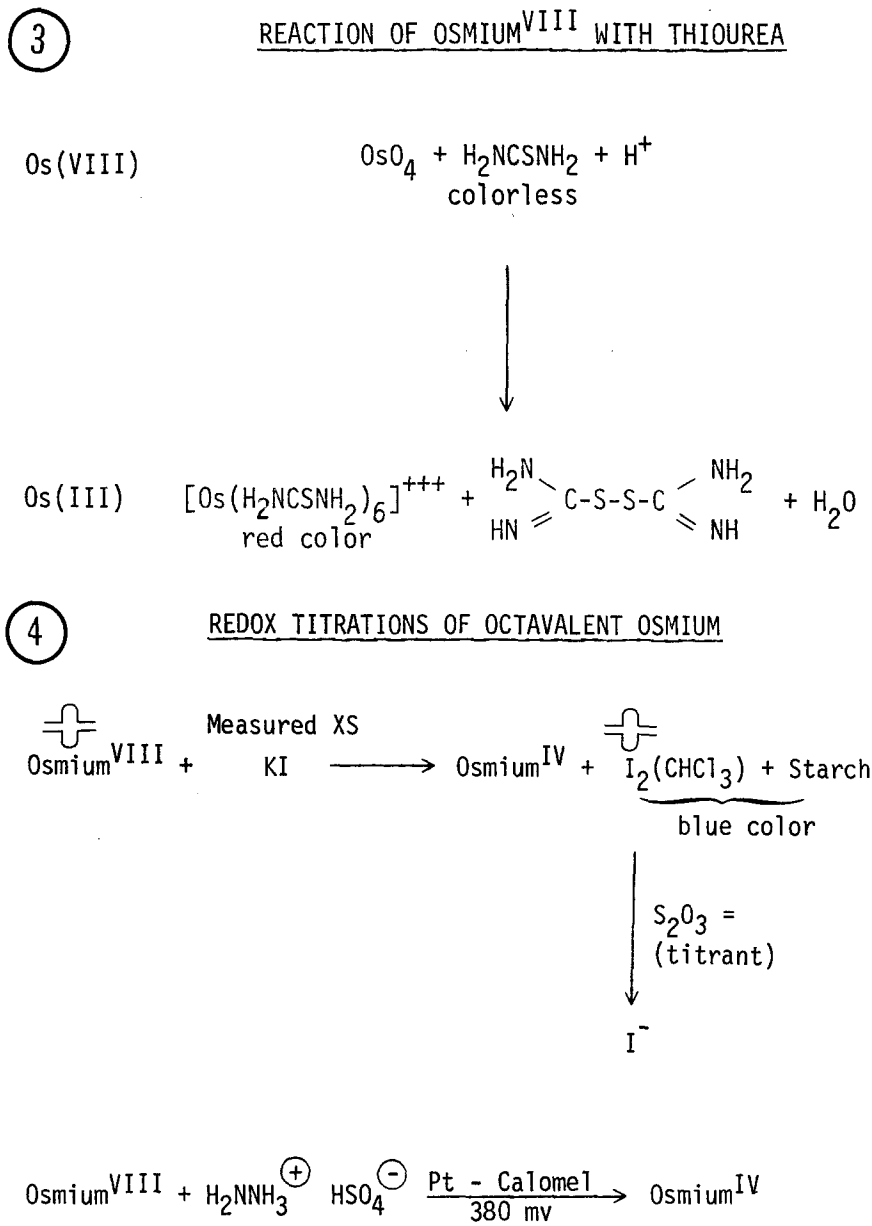


Fig. 3. Reaction for formation of the soluble red complex from excess thiourea and OsO_4 in acidic medium. Note that octavalent osmium is reduced to the trivalent state, not to the tetravalent state as formerly believed. After Sauerbrunn and Sandell, 1953

Fig. 4. The titrimetric characterization of octavalent osmium by redox reactions. In the iodometric titration (upper), an aliquot of osmeth (or OsO_4) solution was treated with a measured excess of KI. The equivalent of iodine liberated was back-titrated with standard thiosulfate until the disappearance of the blue color. The volume of standard thiosulfate consumed is related to the amount of osmeth (or OsO_4) originally present. Potentiometric titration of osmeth or OsO_4 in hydrobromic acid solution with standard hydrazine sulfate solution also results in the reduction of osmium to the tetravalent state. A platinum oxidation-reduction electrode was used versus a KCl-calomel reference electrode

Table 2. Elemental analysis[a] of osmium tetroxide-methenamine mandelate addition compound

	For $C_{14}H_{20}N_4O_7Os$				
	C	H	N	O	Os
Calcd.	30.77%	3.69%	10.25%	20.49%	34.80%
Found	31.28%	3.71%	9.93%	–	31.60%

[a] Determinations by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y. 11377, USA

very soon, if not immediately, after the addition of OsO_4. Some of these decomposition-like reactions were rapid enough to suggest gentle detonation. No attempts were made to isolate or purify these products.

Spectrometric Identification of Osmium Compounds. Because the melting points of the osmium compounds frequently had considerable range, and the elemental analyses were equivocal due to low osmium values, spectroscopy, especially infrared, was utilized to aid in identification and characterization of the compounds. Visible absorption spectra were recorded with a Perkin-Elmer Hitachi Model 124 double beam UV-visible spectrophotometer. Samples for infrared spectroscopy were prepared as potassium bromide pressed discs and the spectra recorded with a Perkin-Elmer Model 457 double beam grating infrared spectrophotometer.

Redox Titrations of Octavalent Osmium Compounds. Potentiometric Titration. Potentiometric titration of OsO_4 and osmeth solutions with standard hydrazine sulfate solutions (Bray and Cuy, 1924) was carried out according to the directions of Crowell and Kirschman (1929), except that titrations were performed on freshly prepared neutral rather than alkaline solutions. Instead of a Leeds and Northrup potentiometer and galvanometer, a Radiometer TTT 60 Titrator and PHM62 Standard pH meter were used with a platinum oxidation-reduction electrode (P101) versus a KCl-calomel reference electrode (K401). A large drop in potential (approximately 50 millivolts per 0.1 ml hydrazine sulfate solution) occurred only when four equivalents of hydrazine sulfate had been added for each gram atom of octavalent osmium (present as OsO_4 or osmeth). This suggests that osmium was reduced from the octavalent to the tetravalent state (Fig. 4).

Iodometric Titration. The method described by Crowell and Kirschman (1929) was modified to facilitate the visualization of iodine present in solution at the end-point by extracting iodine into a small volume of chloroform (approximately 10 ml/50 ml aqueous solution) as suggested by Vogel (1961). The presence of free iodine was confirmed by detecting the small amount of residual iodine in the aqueous layer with starch indicator in the usual manner. The stoichiometry of the reaction is based on the reduction of osmium from the octavalent to the tetravalent state by iodide (Fig. 4) according to the equation:

$$OsO_4 + 4KI + 2H_2SO_4 \rightarrow OsO_2 + 2K_2SO_4 + 2H_2O + 2I_2.$$

II. Osmeth as a Biological Fixative and as a Reagent in Osmium Black Cytochemistry

Biological Fixation. Osmeth was evaluated as a substitute for OsO_4 in the routine double fixation procedure in which osmication follows initial glutaraldehyde fixation. These studies were performed only on tissues from Charles River rats (CD strain) or C57Bl 10 inbred mice which had been prefixed in glutaraldehyde according to the following 2 protocols: (1) Under ether anesthesia, physiological saline was administered through the left ventricle after an incision was made in the right ventricle for drainage. Glutaraldehyde (2.5%, 0.1 M in cacodylate buffer, pH 7.4) was

then perfused by the same route; in some cases the perfusion was gravity assisted by employing an elevated reservoir containing the perfusion fluid. When leakage of the fixative through a small lip incision indicated the attainment of a statisfactory perfusion, the tissues were excised rapidly, cut into blocks of appropriate size (1 mm^3) under fixative, and the fixation process allowed to continue by immersion for an appropriate length of time, usually 2 h. The tissue blocks were then rinsed overnight in buffer prior to postfixation in 0.25% or 0.20% OsO_4 or 0.25% osmeth. Some of the glutaraldehyde-fixed blocks were sectioned at 25–50 μm with a Sorvall TC-2 sectioner (Smith and Farquhar, 1965) prior to the postfixation process. At the end of this process, the tissues were examined for the depth of osmium penetration. (2) Experiments with fixation by immersion were performed using 1 mm^3 blocks of rat diaphragm and uterus. The blocks were fixed for 1 h in 6.25% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. After several rinses in cacodylate-sucrose (pH 7.4), the blocks were osmicated for 1 h in: (a) 0.25% OsO_4 in 0.1 M veronal buffer (pH 7.4) or (b) 0.20 or 0.25% osmeth in 0.1 M veronal buffer (pH 7.4). After osmication, the blocks were rinsed in buffer and then stained en bloc in 0.5% uranyl-acetate (Farquhar and Palade, 1965). Routine dehydration in cold ethanol and embedding in epon was performed. Ultrathin (silver) sections were prepared with a diamond knife and stained with lead and uranium. The electron-micrographs were all printed on Velour Black #4 paper.

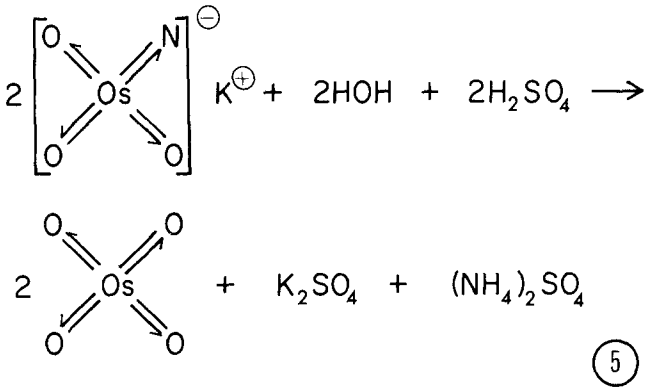
Osmium Black Cytochemistry. Cryostat sections (4–10 μm) of fixed rinsed tissues were prepared according to Pearse (1968) and air-dried on coverslips for light microscopy. Glutaraldehyde-fixed sections were utilized for the demonstration of catalase activity (Novikoff and Goldfischer, 1969). Formaldehyde-fixed sections were utilized to demonstrate nonspecific esterase activity (Hanker et al., 1972b) or cytochrome oxidase activity (Seligman et al., 1968; Hanker, 1975). Alternatively, sections (25–50 μm) of tissues similarly fixed were prepared with a Sorvall TC-2 sectioner (Smith and Farquhar, 1965) and incubated for ultrastructural demonstration of these enzymes. All of these cytochemical reactions involved treatment with 3,3′-diaminobenzidine (DAB) at some stage of the reaction and its oxidative coupling to form an osmiophilic polymer. After rinsing the DAB-treated sections, aliquots of the tissues were osmicated with either 0.20% OsO_4 or 0.25% osmeth solution.

Results

Properties of Potential OsO_4 Substitutes

The only compounds of all those prepared (see Materials and Methods) that could be considered as substitutes for osmium tetroxide in biological fixation and in cytochemical reactions were the osmium tetroxide-methenamine addition compound (osmeth), the osmium tetroxide-methenamine mandelate addition compound, potassium osmiamate, the osmium tetroxide-ammonium hydroxide addition compound, and the osmium tetroxide-pyridine addition compound. There are two outstanding attributes of osmeth and the osmium tetroxide-methenamine mandelate addition compound that warrant their consideration as osmium tetroxide substitutes in tissue fixation and in osmium black formation in cytochemistry. These are the negligible vapor pressure of the OsO_4 component of these molecular addition complexes and the ease with which they dissociate to give OsO_4 in solution once they are dissolved. It is important to realize, however, that their dissociation to give OsO_4 in solution was not complete but only partial.

Potassium osmiamate reacted somewhat less readily with thiocarbohydrazide than osmium tetroxide, although it reacted as readily with acidic thiourea reagent. For this reason it was not evaluated as a fixative at this time. The immediate



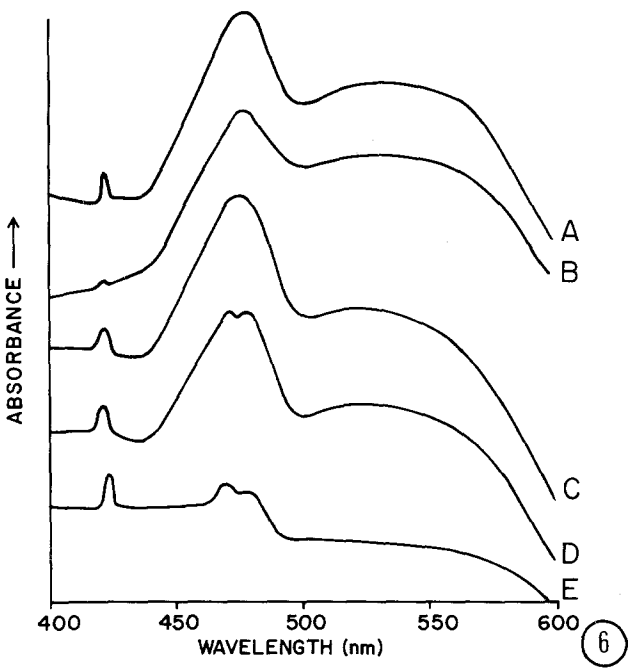
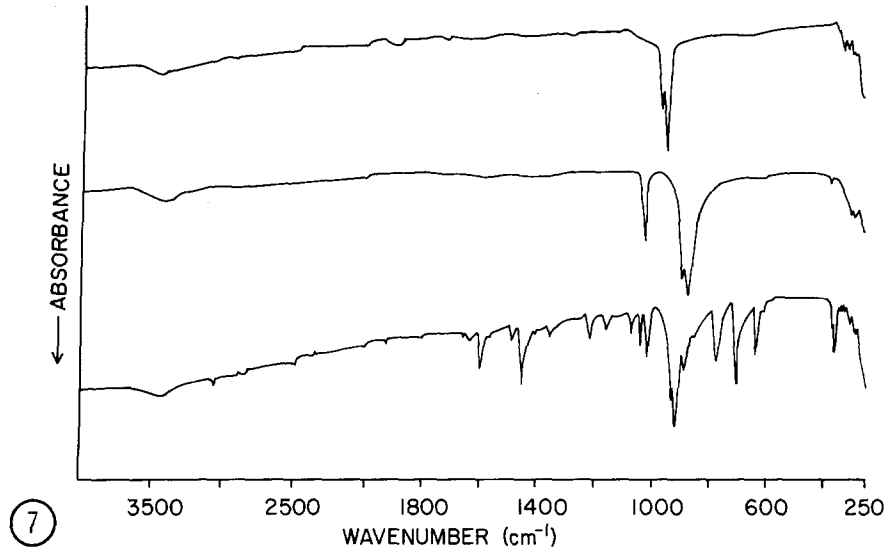
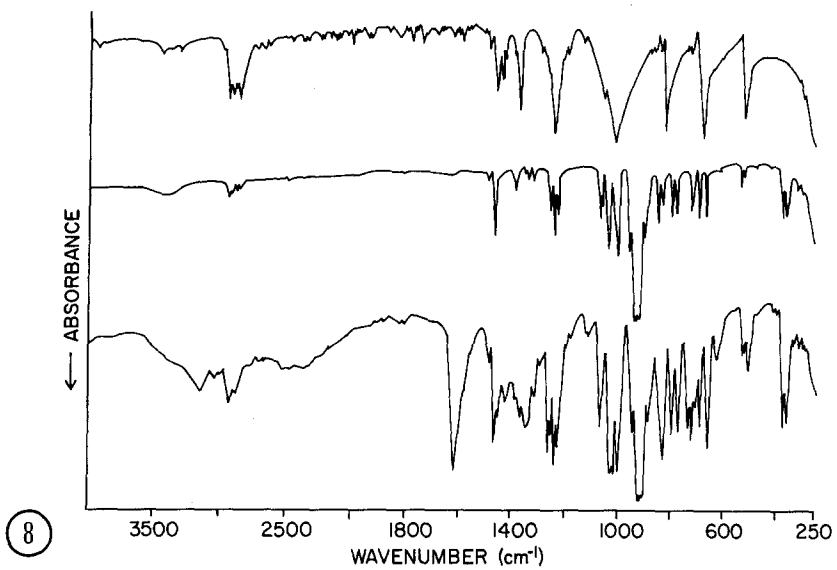


Fig. 5. Hydrolysis of potassium osmiamate resulting in OsO_4

Fig. 6. Visible absorption spectra of octavalent osmium compounds obtained with a Perkin-Elmer Hitachi Model 124 double beam UV-visible spectrophotometer. All solutions were diluted with the required quantity of distilled water to get in the recorder range. Relative absorbancies are not to be taken from this figure. The curves have been staggered so that they would not overlap and, therefore, would be easier to read: They suggest that both osmeth and potassium osmiamate yield OsO_4 in solution. A. 0.20% OsO_4 solution; B. 0.25% osmeth solution; C. 0.23% potassium osmiamate solution heated gently on a steam bath 5 min; D. Same, heated 2 min; E. 0.23% potassium osmiamate solution





Figs. 7 and 8. Infrared absorption spectra of octavalent osmium compounds and methenamine obtained in a Perkin-Elmer Model 457 double beam grating spectrophotometer. All samples were prepared by the potassium bromide pressed disc technique

Fig. 7. Upper, OsO_4; middle, potassium osmiamate; lower, osmium tetroxide-pyridine addition compound

Fig. 8. Upper, methenamine; middle, osmeth; lower, osmium tetroxide-methenamine mandelate addition compound. Note the complex spectrum of osmeth (Fig. 8, middle) relative to the simple spectrum of OsO_4 (Fig. 7, upper). This results from the lack of symmetry of the osmeth molecule, reflects its polarity and its relative inability to penetrate biomembranes

reaction in weak acid with thiourea could result from its hydrolysis to give OsO_4 (Fig. 5). The faint odor of OsO_4 noted when potassium osmiamate was dissolved in 2N H_2SO_4 could have been due to this reaction. Support for this hydrolytic reaction may be obtained by inspection of the visible absorption spectra in Figure 6. The fact that the decomposition was slow would tend to limit its effectiveness as an OsO_4 substitute. The osmium tetroxide-ammonium hydroxide addition complex seemed to offer little advantage, from the point of view of safety, over the use of OsO_4. Its fumes were offensive and irritating and it (or its decomposition products) sublimed readily. It was therefore not evaluated further. The osmium tetroxide-pyridine addition compound was much more stable than the previous addition compound, but since it sublimed so readily at room temperature, it too, was no longer considered.

Characterization of Octavalent Osmium Compounds

Most of the data concerning the identification of the structures of the osmium compounds described under Materials and Methods was included with the preparative directions. The following chemical properties receive further emphasis here, especially as they relate to cytochemical reactions and biologic preservation.

Visible Absorption Spectra. The octavalent nature of osmium in osmiamates is demonstrated by the immediate reaction with acidic thiourea reagent. This has been discussed by Sauerbrunn and Sandell (1953). Although it is generally believed that osmiamates are stable in alkali and that in acid they lose all of their oxygen (Sidgwick, 1950), it would be expected that they undergo hydrolysis to give OsO_4 in weak acid (Milas, 1959). Curves A, and C to E, of Figure 6 indeed suggest that gentle heating may convert potassium osmiamate to OsO_4. Dissolution of potassium osmiamate in 2 N H_2SO_4 also resulted in the slow liberation of OsO_4, but decomposition ensued. Curves B and A suggest that osmeth releases OsO_4 upon solution in water, but a quantitative relationship should not be inferred.

Infrared Absorption Spectra. The spectrum of potassium osmiamate (Fig. 7) is in agreement with previously published studies (Griffith, 1965b; Lewis and Wilkinson, 1958). A satisfactory spectrum could not be obtained of the osmium tetroxide-ammonium hydroxide addition compound, $OsO_4 \cdot 2\ NH_4OH$.

The spectrum of the osmium tetroxide-pyridine addition compound (Fig. 7) shows a singlet at 915 cm^{-1} due to the Os—O stretching mode instead of the doublet seen in the same Figure for Os—O stretch of OsO_4 at 945 cm^{-1} and 968 cm^{-1}. The remaining bands displayed by the spectrum are principally due to vibrational-rotational modes associated with features of the 6-membered heteroaromatic pyridine ring.

Osmeth (Fig. 8) displays a triplet at 908–923 cm^{-1} due to Os—O stretch and a strong sharp band at 1,030 cm^{-1} reminiscent of the Os—N stretching vibration (at 1,023 cm^{-1}) observed with potassium osmiamate. The very weak band observed at 325 cm^{-1} in the OsO_4 spectrum (Fig. 7) is shifted and rep-

resented by a somewhat stronger doublet (353, 371 cm^{-1}). The relatively complex spectrum of osmeth reflects its lack of symmetry; the rather symmetrical OsO_4 and potassium osmiamate have relatively simple spectra (Fig. 7).

The 353, 371 cm^{-1} doublet is stronger in the osmium tetroxide-methenamine mandelate addition compound (Fig. 8) than in osmeth. Most of the other bands of the osmeth spectrum are modified in width, intensity and shape but not shifted in this complex which was prepared in chloroform. The very prominent band at 1,610 cm^{-1} (absent in methenamine and osmeth spectra) is due to the antisymmetrical stretching mode of the carboxylate anion of methenamine mandelate and is evidence that it exists as a true salt in the absence of water. When the synthesis of this addition compound was attempted in aqueous media, a product was obtained which lacked this very intense band and whose spectrum otherwise resembled that of osmeth. This suggests that the desired complex may only be prepared in nonaqueous media.

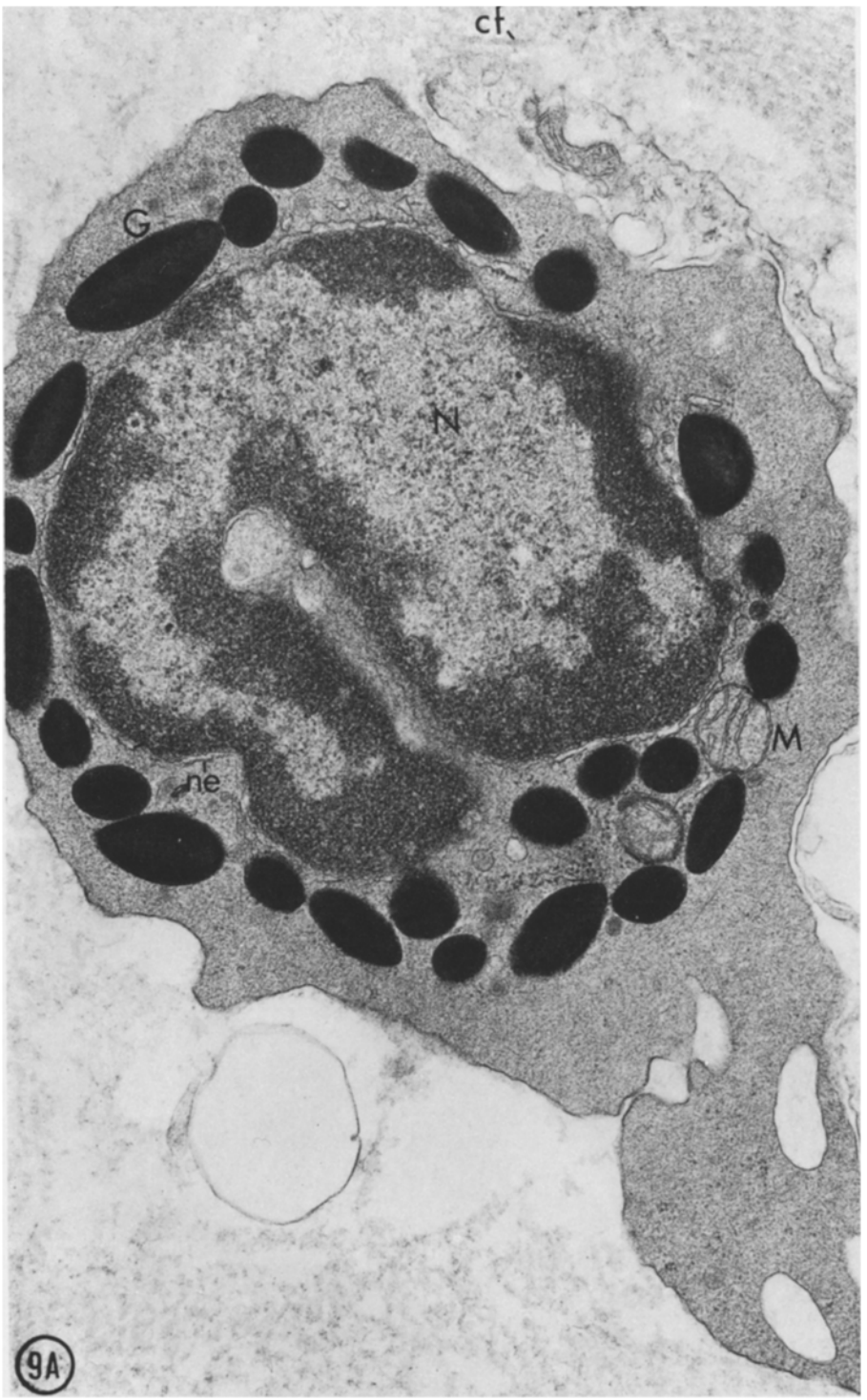
A small amount of light tan precipitate was obtained from a solution of osmeth that had been kept for several months at room temperature. This precipitate gave a spectrum (obtained from a micro KBr disc) which was identical with that of methenamine.

Titrimetric Characterization of Osmeth Solutions. Potentiometric and iodometric titration were performed on 0.25% osmeth solutions and 0.25% OsO_4 solutions prepared as described above. Preliminary studies on OsO_4 solutions indicated no interference by the small amount of dimethylformamide in either titrimetric assay. Values for osmium were consistently low in the analysis of osmeth solutions (Table 3). These stock solutions, moreover, were always losing OsO_4. The values do confirm, however, the availability of OsO_4 in osmeth solutions.

Fig. 9A–C. Tissue eosinophils in blocks of rat uterus prefixed with glutaraldehyde. ×27,500. **A** 0.25% OsO_4; **B** and **C** 0.25% osmeth

Fig. 9A demonstrates that 0.25% OsO_4 yields satisfactory staining of membranes and overall contrast for ultrastructural analysis. However the high opacity to electrons exhibited by the specific granules (*G*) does not allow easy recognition of their protein crystals and matrix as in Figure 9C. In certain areas of osmeth fixed tissue similar high contrast and favorable representation of ultrastructural detail can be obtained (**Fig. 9B**). In areas of osmeth-fixed material that presumably had not blackened as shown in **Figure 9C** the cytoplasmic membranes lack contrast. However, the distinction between the protein crystal (*C*) and matrix (*M*) of the granules can be made more easily than in the well-osmicated cell. The electron opacity that exists here was most likely created by en bloc staining with uranyl acetate or by staining of ultrathin sections with lead and uranium. *N* nucleus; *G* specific cytoplasmic granules; *c* protein crystal of the granules; *m* matrix of granules; *M* mitochondria; *ne* nuclear envelope; *Go* Golgi complex; *cf* collagen fibrils

Fig. 10A and B. Skeletal muscle fibers, in blocks of rat diaphragm, prefixed in glutaraldehyde and then osmicated. ×27,500. **A** 0.25% OsO_4, red fiber; **B** 0.25% osmeth, intermediate fiber. Comparison of these muscle fibers indicates that equally good ultrastructural preservation and contrast can be obtained through osmication of glutaraldehyde-fixed tissue with OsO_4 and osmeth. Z lines (*Z*) are strongly electron opaque. Thick filaments (*A* band) and thin filaments (*I* band) are evident. Membranes of the mitochondria (*M*) and triads (*T*) are easily delineated


ct
G
N
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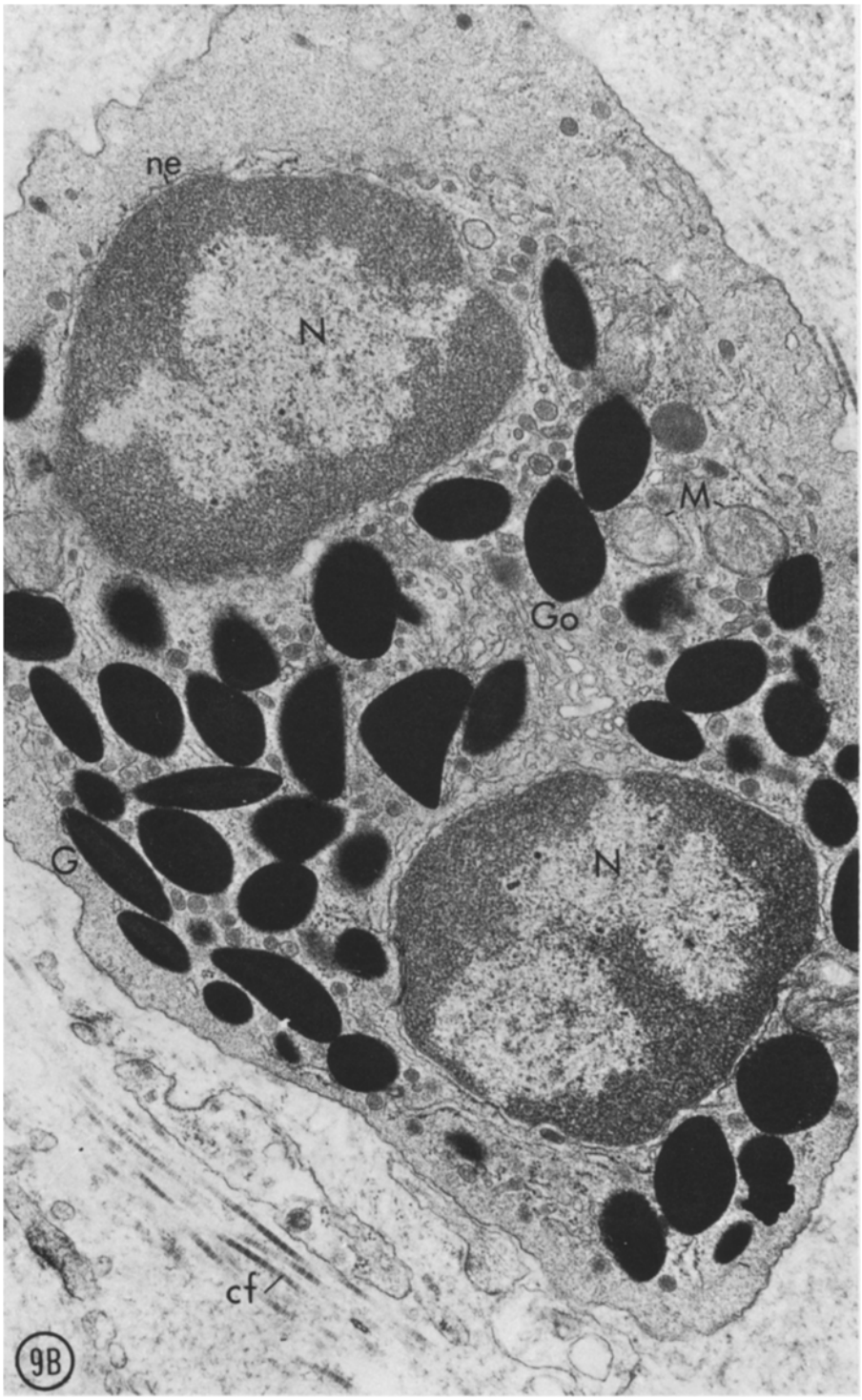
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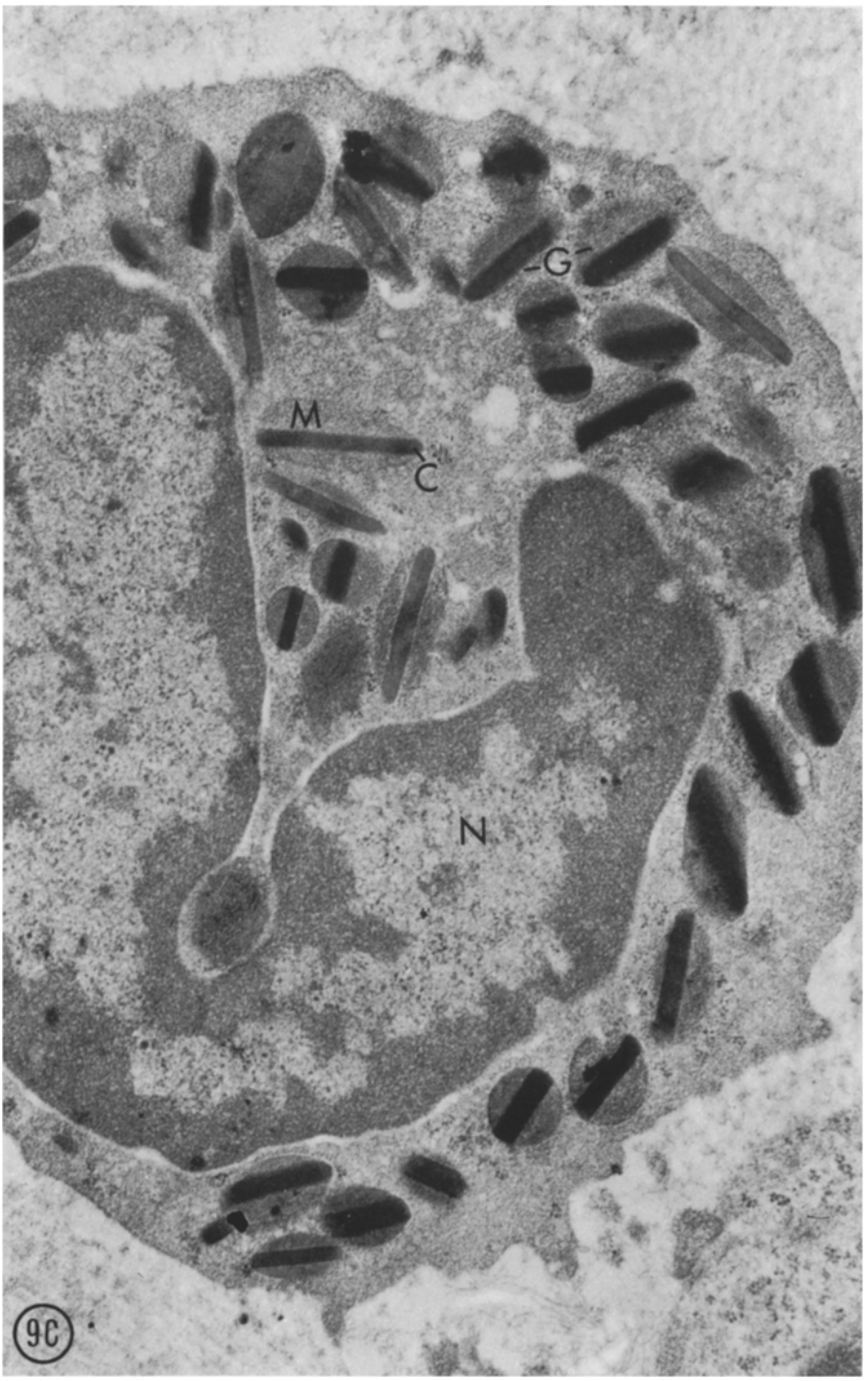
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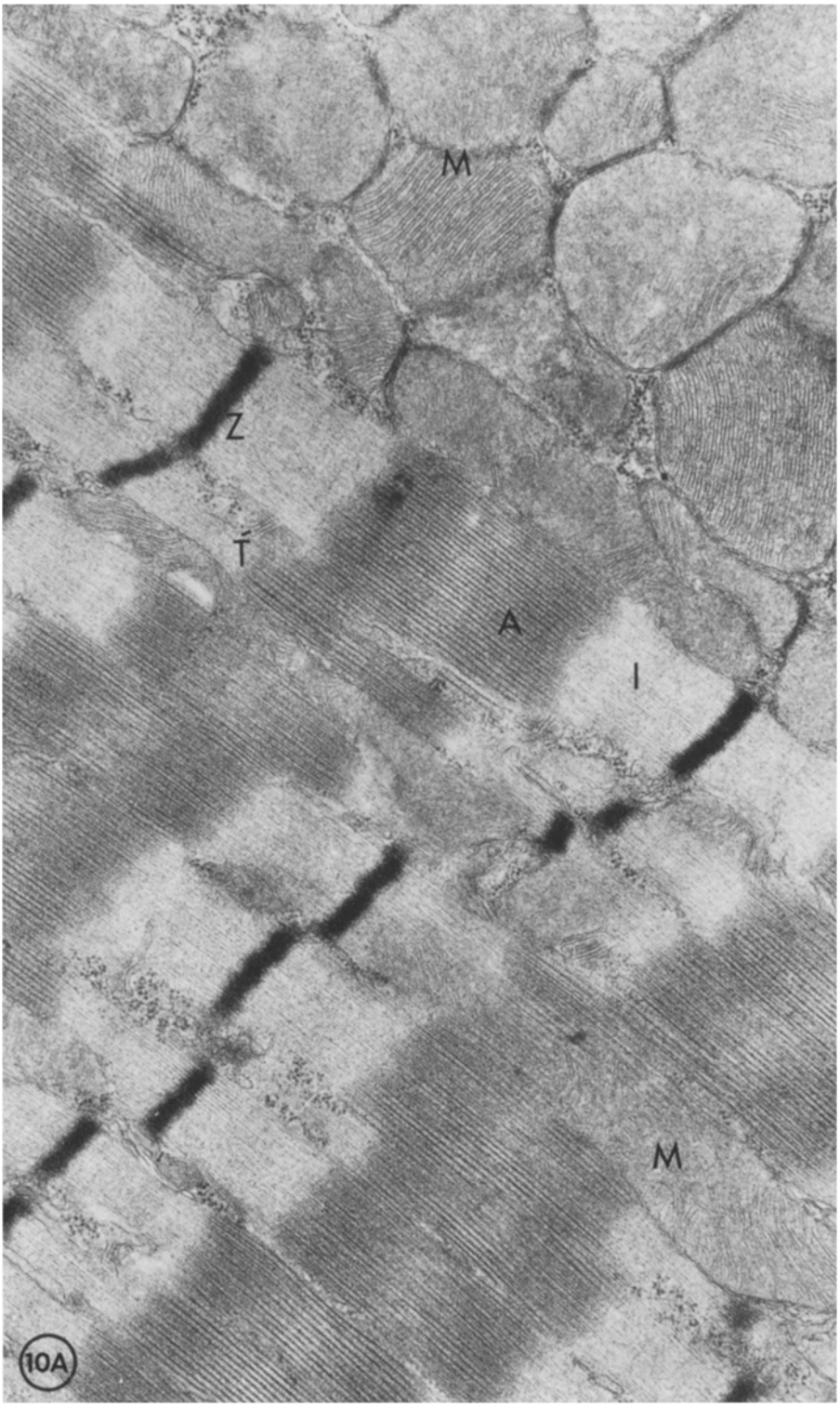
M
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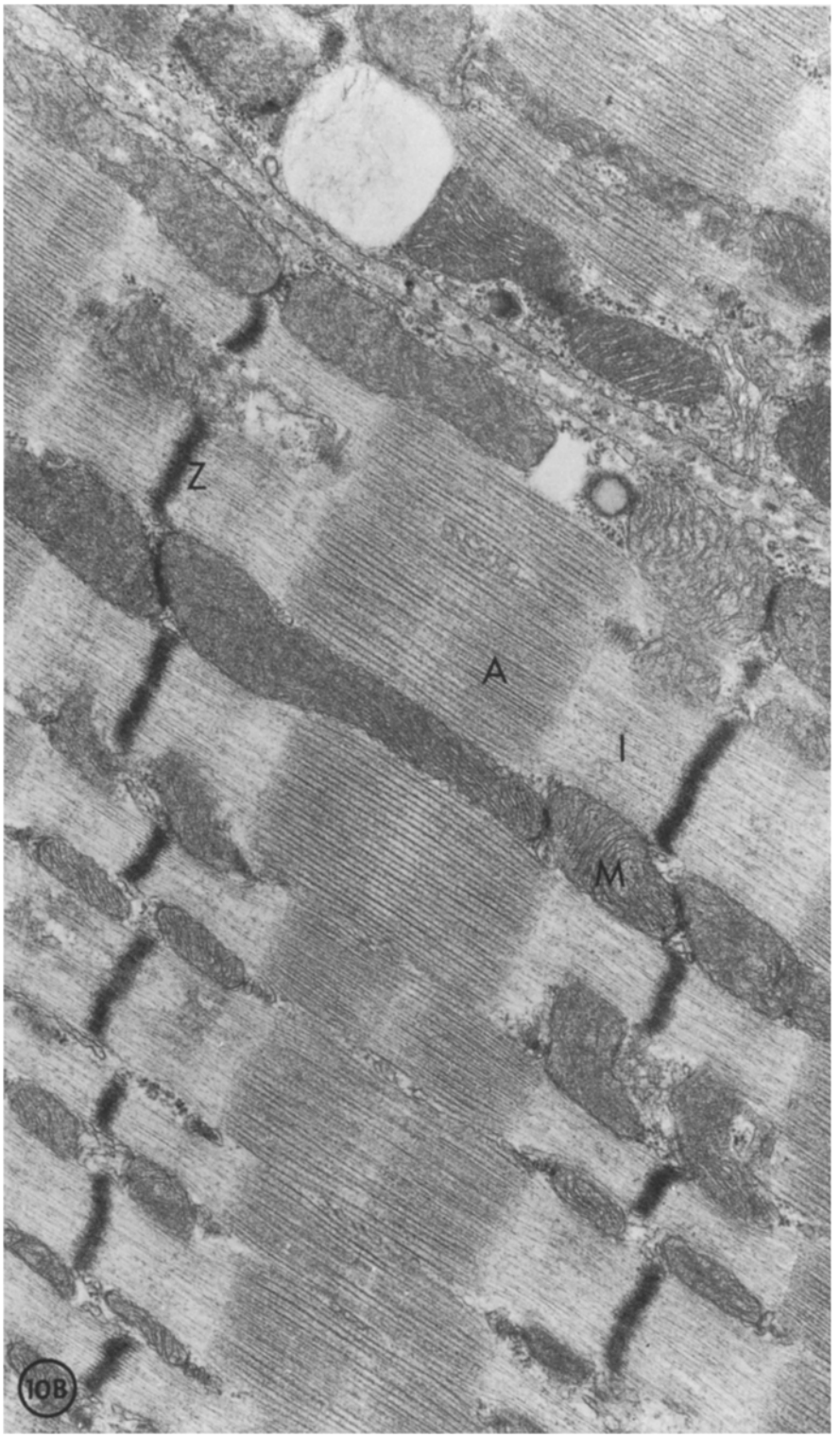
M
10A


Z
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10B

Table 3. Titrimetric analysis of 0.25% osmeth and osmium tetroxide solutions

	Percent[a] osmium by titration			
	Potentiometric		Iodometric	
	Calcd.	Found	Calcd.	Found
Osmeth Solution, 0.25%	0.1466	0.1394	0.1466	0.1332
OsO_4 Solution, 0.25%	0.1871	0.1725	0.1871	0.1598

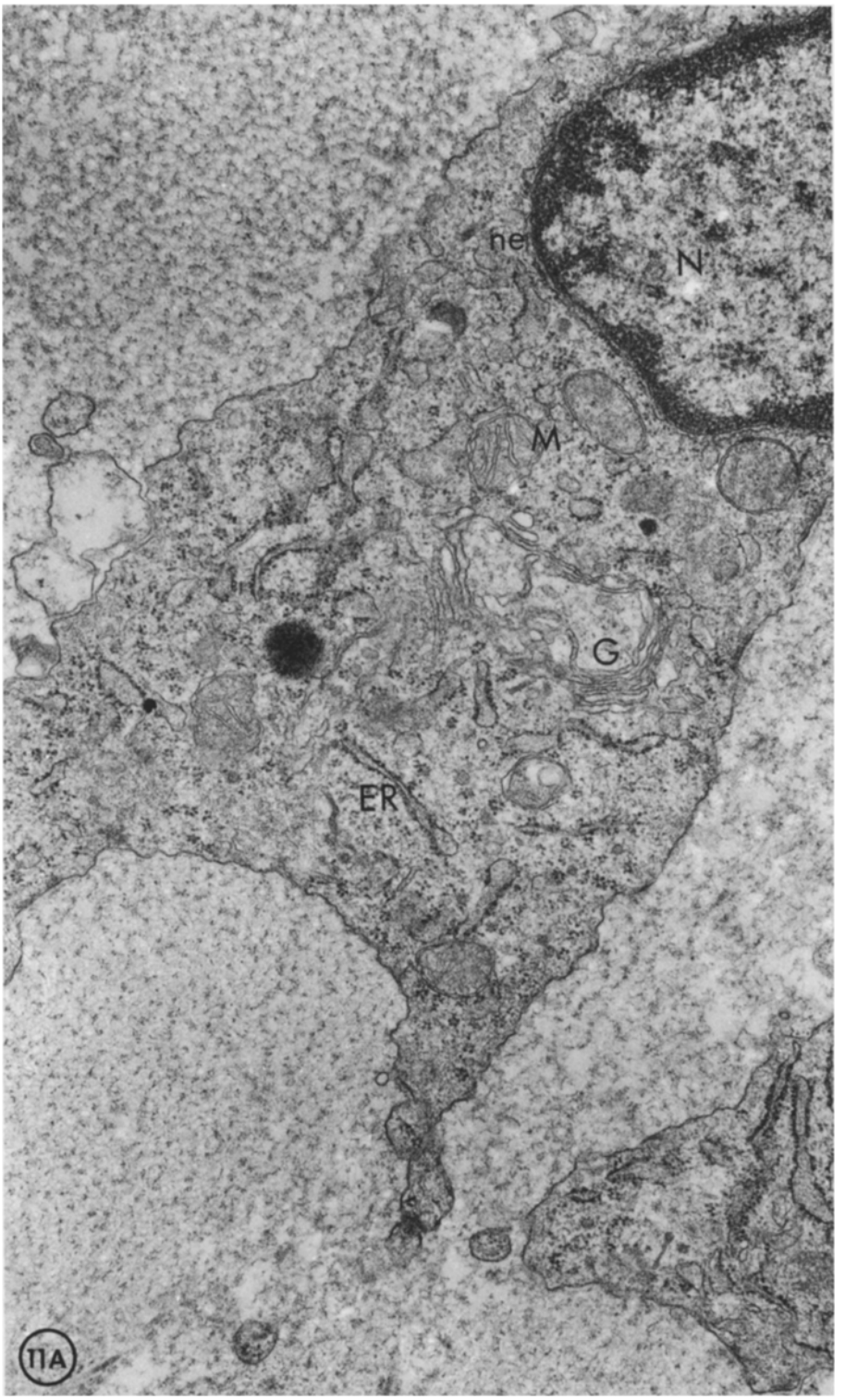
[a] Each value tabulated is the mean of at least 2 determinations on aliquots of the same freshly prepared stock solution

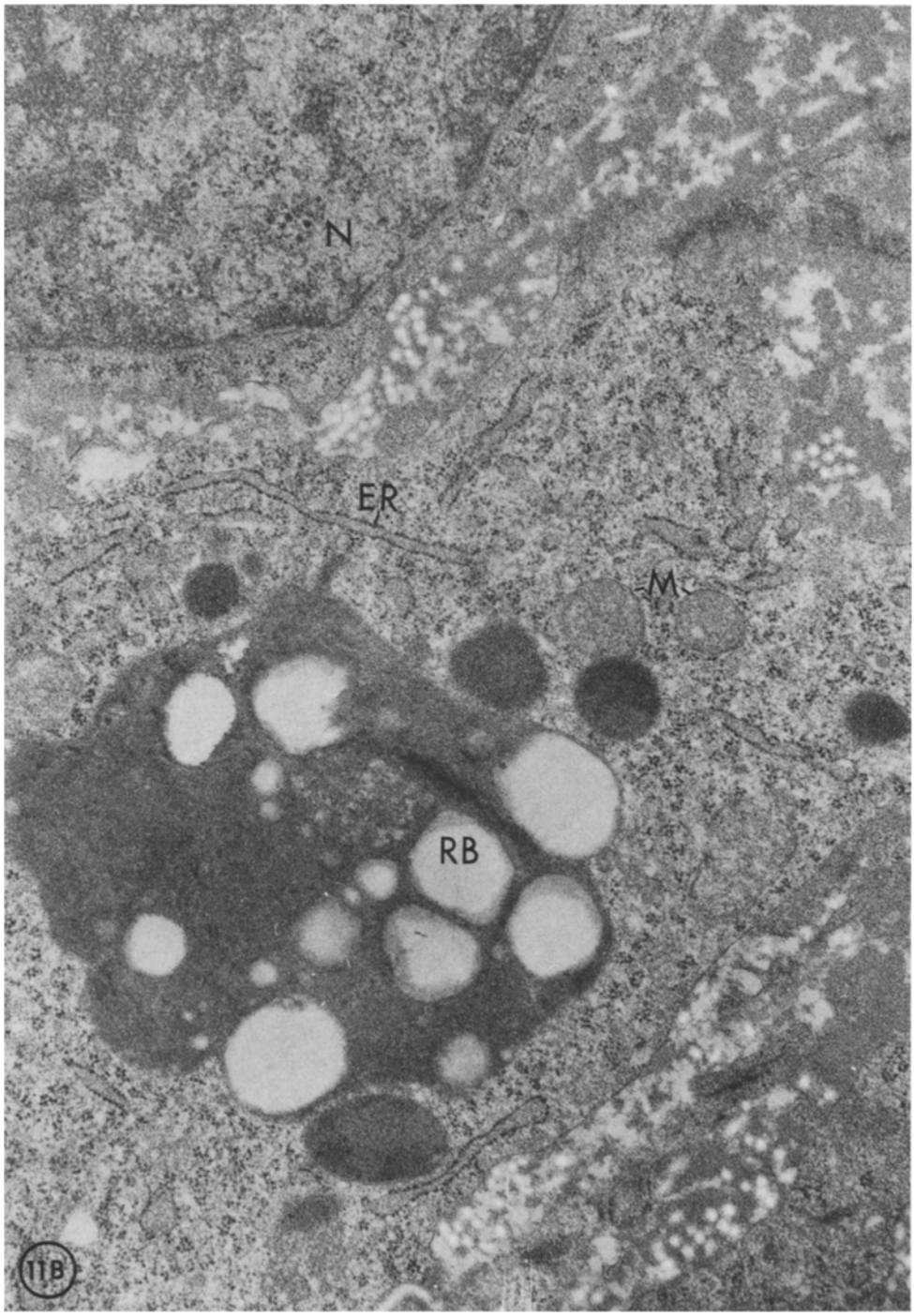
Effectiveness of Osmeth in Double Fixation for Ultrastructural Preservation

0.25% osmeth was less effective than 0.2% OsO_4 in blackening 1 mm^3 blocks of glutaraldehyde-fixed tissues (Figs. 15, 16). After 1 or 2 h of osmication, the OsO_4 blocks were black whereas those in osmeth solutions remained yellow. After standing overnight in osmeth solutions, most tissues blackened superficially but their centers were not osmicated. Furthermore, the OsO_4 solutions blackened with time whereas the osmeth solutions did not. These experiments indicate that the generation (release) of OsO_4 from osmeth occurs slowly and steadily and thus only the surfaces of the blocks will have sufficient opacity to electrons to create ultrastructural contrasts. However, our findings indicate that osmeth creates contrasts in the surface of the block that are comparable to that of OsO_4 solutions. Furthermore, our experiments demonstrate that adequate osmication can be achieved by osmeth using 25–50 μm chopper sections.

Despite this limitation related to penetration, good ultrastructural preservation and contrast is achieved through the use of osmeth. Superficial areas of such blocks yield ultrastructural images of several cell types, such as those shown in Figures 9B, 10B, and 11A, which indicate preservation and contrast favorable for study. The best preservation in osmeth solutions corresponds closely to that obtained in 0.25% OsO_4 (Figs. 9A, 10A, and 12). Thus, where osmeth acts, the results are comparable to that of OsO_4. Furthermore, as a

Fig. 11A, B, and C. Stromal cells, in blocks of rat uterus. ×27,500. **A** Fibroblast; **B** Macrophage; **C** Fibroblast. These three cells in a glutaraldehyde-fixed block were exposed to 0.25% osmeth and reacted differentially in terms of the staining of membranes. In **A**, the membranes of the mitochondria (*M*), Golgi complex (*G*), and rough endoplasmic reticulum (*ER*), and nuclear envelope (*ne*) are distinct. In **11B**, the membranes of the mitochondria (*M*) and rough endoplasmic reticulum (*ER*) can be barely discerned. The large residual body (*RB*) and other dense bodies, probably part of the lysosomal system, have a high electron opacity. In **C**, the position of the rough endoplasmic reticulum (*ER*) can be delineated through the alignment of the ribosomes which have strong opacity to electrons. Grey cytoplasmic bodies (*x*) may represent mitochondria or components of the lysosomal system. Cytoplasmic filaments (*f*), probably microfilaments, are recognizable. *N*, nucleus


he
N
M
G
ER
11A


N
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M
RB
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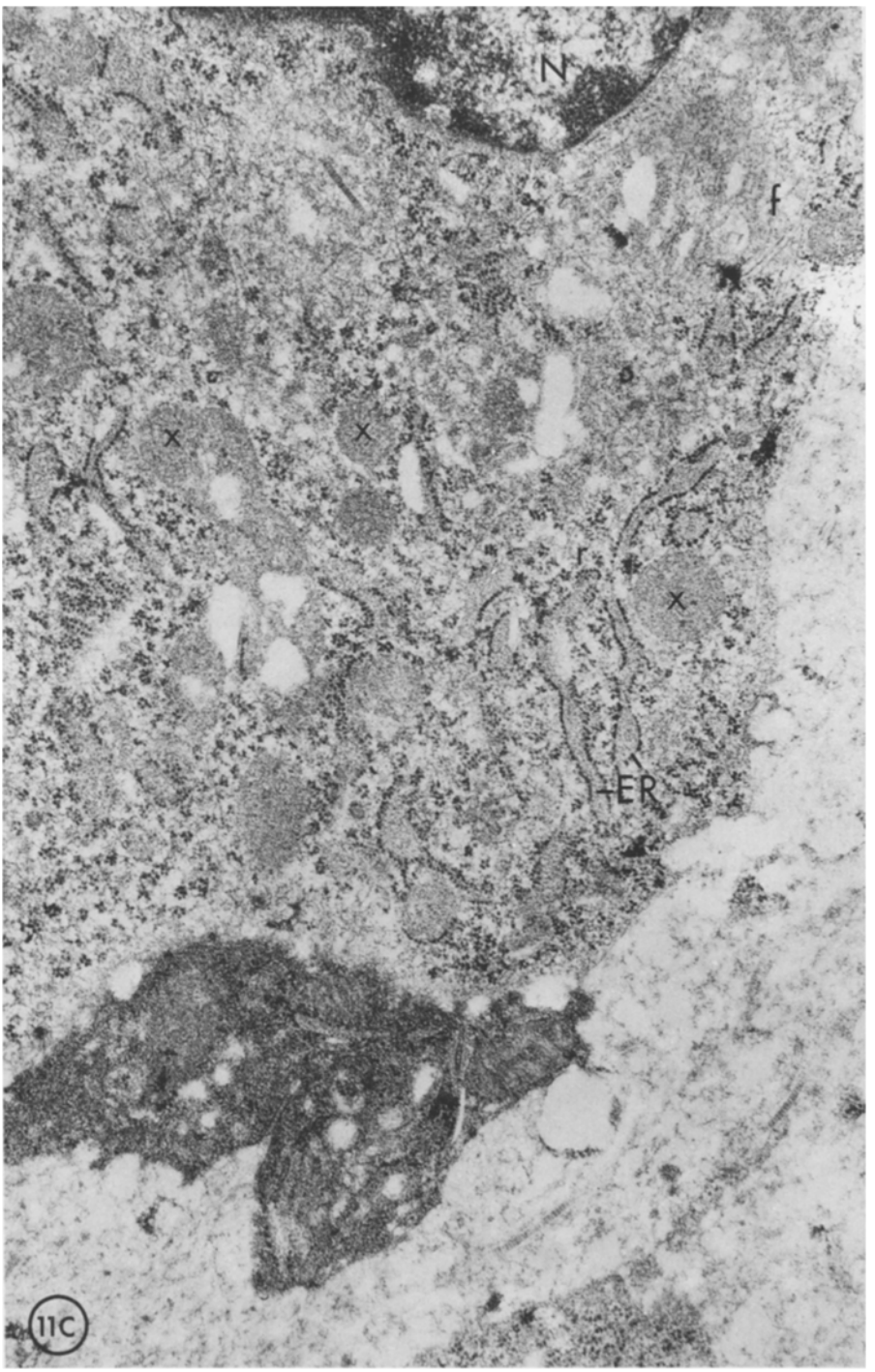

N
f
X
X
X
ER
11C

corollary of this comparison, it seems likely that 0.25% OsO_4 solutions may be used for ultrastructural preservation in place of the usual 1 or 2% OsO_4 fixatives which are routinely used. This reduction in concentration could result in considerable economy in the use of the relatively expensive OsO_4 reagent.

It is important to be aware that the deeper portions of the osmeth fixed blocks may suffer from lack of contrast, especially because of the absence of staining in the membranes (See Figs. 9C, 10B, 10C, and 16). These preparations (Figs. 9C, 10B, and 10C) allow one to visualize the degree of contrast that is imparted by en bloc staining with uranyl acetate followed by staining ultra-thin sections with lead and uranium.

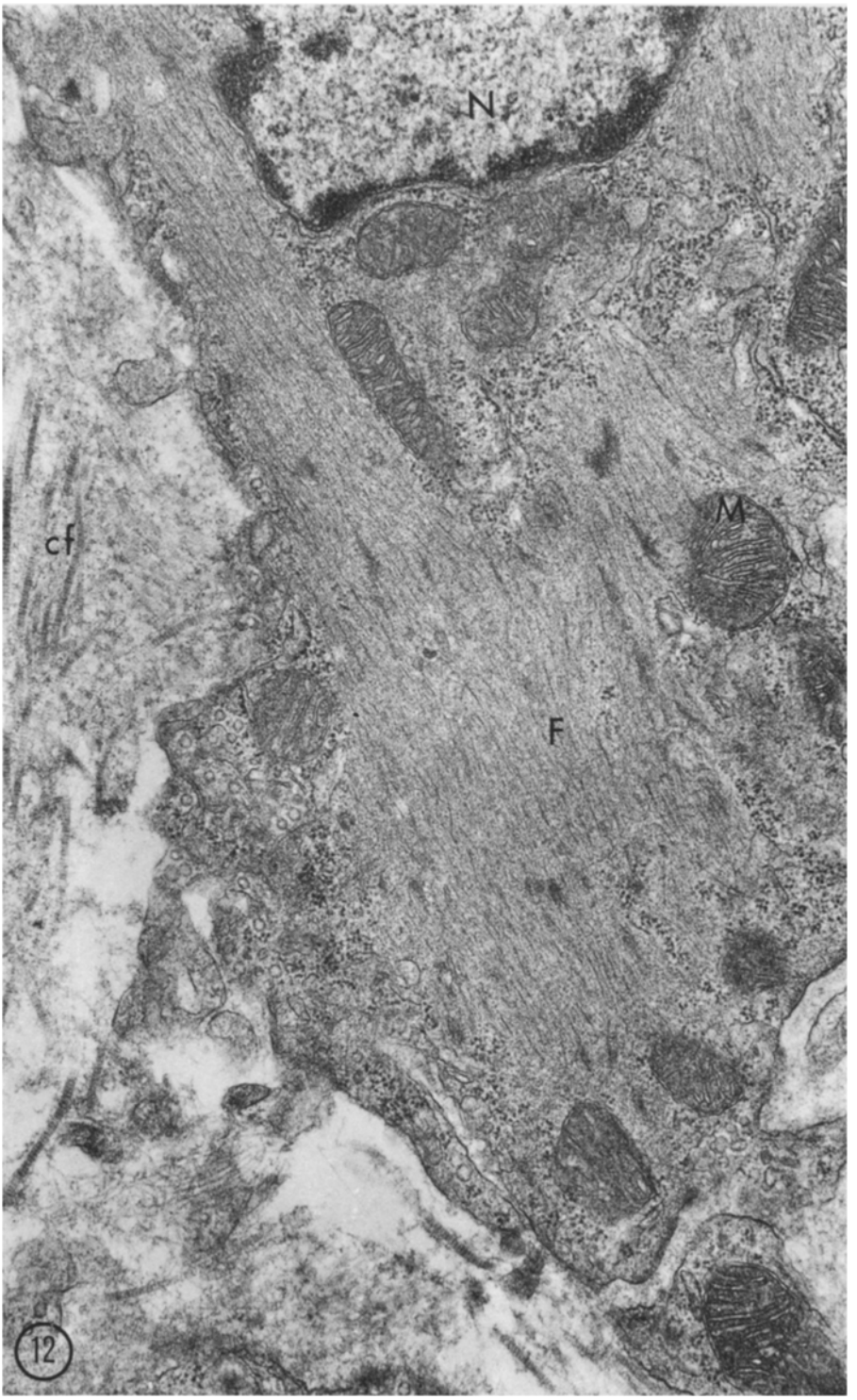
Osmeth in Cytochemical Reactions Resulting in Osmium Blacks

When equivalent concentrations of osmeth and OsO_4 solutions were used (0.25% osmeth and 0.20% OsO_4) on cryostat sections, there was no detectable difference in the intensity of the osmium blacks formed with the indomine polymers resulting from oxidation of DAB. Thus in the demonstration of non-specific esterase by catalytic osmiophilic polymer generation (Hanker et al., 1972a, b), osmeth may be substituted for OsO_4 (Fig. 13). Likewise in the demonstration of horseradish peroxidase tracer by the procedure of Graham and Karnovsky (1966a, b) or catalase by the procedure of Novikoff and Goldfischer (1969) (Fig. 14) no differences were noted when osmeth solutions of equivalent concentration with respect to OsO_4 were used.

Discussion

Osmeth has two drawbacks that may tend to limit its use as an OsO_4 substitute; its low solubility and, secondly, the relative lack of penetration of tissue blocks must be considered in its application. The latter limitation was not operative however, when 0.25% osmeth solutions were compared with 0.20% OsO_4 solutions in osmium black cytochemistry on cryostat sections or on 25–50 μm chopper sections. When osmeth was used as a fixative, better penetration was also achieved in the thin chopper sections than in tissue blocks. A possible advantage of osmeth solutions in ultrastructural fixation is that its solutions do not blacken as do OsO_4 solutions (Fig. 15). This could tend to limit spurious osmium black deposits as a result of postfixation of aldehyde-fixed tissues (Hopwood, 1970). In other words, osmeth may allow slow local generation of OsO_4 that would react with macromolecular constituents of the tissues. The dissociation of osmeth

Fig. 12. Smooth muscle cell, in blocks of rat myometrium. ×27,500. Double fixation, using only 0.25% OsO_4, yielded highly satisfactory preservation and contrast in the organelles and filaments (*F*) of the smooth muscle cells as well as in the extracellular components. *N* nucleus; *M* mitochondria; *cf* collagen fibrils. This representation, along with Figures 9A and 10A, suggest that this low concentration of OsO_4, used after glutaraldehyde stabilization of macromolecular form, may be adequate for ultrastructural analysis of biological form


N
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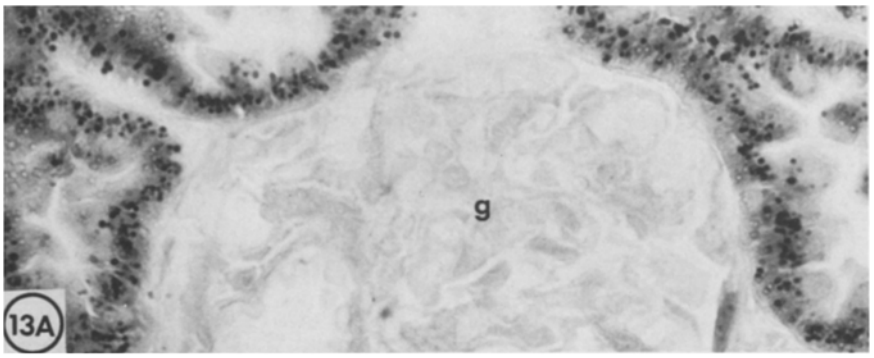
F
12


g
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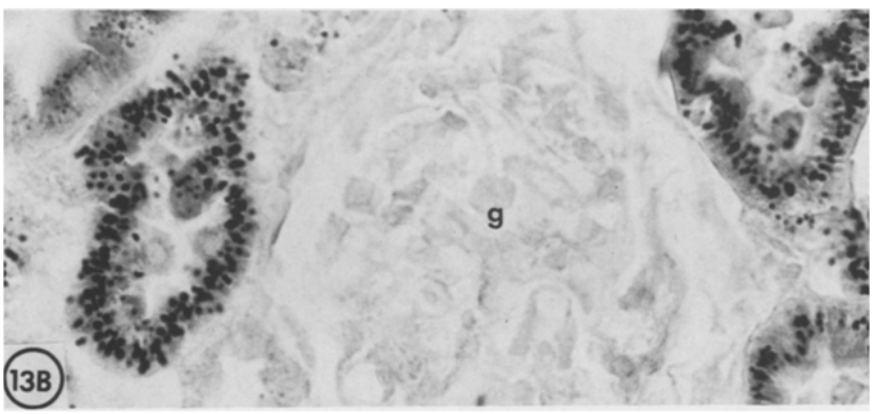
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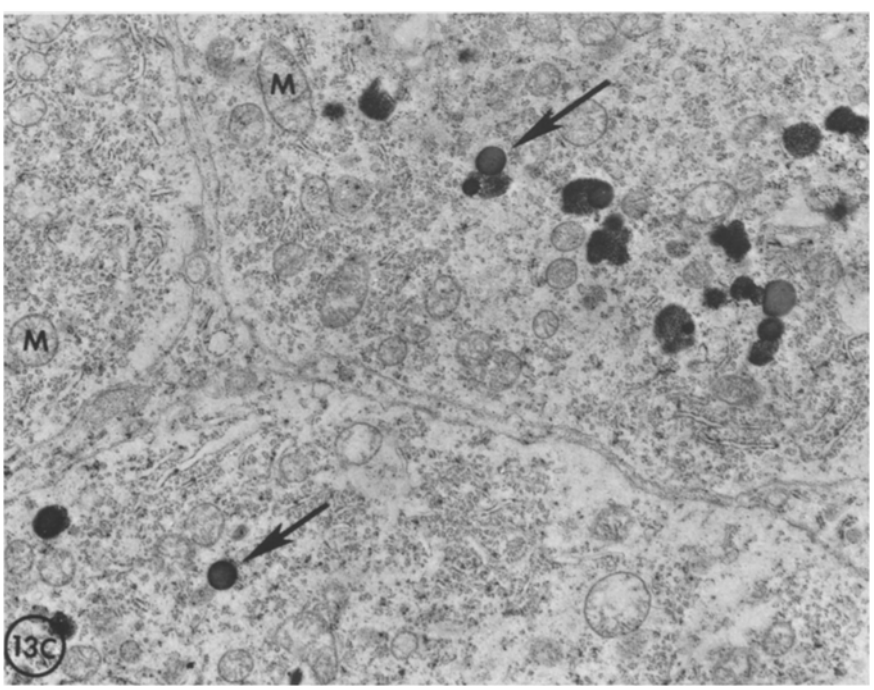

M
M
13C

to give OsO_4 is probably stepwise and may be represented by the expression:

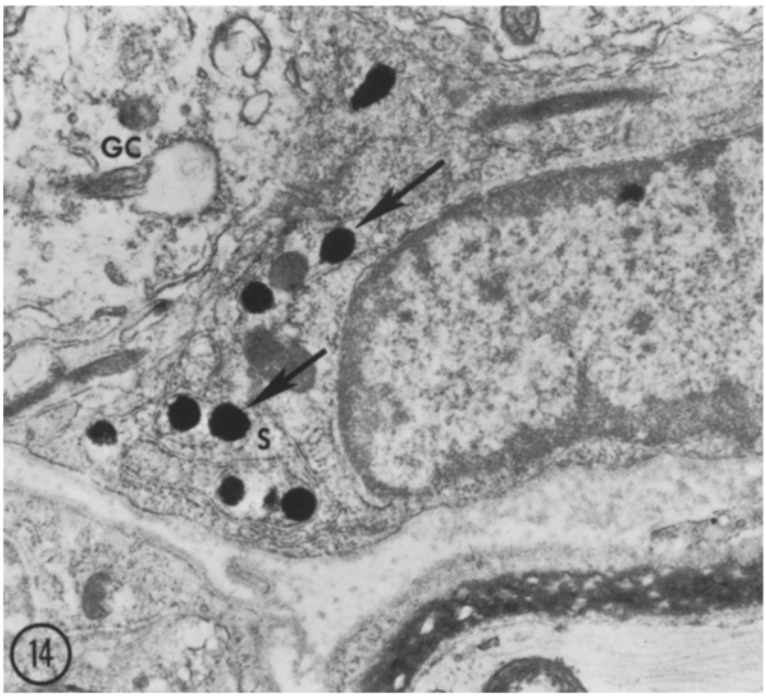
$$\text{methenamine} \cdot 2\ OsO_4 \rightleftharpoons \text{methenamine} \cdot OsO_4 + OsO_4.$$

The relative size of the arrows suggests that most of the dissolved osmeth is not dissociated. This is congruous with our observation of the rather poor penetrability of tissue blocks to osmeth (Fig. 16). On the other hand, this expression also accounts for the especial utility of osmeth in cytochemical reactions resulting in osmium black formation. Ligands which are better competitors than methenamine such as the 3,3′-diaminobenzidine oxidation product or thiocarbohydrazide were found to react very readily with osmeth solutions. In fact, no difference could be noted when equivalent concentrations of osmeth (0.25%) and OsO_4 (0.20%) were used for osmication of these substances. The greater drawback to the complete replacement of OsO_4 by osmeth is not its incomplete dissociation but rather its more limited solubility in water. The greater solubility of the osmium tetroxide-methenamine mandelate addition complex over osmeth is noteworthy. The lower osmium content, however, could negate this advantage. The properties of this compound in fixation and in cytochemical reactions were not evaluated as completely as those of osmeth.

No attempts were made in these studies to compare osmeth with OsO_4 as a primary fixative. All of our studies of fixation by osmeth solutions were performed on tissues which had been prefixed in aldehydes. These studies, in which 0.2% OsO_4 solutions were compared with 0.25% osmeth solutions, showed that 0.2% OsO_4 is adequate for most postfixation purposes. It has long been felt by some investigators that the concentrations of OsO_4 solutions used as biological fixatives might be excessive. Our electron micrographs (Figs. 9A, 10A and 12) are in accord with the suggestions of the adequacy of low concentrations of OsO_4 in fixation.

In this regard Shigenaka et al. (1973) investigated the fine structural preservation of various hypotrichous ciliates with primary OsO_4 fixative solutions varying in concentration from 0.1–2%. Euplotes were fixed satisfactorily even at 0.1% OsO_4 concentration, and it might be expected that many other tissues could be fixed adequately with such weak OsO_4 solutions. Although other ciliates showed cytolysis especially at the lateral edges in 0.1% solution and slight shrinkage at 0.5% solution, no attempt was made to compensate for the change in osmolality of the weaker OsO_4 solutions in those studies. In the cytochemical

Fig. 13A–C. Lysosomal nonspecific esterase localized in rat tissues. Catalytic osmiophilic polymer generation method (Hanker et al., 1972b) employing 2-thiolacetoxybenzanilide (TAB)[1] as substrate. The osmiophilic polymer produced on DAB oxidation at the sites of enzymic activity was osmicated with 0.25% osmeth or 0.2% OsO_4 as noted[1]. Available from Polysciences, Inc., Paul Valley Industrial Park, Warrington, PA, 18976, USA. **13A** Cryostat section, kidney tubules, calcium-formalin fixation. Nonspecific esterase activity is limited to lysosomes in the tubular epithelium. Note that glomerulus (*g*) does not contain reactive lysosomes. Osmication with 0.2% OsO_4. ×900. **13B** Adjacent section. Same treatment with the exception of osmication with 0.25% osmeth. ×900. **13C** Electron micrograph demonstrating nonspecific esterase in the lysosomes (arrows) of trigeminal ganglion cells. Note absence of reaction product in the mitochondria (*M*). Fixation with 3% glutaraldehyde, 2% depolymerized paraformaldehyde. Osmication with 0.25% osmeth. ×10,000



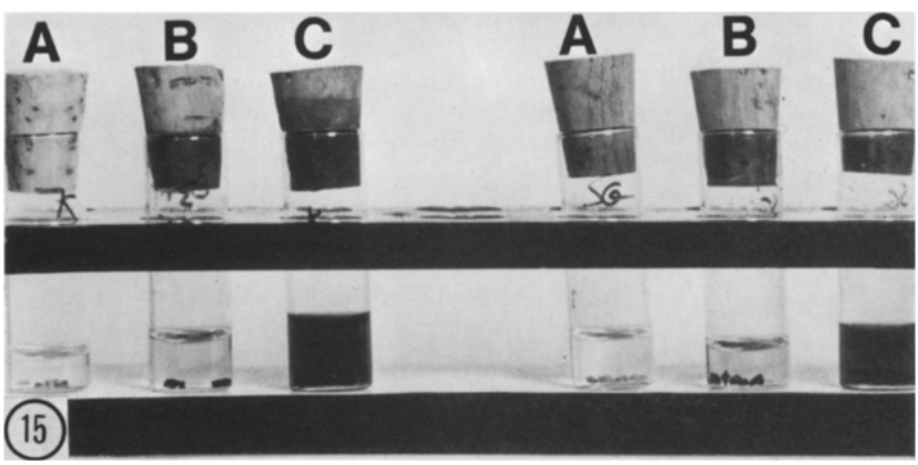


Fig. 14. Electron micrograph of ultrathin section of a chopper section of mouse grigeminal ganglion fixed in glutaraldehyde and reacted for catalase activity with diaminobenzidine (DAB)[1] and H_2O_2 according to the procedure of Novikoff and Goldfischer (1969). Microbodies (arrows) are prominent in sheath cells, shown here (*S*) but not in adjacent ganglion neurons (*G C*). Note poor preservation of myelin. Osmication with 0.25% osmeth[1]. ×9,500[1]. Available from Polysciences, Inc., Warrington, PA 18976, USA

Fig. 15. Photograph of tissue blocks of mouse kidney (left) and submandibular gland (right) fixed in 2.5% glutaraldehyde buffered to pH 7.4 with 0.1 M cacodylate buffer, rinsed in buffer and then osmicated with either 0.2% OsO_4 or 0.25% osmeth 72 h. The glutaraldehyde-fixed tissues in *A* vials have been kept in buffer rinse only. *B* vials in 0.25% osmeth. *C* vials in 0.20% OsO_4. Note that osmeth has blackened the surface of the kidney blocks more than the blocks of the submandibular gland. Note especially blackness of the OsO_4 solutions and the relative clarity of the osmeth solutions

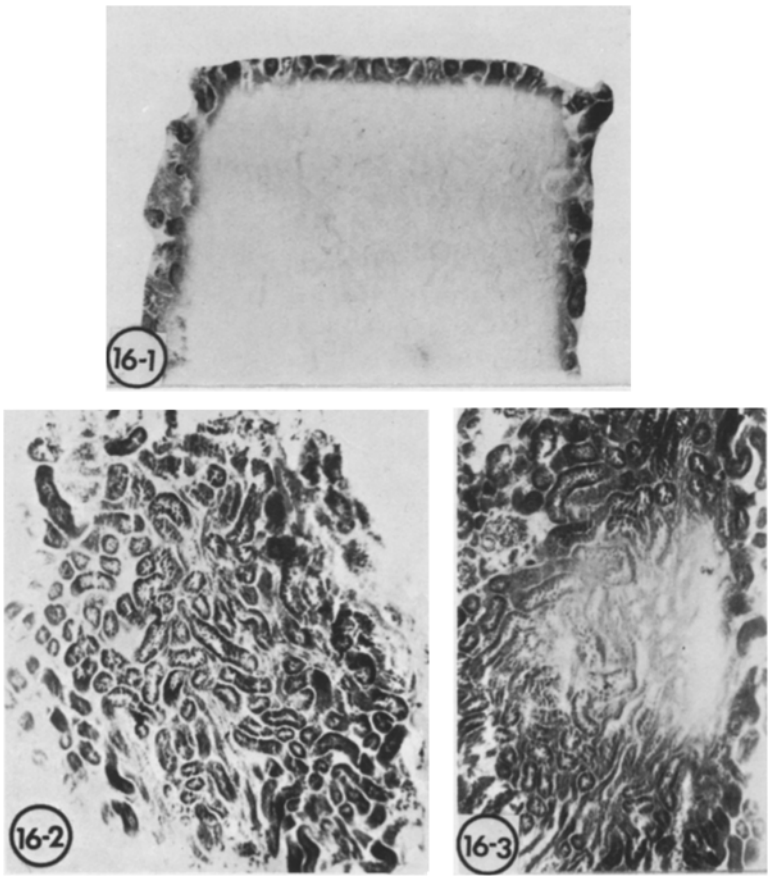


Fig. 16. Photomicrographs of 10 μ cryostat sections of blocks of mouse kidney from vials *B* and *C* of Figure 15. ×100. Section 1 is from a block fixed in vial *B* (0.25% osmeth). Sections 2 and 3 are from blocks fixed in vial *C* (0.20% OsO_4). Section 2 is taken closer to the surface of the block than section 3 which is taken from approximately the middle

studies resulting in osmium black formation discussed below, no essential differences were noted when osmeth solutions (0.25%) were compared with OsO_4 solutions (0.2%) having equivalents of osmium.

Several factors must be considered in any explanation of why osmeth solutions appear to be as satisfactory as OsO_4 solutions of equivalent concentration in osmium black cytochemistry although they are not nearly as effective in the fixation of tissue blocks. First, all of the former studies were performed either on cryostat sections or on 25–50 μm chopped sections which offer less obstacle to penetration than the 1 mm^3 tissue blocks. This possibility is supported by the better penetration observed in chopper sections. Another factor could be that the osmiophilic end products of the cytochemical reactions are relatively much more osmiophilic than either methenamine or reactive groupings

in tissue constituents. These end products of the cytochemical reactions, therefore, might effectively compete with methenamine for the OsO_4 produced upon the dissociation of osmeth in solution and, indeed, appear to facilitate its dissociation. The reactive groupings in tissue, on the other hand, may be unable to compete effectively with methenamine for OsO_4. In this situation, the effective concentration of OsO_4 would be greatly diminished.

Still, another important consideration stems from the fact that the dissociation of osmeth to OsO_4 in aqueous solution (Fig. 2) is reversible and incomplete. Although each Os–O bond of OsO_4 is polarized ($\overset{8+}{Os}\rightarrow\overset{8-}{O}$), the OsO_4 molecule is tetrahedral (Woodward and Roberts, 1956), perfectly symmetrical and, therefore, as a whole nonpolar. As a consequence it could be expected to readily penetrate charged tissue, cell, and organelle surfaces. The osmeth molecule, on the other hand, from infrared spectroscopy studies, appears to be much less symmetrical (Fig. 2), and to that extent, polar. It could, therefore, be expected to penetrate biomembranes less readily than OsO_4.

The use of osmeth as a substitute for OsO_4 should be considered in cytochemical reactions involving osmiophilic end products which have been performed on cryostat sections or relatively thin (25–50 μm) chopper sections especially where access to a fume hood is limited. In fixation, the use of osmeth should be restricted to the 25–50 μm chopper sections or isolated cells, or to study the surfaces of blocks.

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