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7. This research was accomplished at Scripps Institution of Oceanography, La Jolla, California. Field work was performed with the cooperation of Instituto Nacional de Pesquisas da Amazônia, Manaus-Amazonas, Brazil.

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Coordination Polymers of Osmium: The Nature of Osmium Black

Abstract. *The design of cytochemical reagents that yield osmiophilic products from which an osmium black may be derived on exposure to osmium tetroxide has resulted in new methods described previously for the ultrastructural demonstration of enzyme activity and functional groups of macromolecules with the electron microscope. Attempts to determine the nature of the osmium black end products have been frustrated by their insolubility. The preparation of water-soluble analogs and their characterization as polymers suggest that the insoluble osmium blacks are coordination polymers. This is consonant with the unusually favorable properties of osmium black in electron microscopy. Although polymers of osmium have frequently been postulated as the end products of reaction of osmium tetroxide with tissue constituents or with other organic compounds, this is the first example of their characterization.*

Osmium tetroxide has become an important reagent for the electron microscopy of biological material. According to the old literature, osmium black is finely divided osmium metal which is formed by the reduction of osmium tetroxide (OsO_4) in solution (*1*). However, other investigators believed that reduction of osmium tetroxide by the unsaturated lipid components of tissue yielded black, hydrated $OsO_2 \cdot nH_2O$ (*2*). More recent work (*3*) indicates that cyclic osmate esters, first postulated by Criegee (*4*), are probably the major reaction products and that some $OsO_2 \cdot nH_2O$ is formed as a by-product. However, there is no evidence to substantiate its presence.

The osmium blacks which are used in the ultrastructural chemical demonstration of enzymes and functional groups with the electron microscope (*5*) are amorphous; they are generally insoluble in tissue constituents, in the organic solvents used for dehydrating tissue, and in the acrylic and epoxy monomers used in the preparation of ultrathin sections required for electron microscopy.

In the course of our study of the reactivity of osmiophilic reagents and their end products with OsO_4, it became apparent that the osmium blacks were not a single, uniform substance, but vary in properties, depending upon the sulfur-containing organic reductant.

Several of these osmium blacks were prepared (*6*) by reaction of OsO_4 with thiosemicarbazide, thiocarbohydrazide, thiophenol, *p*-chlorothiophenol, and 2-naphthalenethiol. Conductivity measurements on these samples ruled out the presence of significant amounts of osmium metal. Infrared and elemental analyses confirmed that these materials were not hydrated OsO_2 but rather coordination compounds of osmium with organic sulfur ligands.

The compound formed by reaction of OsO_4 with *p*-chlorothiophenol had considerable solubility in pyridine. Nuclear magnetic resonance examination 5 kc up- and downfield from pyridine showed no proton peaks. Since the presence of hydrogen had been confirmed by infrared and elemental analyses, we suspected that this particular osmium black did not truly dissolve in pyridine but formed a colloidal suspension. This was affirmed by the high viscosity of the solutions. These results, in conjunction with the demonstrated ability of organic sulfur ligands to bridge osmium to osmium as well as to other metals (*7*) led us to suppose that the osmium blacks are coordination polymers of osmium.

Determinations of molecular weight could not be performed by colligative methods on the existing samples of osmium blacks, owing to their extreme insolubility. Attempts to obtain products which would be more soluble in organic solvents by using ligands with a greater carbon-hydrogen content were unsuccessful. However, it proved possible to form a water-soluble osmium black by using a hydrophilic ligand. To a solution of 3-mercapto-1,2-propanediol (*8*) (86 mg, 0.0008 mole) in 25 ml of water, 10 ml of 2 percent OsO_4 solution (0.0008 mole) was added by drops, with stirring, at room temperature. During the addition, the solution changed from brown to black. The addition of acetone caused the separation of a very fine dark brown precipitate which coalesced into larger particles after brief digestion on a steam bath. The mixture was then cooled in an ice bath, whereupon the precipitate settled out. It was collected by suction filtration, washed with acetone, and allowed to dry in air. The product was a dark brown amorphous powder (67 mg). It was readily soluble in water and changed from brown to black on heating to 300°C, but did not melt. This product is probably the tetramer $[Os(SCH_2CHOHCH_2OH)_4]_4$. The analyses (*9*) were: calculated for the monomer $C_{12}H_{28}O_8S_4Os$: C, 23.3; H, 4.6; O, 20.7; S, 20.7; Os, 30.7; found: C, 23.1; H, 4.6; O, 20.9; S, 19.7; Os, 30.5. The material gives a vapor-pressure osmometric molecular weight of 2555 in water (calculated, 2475).

By repeating the preparation but dissolving the 3-mercapto-1,2-propanediol in 5 ml of water instead of 25 ml, a blacker product (about 200 mg) was obtained. This has essentially the same properties, and gave the same elemental analyses. However, it showed an osmometric molecular weight of 3040, in fairly good agreement with the calculated molecular weight of 3093 for the pentamer $[Os(SCH_2CHOHCH_2OH)_4]_5$.

A relationship between the more soluble and less soluble osmium blacks and the importance of bridging in polymer formation can be demonstrated by treating an aqueous solution of the pentamer with excess OsO_4; a black precipitate is obtained which is insoluble in water or organic solvents, which presumably indicates further polymerization.

The infrared spectrum of the tetramer or pentamer is similar to that of 3-mercapto-1,2-propanediol except that the SH stretching absorption at 2540 cm^{-1} has disappeared and the CH_2 deformation peak (*10*) of 3-mercapto-1,2-propanediol is broadened and shifted to 875 cm^{-1} from 864 cm^{-1}. Evidence that this is not due to an Os—O stretching mode is provided by the fact that the same shift and broad-

ening are demonstrated by the infrared spectrum of the mercuric mercaptide of 3-mercapto-1,2-propanediol. This is in agreement with the expected reduction of osmium tetroxide by the sulfhydryl groups in forming the polymeric mercaptide. The 60-Mc nuclear magnetic resonance proton spectrum of the tetramer or pentamer shows a sharp peak at $\delta = 4.67$ parts per million (ppm), which is indirectly attributable to the OH protons of the polymers; protons from SH were ruled out by the infrared spectra of the tetramer and pentamer mentioned above, and protons from the solvent (D_2O) accounted for only one-fifth of the peak integral. In addition, there is an extremely broad peak extending from $\delta = 1$ ppm to $\delta = 7$ ppm, with its summit at $\delta = 3.7$ ppm, which is attributable to OCH and OCH_2 protons. The broadness of this peak is probably due to the paramagnetism of the polymers, which was confirmed by electron spin resonance spectra. The nuclear magnetic resonance spectra indicate that these polymers are in solution.

One milliliter of an aqueous solution containing 40 mg of the tetramer and 40 mg of sucrose was injected intravenously into each of several albino mice. The dark brown solution colored the blood, skin areas rich in blood vessels, and the eyes. Within 45 minutes the blood cleared, the eyes returned to normal color, and the dark material was found in the urine. This is biological evidence that the polymer is in true solution. No acute toxicity was noted.

Although the formation of polymers of osmium have frequently been postulated (*11*, *12*), this is the first example of their characterization. The synthesis of other soluble coordination compounds of osmium, including some with multiple ionic charges, and their use as stains for light and electron microscopy is the subject of a separate communication (*13*).

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Dimethyl Sulfoxide Protects Tightly Coupled Mitochondria from Freezing Damage

Abstract. *Dimethyl sulfoxide prevented loss of respiratory control and decrease in efficiency of oxidative phosphorylation when plant mitochondria were stored in liquid nitrogen. Respiration was severely inhibited and was not stimulated by adenosine diphosphate when mitochondria were frozen in liquid nitrogen without dimethyl sulfoxide. Thus, isolated mitochondria provide a model system for the study of the effects of freezing on biological membranes and of the prevention, by dimethyl sulfoxide, of freezing damage.*

Freezing causes extensive disorganization of mitochondrial and other membranes within living cells, and cryoprotective agents such as dimethyl sulfoxide (DMSO) prevent this damage (*1*). According to Trump *et al.* (*1*), there is no satisfactory theory to explain such protective treatment. Damage to isolated mitochondria by freezing includes rupture of membranes, loss of soluble enzymes, and increase in the activity of adenosine triphosphatase (*2*). Freezing abolishes respiration and phosphorylation of isolated spinach mitochondria, but sucrose provides partial protection (*3*). Glycerol and DMSO prevent decreases in respiration and oxidative phosphorylation when rat-liver mitochondria are frozen (*4*). There are no reports on whether freezing affects respiratory control of tightly coupled mitochondria. We have tried to develop a method for storing tightly coupled mitochondria and to learn whether isolated plant mitochondria can be used for studies on the mode of action of cryoprotective agents.

Mitochondria were isolated from wall tissue of mature green tomato fruits (variety Kc146) with the methods of Drury and Garrison (*5*). Isolated mitochondria were suspended in a solution containing 0.5 mole of mannitol, 1.5 g of bovine serum albumin, and 5 mmole of sodium barbital per liter at *p*H 7.5. Oxygen uptake was measured polarographically (*6*) with a Clark platinum electrode. The ratio of adenosine diphosphate to oxygen consumed (ADP/O) and the respiratory control ratios were calculated according to Chance and Williams (*6*). The latter ratio is equal to the rate of oxygen uptake stimulated by ADP divided by the subsequent rate limited by ADP.

The reaction mixture contained a solution with 0.5 mole of mannitol, 5 mmole of $MgCl_2$, 10 mmole of tris(hydroxymethyl)aminomethane, 10 mmole of KH_2PO_4, and 0.5 mmole of ethylenediaminetetraacetate per liter plus mitochondria, substrate, and ADP in a final volume of 2.95 ml at *p*H 7.5 and 23°C. For the freezing experiments, 0.5-ml samples of mitochondrial suspension were placed in 50-ml polycarbonate tubes or 1-dram glass vials. To obtain various cooling rates and storage temperatures we placed the samples in a deep freeze (−18°C), partially immersed them in liquid nitro-