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A Chemical Mechanism for Tissue Staining by Osmium Tetroxide-Ferrocyanide Mixtures[1]

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The presence of $Fe(CN)_6^{-4}$ provides sequential, one-electron reduction pathways for OsO_4. An equilibrium is established containing OsO_4, $Fe(CN)_6^{-4}$, $Fe(CN)_6^{-3}$, $OsO_2(OH)_4^{-4}$, and labile cyano-bridged Os-Fe species containing Os in nominal oxidation states of VIII, VII, and VI. These osmium complexes are chelated by appropriately placed donor atoms in the macromolecular tissue matrix, and chelation facilitates the reduction of osmium *in situ* to lower oxidation states (predominantly IV) that are relatively nonlabile. The greater reactivity and concentration of the Os(VII and VI) intermediates in this system leads to more Os deposition than OsO_4 alone; the chelation is responsible for the immobilization of Os and the observed staining pattern in electron micrographs. Chemical data from model systems and electron micrographs of tissue are presented in support of this mechanism.

A number of reports have recently appeared describing the cytochemical applications of various osmium-ferrocyanide [$Fe(CN)_6^{-4}$]/ferricyanide [$Fe(CN)_6^{-3}$] staining systems: OsO_4—$K_4Fe(CN)_6$ for enhanced staining of membranes in general (14), sarcoplasmic reticulum in particular (25, 29), and glycocalyx (15); and OsO_4-$K_3Fe(CN)_6$ (4) or $K_2OsO_2(OH)_4$—$K_4Fe(CN)_6$ (5, 6) for the selective contrasting of glycogen in muscle and liver. De Bruijn and Den Breejen (7) have identified the C_2-C_3 hydroxyl groups as the reaction site on glycogen, but the mechanism of staining of other cellular components has remained obscure. An important observation about these systems, reported previously (6, 9) and confirmed by us, is that the osmium and iron compounds must be present simultaneously to give enhanced contrast. This indicates that an intermediate formed by the reactants is responsible for the observed enhanced staining. Various authors have referred to the intermediate(s) as "ferrocyanide-reduced osmium" (14), "potassium osmium cyanide complex" or "POCC" (9, 10), and De Bruijn and Den Breejen proposed for its structure a bridged cyano complex of osmium and iron (5, 6): $\{Os^{VI}O_2(OH)_2 \cdot [Fe^{II}(CN)_6]_2 \cdot Os^{VI}O_2(OH)_2\}$. However, such a structure seems unlikely on chemical grounds (3).

In order to rationalize the results reported in the literature for the different Os-Fe mixtures with various substrates and to efficiently extend their use to other systems, we have undertaken the investigation reported herein. Various electrochemical and spectroscopic techniques have been used to chemically characterize the Os-Fe mixtures. The details of the experiments and results of this phase of the study are reported elsewhere[4]; the conclusions are repeated here to help explain the cytochemical results. We have also used Electron Spectroscopy for Chemical Analysis (ESCA) (30) to examine some stained substrates and have synthesized some model compounds to illustrate the proposed mechanism.

METHODS AND MATERIALS

All chemicals used were of reagent grade. OsO_4 was obtained from Steven Metallurgical Corp., New York, N.Y. Ir spectra were recorded on Perkin Elmer model 21 and Beckman model 4250 spectrometers, and uv-vis spectra on a Beckman model DB-G. The ESCA spectrometer system was described in an earlier publication (30). Samples were prepared for ESCA examination by evaporation of the solution or suspension directly on the sample plate.

Staining of poly-L-lysine: A suspension of 10 mg of poly-L-lysine hydrobromide (m.w. 70,000; Sigma No. P2636 (Sigma Chem. Co., St. Louis, Mo.)) in 1 ml of 0.1 M phosphate buffer, pH 8.0, was treated with 1 ml of 0.34 M $K_4Fe(CN)_6$ followed by 1 ml of 0.14 M OsO_4. A brown flocculent solid immediately appeared. The mixture was allowed to stand at 25°C for 5 min. The precipitate then was collected by centrifugation and washed three times by resuspension in 1 ml of water. A second sample was treated as above, except that water was substituted for the $K_4Fe(CN)_6$ solution. A yellow flocculent solid was formed.

SR vesicles: SR membrane vesicles were prepared from rat skeletal muscle by the method of MacLennan (16). An aliquot was treated with deoxycholate (DOC) to remove the acidic extrinsic proteins (16). Untreated and DOC-treated SR vesicles were fixed in a solution containing 3% glutaraldehyde, 3% dextrose, 3% dextran (Pharmacia T70, average m.w. 70,000 daltons), and 5 mM $CaCl_2$ at pH 7.1 (21) for 30 min at room temperature. Following fixation, the vesicles were centrifuged at 150,000 × *g* for 30 min at 4°C in a Beckman 50Ti rotor.

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[4] White, DL: Spectroscopic and electrochemical characterization of OsO_4-$K_4Fe(CN)_6$ mixtures. Inorganic Chemistry (manuscript in preparation).

The pellet was resuspended in distilled water, and portions of it were reacted for 1 hr at room temperature with one of the following solutions: a) 0.8% $K_4Fe(CN)_6$ in 2% aqueous OsO_4 or b) aqueous 2% OsO_4. The reacted vesicles were again pelleted and washed as above. For electron microscopy, the pellets were dehydrated in a graded ethanol series and embedded in Spurr's medium. For ESCA studies, they were resuspended in distilled water and aliquots were examined as described above and in reference (41).

Erythrocyte ghosts: Erythrocyte ghosts from rat were prepared by the method of Wood (31). The isolated ghosts were fixed, stained, and examined as described above for the SR vesicles.

Electron microscopy of tissue: Rat skeletal muscle and liver tissue samples were used. They were minced into 1 mm^3 cubes, fixed in 2.5% glutaraldehyde in 100 mM cacodylate-HCl buffer containing 5 mM $CaCl_2$ at pH 7.4 for 6 hr at 4°C. Following fixation, the tissue samples were washed in cacodylate buffer and immersed in one of the following solutions: a) 2% OsO_4 in 100 mM cacodylate buffer at pH 7.4; b) 1.8% $K_4Fe(CN)_6$ in 2% aqueous OsO_4 (the pH of the mixture was 11.5); c) 50 mM $K_2OsO_2(OH)_4$ + 50 mM $K_4Fe(CN)_6$ at pH 8.5; or d) 40 mM $K_3Fe(CN)_6$ + 20 mM $K_2OsO_2(OH)_4$ at pH 9.5. Muscle was treated for 18 hr while liver was treated for 3 hr, both at room temperature. After reaction with one of the above solutions, the samples were rinsed in cacodylate buffer, dehydrated in ethanol, and embedded in Spurr's medium. Tissue sections were observed in a Hitachi HU 11E electron microscope without counterstaining.

Model compounds: 1,10-Phenanthroline (phen) chelate, $[phenOs(OH)_3]_2O$. A solution consisting of 5 ml of 0.137 M aqueous OsO_4 169 mg (0.686 mmole) of 1,10-phenanthroline monohydrate monohydrochloride (Aldrich Chemical Co., Milwaukee, Wisc.), and 6.9 ml of 0.1 N HCl was added dropwise to a solution of 609 mg (1.37 mmoles) of $K_4Fe(CN)_6 \cdot H_2O$ in 10 ml of 0.05 M, pH 8, phosphate buffer. This reaction mixture was stirred at room temperature for 4 hr. The resulting tan precipitate (235 mg; 80% yield) was isolated by filtration, washed with water and ether, and then dried *in vacuo* at 50°C. Spectrophotometric assay of the filtrate and washes indicated that 83% of the $Fe(CN)_6^{-4}$ was converted to $Fe(CN)_6^{-3}$. A sample of the filtrate was purified for analysis by recrystallization from water. Calculated for $C_{24}H_{22}N_4O_7Os_2$: C, 33.56; H, 2.58; and N, 6.52%. Found: C, 33.29; H, 2.55; and N, 6.41%. ESCA Os 4f emissions: 54.8 and 52.2 eV. The IR spectrum showed in addition to the phenanthroline absorptions an -OH stretch at 3356 cm^{-1} and a metal-ligand stretch at 821 cm^{-1}.

The 2,2′-dipyridyl (bipy) chelate, $[bipyOs(OH)_2O]_n$, was prepared in an analogous fashion. The green amorphous solid was obtained in 80% yield; it was insoluble in water. An 85% conversion of $Fe(CN)_6^{-4}$ to $Fe(CN)_6^{-3}$ was observed. Calculated for $C_{10}H_{10}N_2O_3Os$: C, 30.30; H, 2.54; and N, 7.07%. Found: C, 29.66; showed in addition to the dipyridyl absorptions an -OH stretch at 3279 cm^{-1} and a metal-ligand stretch at 823 cm^{-1}.

Chromatography of the aqueous filtrate from the bipyridyl reaction on AG-1 × 8 chloride form ion exchange resin gave a broad osmium-containing band. Concentration of this solution gave *ca.* 5 mg of orange needles. ESCA Os 4f emissions: 54:7 and 52.0 eV. The IR spectrum of this compound was similar to that of the insoluble green product, but with additional bands at 935, 843, and 787 cm^{-1}. This material was converted into the green solid described above when its solutions were heated and concentrated.

When the reactants were present in millimolar or less concentrations, no precipitate was formed. Spectrophotometric analysis of such solutions with the reactants present in various ratios indicated a stoichiometry of 1 bipy : 1 Os : 2 $Fe(CN)_6^{-4}$.

The same phenanthroline and dipyridyl osmium chelates were obtained by addition of their aqueous solutions to aqueous $K_2OsO_2(OH)_4$. This was demonstrated by TLC on cellulose-coated plastic sheets with water, and by ESCA and IR spectroscopy.

RESULTS AND DISCUSSION

Characterization of the Os-Fe mixtures[4]: Admixture of aqueous solutions of OsO_4 and $K_4Fe(CN)_6$, both almost colorless and at acid pH, results in a strongly alkaline, dark amber solution. Although it is obvious that a reaction has taken place, the osmium-containing products have remained elusive. Each attempt to examine the reaction mixture by, for example, crystallization, chromatography, or removal of volatiles resulted in loss of OsO_4 and recovery of $K_4Fe(CN)_6$, or consumption in a side reaction of OsO_4 and the osmium-ferrocyanide intermediate by direct reaction or reversal of its formation reaction. However, two electrochemical techniques, cyclic voltammetry and AC polarography (2), have revealed some of the details of a complex set of facile equilibria (Fig. 1) involving hexacyanoferrate and osmium oxo-complexes.

In this scheme, the number of possibilities have been somewhat arbitrarily limited by assuming that: 1) the only osmium oxidation states involved are Os (VIII), Os (VII), and Os (VI) (there is no evidence of others[4]); 2) $Fe(CN)_6^{-4}$ and $Fe(CN)_6^{-3}$ only undergo one electron redox reactions (1, 3); and 3) that only 1:1 and 1:2 Os:Fe complexes are formed (this best fits the data[4]). Even with these restrictions, there are 21 possible equilibria. Characterizing such a large number of simultaneous equilibria is a formidable task and is probably unnecessary for cytochemical applications. The important result is that the presence of $[Fe(CN)_6]^{-4}$ and $[Fe(CN)_6]^{-3}$ provides sequential one-electron pathways for oxidation-reduction reactions of osmium complexes. Such reactions would be expected to have lower energies of activation than the usual reduction reactions of OsO_4 alone, reactions that involve two-electron processes (8). This is illustrated by the fact that in mixtures of OsO_4 and $K_4Fe(CN)_6$ intermediates are formed whose polarographic reduction potentials are more anodic than that of OsO_4, i.e., they are more easily reduced.[4]

Reaction mechanism in model systems and tissue substrates: The additional contrast given to SR is one of the most striking effects of the OsO_4-$K_4Fe(CN)_6$ stain. Likewise, isolated SR vesicles also displayed densely contrasted membranes and content and thus were chosen as one of the model substrates for this investigation. They were examined by ESCA spectroscopy (30), and this technique revealed that osmium was present in OsO_4—$K_4Fe(CN)_6$-stained vesicles almost entirely as Os(IV) (Fig. 2a). This is in marked contrast to SR vesicles conventionally stained with OsO_4 or red blood cell ghosts stained with either OsO_4 or OsO_4—$K_4Fe(CN)_6$. These gave mixtures of Os(VI), Os(IV), and Os(III) (Fig. 2b–e, also see Ref. 30). It is reasonable to correlate the additional contrast of the OsO_4—$K_4Fe(CN)_6$-stained SR vesicles to the greatly heightened porportion of Os(IV) in the stained membrane. That is, more osmium must have been immobilized at the SR membrane, and the bulk of this additional osmium is present as Os(IV).

A significant difference between SR membrane and plasma membrane is the presence in the former of extrinsic proteins, e.g., calsequestrin, that are effective at sequestering metal ions (11, 19). Calsequestrin is located on the inner surface of the SR (17) and can be extracted by DOC, leaving the membrane intact (18, 20). Following DOC treatment, the cisternae of osmium tetroxide-$K_4Fe(CN)_6$-stained vesicles lacked contrast

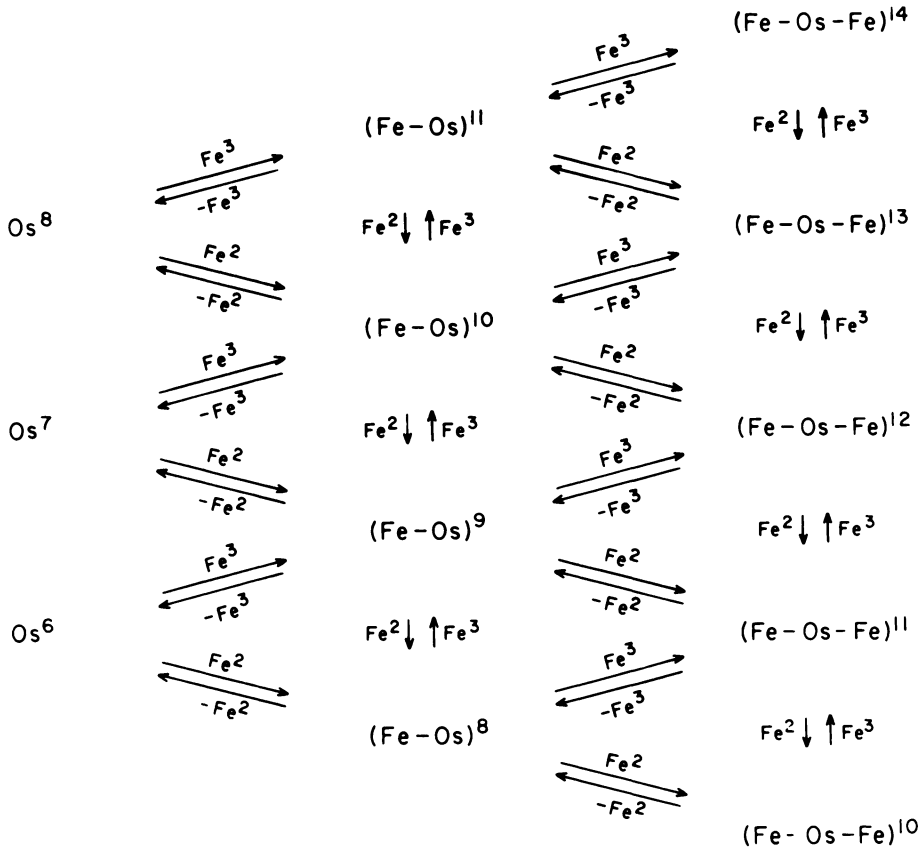


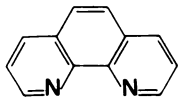
FIG. 1. Scheme illustrating the various equilibria that can be set up in mixtures containing OsO_4 and $Fe(CN)_6^{-4}$ or $Fe(CN)_6^{-3}$. The superscripts give the sum of the ionic charges of the metal atoms, but are not meant to indicate a particular electron distribution (valence electrons are probably delocalized over all of the metals). The Os oxo and hydroxy ligands and Fe cyano ligands have been omitted for clarity: thus Os^6 equals $OsO_2(OH)_4^{-2}$, and Os(VI) complex; and Fe^2 equals $Fe(CN)_6^{-4}$, an Fe(II) complex. Bridging ligands have also been omitted: thus $(Fe\text{-}Os\text{-}Fe)^{11}$ equals $[(CN)_5FeCNOsO_2(OH)_2NCFe(CN)_5]^{-7}$.

(Fig. 3), and ESCA indicated that the proportions of Os(VI) and Os(III) had increased at the expense of Os(IV) (Fig. 2).

The above results suggested that sequestration, or chelation, of osmium might be important in the mechanism of OsO_4—$K_4Fe(CN)_6$ staining. In order to test this idea, the two chelates 1,10-phenanthroline (phen) and 2,2′-dipyridyl (bipy) were allowed to react with the OsO_4—$K_4Fe(CN)_6$ mixture. Neither reacts with either component of the mixture alone. With the mixture, however, there are immediate reactions. 1,10-Phenanthroline gives a yellow-brown, crystalline product formulated as I on the basis of spectroscopic and analytical data.

$$(phen)Os(OH)_3—O—Os(OH)_3(phen) \quad \text{I}$$

phen =

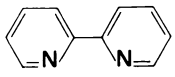


The major product with 2,2′-dipyridyl is a green, amorphous solid. In some preparations a small amount of orange crystals was also isolated. This solid was not isolated in a pure state. In fact, heating or concentrating its solutions caused the precipitation of the above mentioned green solid. This solid was soluble in concentrated sulfuric acid and could be recovered upon dilution and neutralization of the acid solution. These observations, plus analytical and spectroscopic data, suggest the reaction and structures shown in Eq. 1 below.

$$\underset{\text{III}}{n(bipy)Os(OH)_4} \longrightarrow \underset{\text{II}}{[(bipy)Os(OH)_2—O—]_n} + -\frac{n}{2}H_2O \qquad (1)$$

Orange Green

bipy =



The ESCA spectra of the compounds I-III indicated that the osmium was present in each as Os(IV). Uv-vis spectra of the reaction mixtures containing these chelates showed that 1 mole of chelate reacted with 1 mole of osmium tetroxide and 2 moles of ferrocyanide to yield 1 mole of product. The oxidation of Fe(II) is only sufficient to reduce Os(VIII) to Os(VI). Its further reduction to Os(IV) is presumably brought about through the oxidation of hydroxide ion (Eq.2).

$$4\ Fe(CN)_6^{-4} + 2\ chel + 2\ OsO_4 + 5\ HOH \rightarrow [(chel)Os(OH)_3]_20 + 4\ Fe(CN)_6^{-3} + O_2 \qquad (2)$$

chel = phen or bipy

Although this may not be the exact order of the reduction,

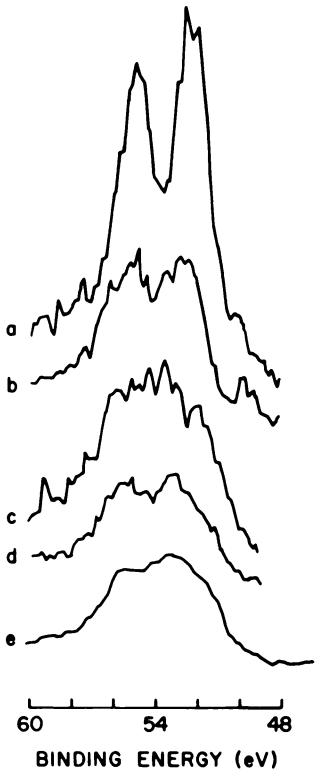


FIG. 2. ESCA spectra of reaction of SR vesicles and erythrocyte ghosts with OsO_4 and OsO_4-$K_4Fe(CN)_6$: a) SR vesicles treated with OsO_4-$K_4Fe(CN)_6$; the doublet at 55.2 and 52.4 eV indicate that osmium is predominantly present as Os(IV). It is possible that this pattern obscures a weak Fe 2p singlet at 53.8 eV, but no unequivocal Fe 3p emissions in the 725-705 eV region could be detected, even after long accumulation times. The Fe 3p emissions do not occur near any Os emissions and are easily observed for $Fe(CN)_6^{-3}$ and $Fe(CN)_6^{-4}$; b) DOC extracted SR vesicles reacted with OsO_4-$K_4Fe(CN)_6$; c) SR vesicles reacted with OsO_4; d) erythrocyte ghost reacted with OsO_4-$K_4Fe(CN)_6$ and e) erythrocyte ghosts reacted with OsO_4. Samples in (b)–(e) give mixtures of Os(VI), Os(IV) and Os(III).

it is supported by the fact that compounds I and III are formed by the reaction of $K_2OsO_2(OH)_4$ with phen and bipy, respectively.

Thus aqueous, alkaline solutions containing OsO_4 and 1,10-phenanthroline or 2,2′-dipyridyl are stable. The addition of $Fe(CN)_6^{-4}$ provides an alternative lower energy pathway that allows the reduction of OsO_4 under these conditions. Although there is as yet no detailed evidence for a particular mechanism, a reasonable pathway can be proposed based on the known behavior of osmium complexes (8):(a) OsO_4 is tetrahedral in aqueous solution at pH's less than 10; (b) no octahedral complexes with unhindered bases such as pyridine have been isolated (26); and (c) the interaction with 2,2′-dipyridyl and 1,10-phenanthroline is minimal (*vide infra*). Thus the first step in the reaction sequence is probably a reduction of Os(VIII) by ferrocyanide to Os(VII). Oxo-Os(VII) would presumably be octahedral (8), and osmium in this oxidation state could be complexed by the chelate, although a second reduction by $Fe(CN)_6^{-4}$ to Os(VI) may occur first. In either event, neutral chelating ligands stabilize lower oxidation states relative to higher ones, in part due to the lower nuclear charge in the former (3, 8). This is in addition to the stabilization due to the entropy effects of chelating ligands *versus* similar unconnected ligands. Although the stabilization energies are usually modest, they can have profound effects in systems such as osmium with several energetically closely placed oxidation states. Thus aqueous $K_2OsO_2(OH)_4$ is stable in the presence of pyridine, but reacts with 2,2′-dipyridyl to give compound III. Likewise, the reduction potential of the presumptive Os(VI) chelate in the OsO_4—$K_4Fe(CN)_6$-chelate mixture is low enough that reduction by hydroxide ion to Os(IV) occurs (Eq. 2).

The implications of this model for cytochemical staining are as follows. OsO_4—$K_4Fe(CN)_6$ mixtures have a significant equilibrium concentration of Os(VI) and reactive Os(VII) (or its equivalent) species. When these encounter chelating sites in the macromolecular matrix of the substrate, the osmium is not only immobilized, but the energy of activation for further reduction to lower oxidation states (IV, III, II) is made smaller. It is also possible (even probable) that oxo-bridged osmium polymers are initiated at these sites, increasing the amount of osmium deposition at or near them.

Without the presence of ferrocyanide, the concentration of Os(VI) compounds in solution is greatly reduced and Os(VII) complexes are not present at all (22). The Os(VI) compounds that are present are probably the result reduction of OsO_4 by the substrate (e.g., at a carbon-carbon double bond) and hydrolysis of these initially formed compounds (osmate esters) (27). Thus one would expect a lower overall contrast relative to that produced by OsO_4—$K_4Fe(CN)_6$.

Several other observations can be cited in support of this mechanism. Poly-L-lysine reacted rapidly with OsO_4—$K_4Fe(CN)_6$ solution to give products containing osmium predominantly as Os(IV) (Fig. 4), as was the case with SR vesicles, bipy, or phen. OsO_4 alone reacted more slowly and gave the usual mixture of Os(VI), Os(IV), and Os(III). These results illustrate the efficacy of amino groups in the reduction and complexation of osmium, and further indicate that proteins in general are important in these processes.

The selective contrasting of glycogen as demonstrated by De Bruijn and Den Breejen (5–7) represents a special case of the scheme that we have proposed. First, it should be noted that their systems are mixtures of oxidized Os with oxidized Fe [Os(VIII)-Fe(III)] or reduced with reduced [Os(VI)-Fe(II)]. In contrast, the systems used by others are mixtures of oxidized Os with reduced Fe [Os(VIII)-Fe(II)]. As De Bruijn and Den Breejen emphasized, however, many pathways exist for redox reactions with a tissue substrate present (6). Given the number of possible oxidation states of osmium, the relative ease of interconversion of many of them, and the facile interconversion of ferro- and ferricyanide, it can be seen that a set of equilibria like that in Figure 1 could be established, although the relative concentrations of individual species might differ given the different starting concentrations. De Bruijn and Den Breejen (7) convincingly demonstrated that the glycogen C_2–C_3 hydroxyl groups are the tissue ligands involved in the staining reaction, and these presumably immobilize the osmium by chelation. The resulting chelated, nonlabile, lower-valent osmium complexes would thus be responsible for the observed contrast, and not a stoichiometric osmium-ferro-

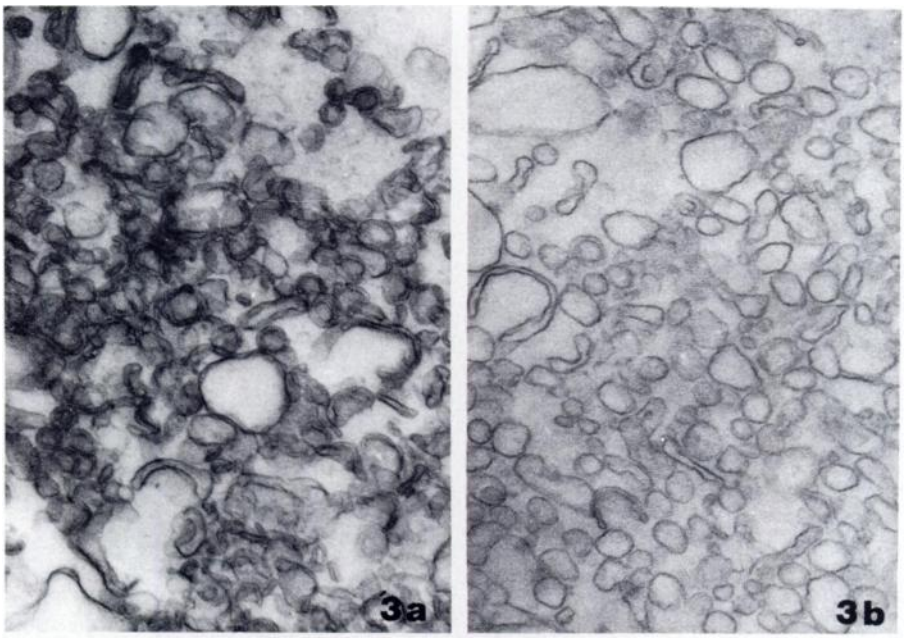


FIG. 3. Electron micrographs of SR vesicles isolated from rat skeletal muscle: (a) vesicles reacted with OsO_4-$K_4FE(CN)_6$. Note contrast of cisternal content and general overall contrast compared to (b), in which the vesicles were extracted with DOC before reaction with OsO_4-$K_4Fe(CN)_6$. No counterstain. Figures 3a and 3b ×62,000. In the case of all electron micrographs, silver sections were used and the exposure and processing of negatives and prints were identical for both stained and control tissues.

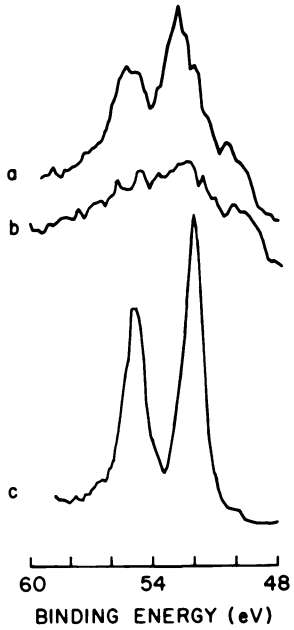


FIG. 4. ESCA spectra of products of reaction of OsO_4 and OsO_4-$K_4Fe(CN)_6$ with poly-L-lysine: (a) poly-L-lysine reacted with OsO_4-$K_4Fe(CN)_6$—Os(IV) is the predominant osmium species; (b) poly-L-lysine reacted with OsO_4 alone—Os(VI), Os(IV), and Os(III) were present in the mixture; and (c) spectrum of K_2OsCl_6, an Os(IV) standard.

cyanide complex as proposed by De Bruijn and Den Breejen (5, 6).

Electron microscopic examination of tissues reacted with OsO_4—$K_4Fe(CN)_6$, $K_2OsO_2(OH)_4$—$K_4Fe(CN)_6$, and $K_2OsO_2(OH)_4$—$K_3Fe(CN)_6$ support this conclusion. Glycogen was contrasted by all three mixtures (Figs. 5, 6). Membranes (Fig. 6) and in the case of muscle, SR cisternae (Fig. 5), and myofilaments were given additional contrast only by the first or the last mixture. Spectroscopic and electrochemical data demonstrate that these two starting mixtures yield identical product solutions.[4] Os(VI), either added as $K_2OsO_2(OH)_4$ or generated *in situ*, appears to suffice for the reaction with glycogen. To stain membranes, the Os(VII) appears to be necessary and can be provided in sufficient quantity only when OsO_4—$K_4Fe(CN)_6$ or $K_2OsO_2(OH)_4$—$K_3Fe(CN)_6$ is used. This also may reflect a reaction of OsO_4 (present in the equilibrium mixture) with unsaturated lipids in the membrane.

Cyano complexes work well not because of the formation of a long-lived bridged complex, but because they are stable in alkaline solutions and can participate in electron exchange reactions via the ligand (1, 3). Thus the Fe, Ru, and Os cyano complexes that De Bruijn and Den Breejen (6) found to give glycogen contrast with $K_2OsO_2(OH)_4$ presumably have reduction potentials that lie in the proper range under the conditions of the staining reaction. That is, the compounds are neither so strongly oxidizing that only OsO_4 is found in significant amounts, nor so strongly reducing that intermediate osmium states (VII, VI, V, IV) do not survive long enough to react with tissue. The very weak signals that De Bruijn and Den Breejen observed by electron microprobe (EDAX) examination of stained tissue sections could have been due to occlusion of a small amount of metal salt in the stained tissue matrix, and not a stoichiometric Os-Fe (Ru, Os) complex. The relative ability of two different metal atoms to impart contrast to tissue is proportional to their atomic numbers raised to the

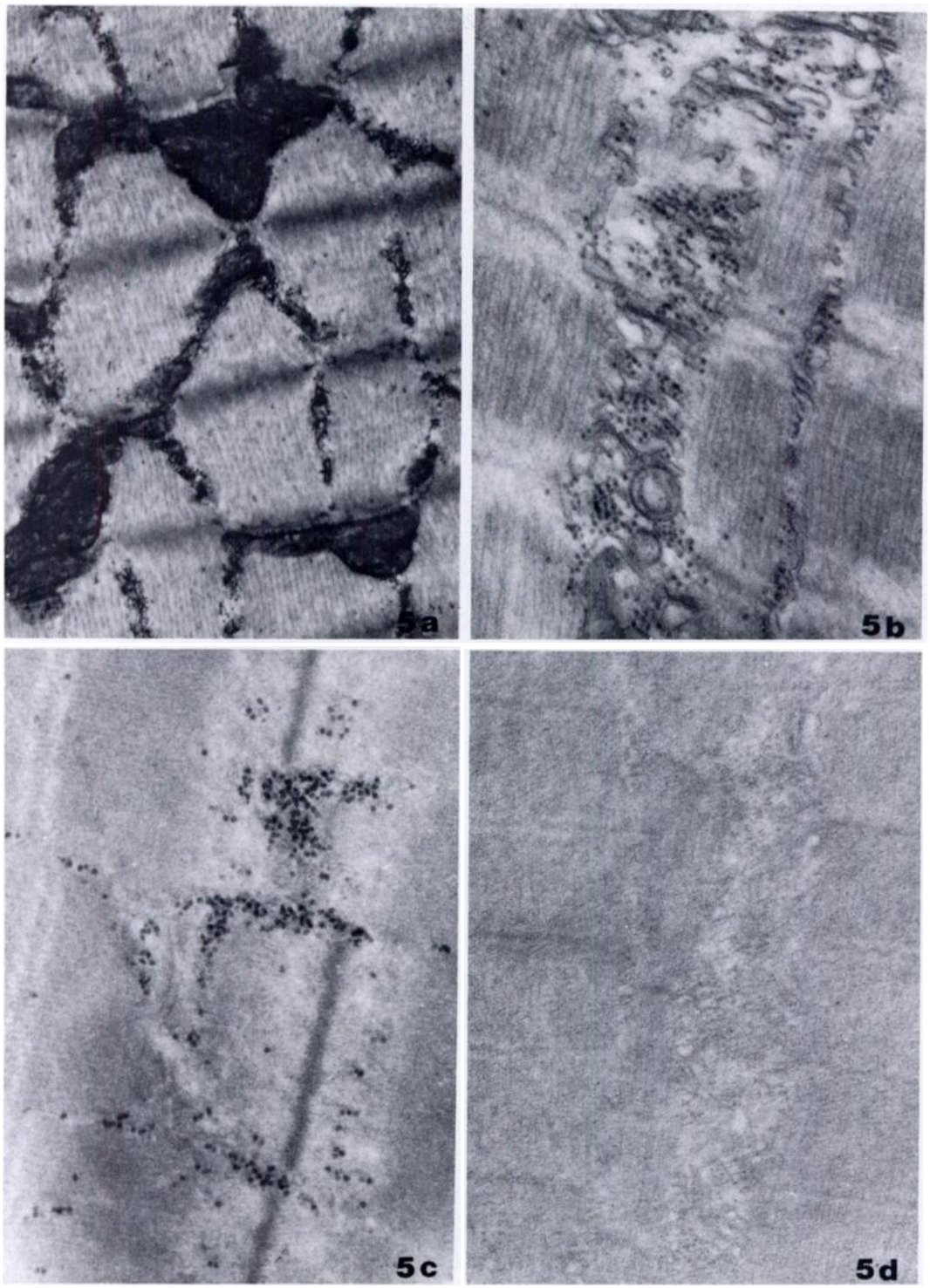


FIG. 5. Rat skeletal muscle fixed in glutaraldehyde and treated with osmium compounds: (a) OsO_4-$K_4Fe(CN)_6$; (b) $K_2OsO_2(OH)_4$—$K_3Fe(CN)_6$; (c) $K_2OsO_2(OH)_4$-$K_4Fe(CN)_6$ and (d) OsO_4 alone. Tissue in (a) and (b) have similar contrast. However the SR in (a) is more densely contrasted, while SR in (c) and (d) is not contrasted. The membranes in (a) and (b) were contrasted as well, while membranes in (c) resembled membranes of OsO_4 treated tissue in (d). Glycogen was stained in all preparations except for OsO_4 alone. No counterstain. (a) × 30,000; (b)–(d) × 47,000.

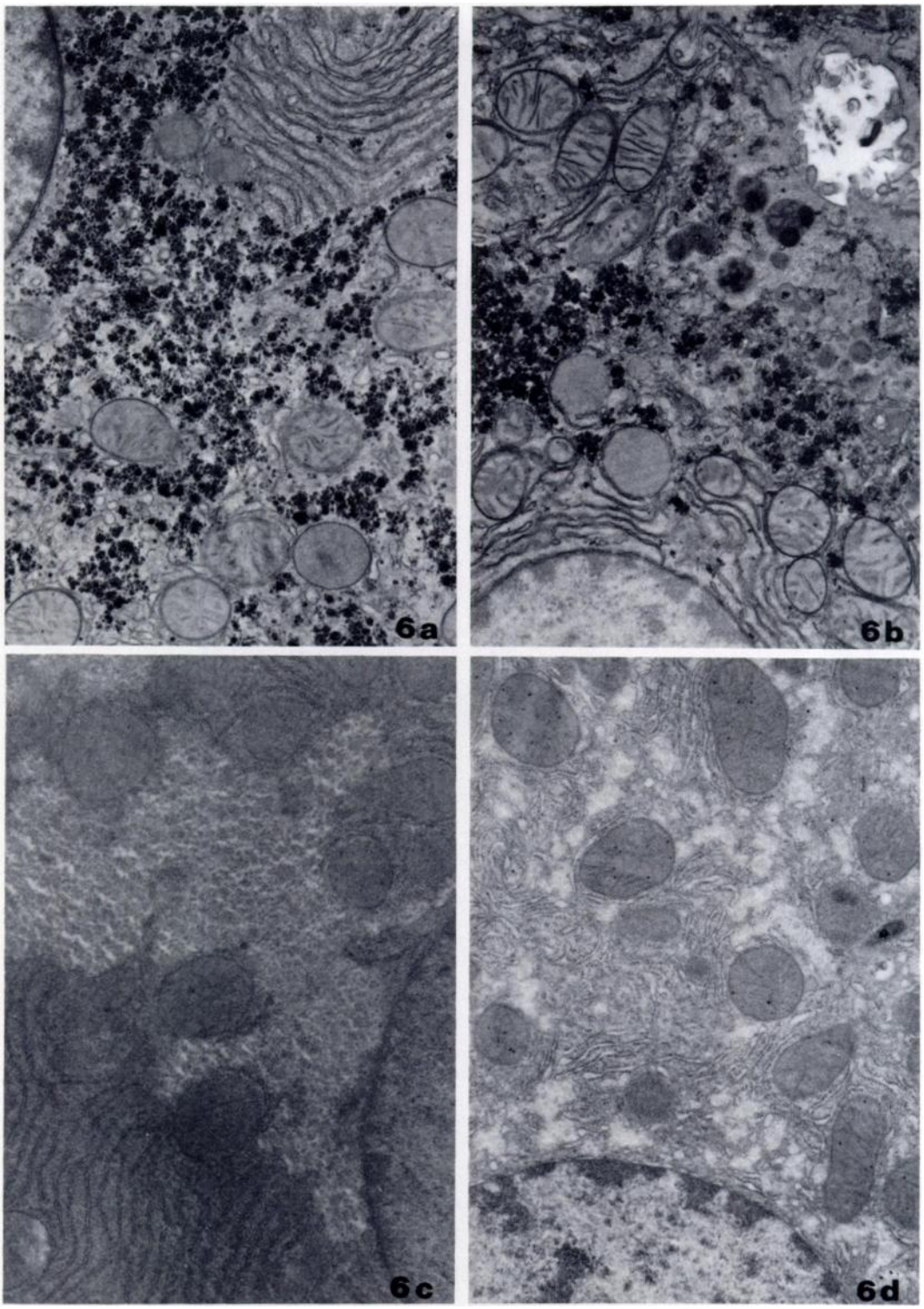


FIG. 6. Rat liver fixed in glutaraldehyde and treated with osmium compounds: (a) OsO_4-$K_4Fe(CN)_6$; (b) $K_2OsO_2(OH)_4$-$K_3Fe(CN)_6$; (c) $K_2OsO_2(OH)_4$—$K_4Fe(CN)_6$ and (d) OsO_4 alone. As with the muscle tissue (Fig. 4a–d) tissue in (a) and (b) were densely contrasted, with all membranes and glycogen darkly staining. Endoplasmic reticulum content is uncontrasted. The membranes in (c) are contrasted much like the OsO_4 treated tissue in (d), with glycogen only slightly more stained than in (d), where there is no glycogen staining. The density of glycogen staining by $K_2OsO_2(OH)_4$-$K_4Fe(CN)_6$ increased with time such that the glycogen was as dark as that in (a) and (b) at the end of 18 hrs. No counterstain. (a)–(d) × 17,000.

four-thirds power (28). Thus Fe in a 1:1 Os:Fe complex would theoretically give rise to only ~24% ($26^{4/3}/76^{4/3}$) or the contrast due to osmium alone. Even if a stoichiometric complex were bound to the tissue, it is doubtful that Fe, *per se*, would contribute significantly to the dramatic enhancement of contrast observed with these systems.

The osmium tetroxide-potassium ferrocyanide mixture gives sufficient contrast to tissues to render unnecessary counterstaining with heavy metals. It also gives additional contrast to certain cellular components; e.g. glycocalyx, sarcoplasmic reticulum and glycogen. It is inexpensive (relative to osmium alone), easily prepared, and stable. Two major factors that may have discouraged more widespread use are 1) lack of a clear understanding of the staining mechanism, and 2) the absence of a large fund of experience with this system. This investigation has addressed the first point; the second should be obviated with time.

The proposed mechanism suggests further experiments, among them the use of mordanting (chelating) agents such as tannic acid (12, 13, 23, 24) followed by the OsO_4—$K_4Fe(CN)_6$ staining mixture. Such extensions of existing techniques are currently under investigation in these laboratories.

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