Osmiophilic Polymer Generation: Catalysis by Transition Metal Compounds in Ultrastructural Cytochemistry

Abstract. *A transition metal compound that is bound in tissues by any appropriate cytochemical reaction may catalyze the generation of an insoluble osmiophilic polymer from organic monomers such as 3,3'-diaminobenzidine. When the polymers are treated with osmium tetroxide, electron-opaque, insoluble osmium* blacks *(coordination polymers of osmium) are formed at the sites of the particular macromolecule or enzyme permitting its light, and electron, microscopic localization. This approach represents a distinct advantage over earlier cytochemical methods because the shorter incubation time needed here results in less artifactual deposition of metal ions, and less tendency to crystallize the reaction product. In addition, the shorter incubation times permit longer fixation of tissues and hence less artifact due to enzyme diffusion.*

Coordination polymers of osmium *(1),* osmium *blacks,* can be deposited selectively (*2*) by macromolecules or sites of enzyme activity. Because these polymers generally remain unaltered during the processes of dehydration and plastic embedding, they are quite useful in ultrastructural cytochemistry (*3*).

Localization of enzymes was achieved by the formation of osmiophilic polymers in demonstrations of esterase (*4*) and in the demonstration of oxidative enzymes (*5*) such as peroxidases, cytochrome oxidase (*6*), and cytochrome c. In the case of the oxidative enzymes, the polymerization of 3,3'-diaminobenzidine (DAB) to an indamine-type polymer (*6*) was found to be catalyzed by the transition metal of the prosthetic group of the enzyme.

We report a new approach to ultrastructural cytochemistry. Small quantities of transition metal compounds can be localized at tissue sites by their selective affinity for particular macromolecules, or by their selective deposition at sites of enzyme activity. Upon subsequent treatment with organic monomers such as DAB, the transition metal compound generates an osmiophilic polymer. When treated with osmium tetroxide, the site of the original biopolymer or the site of enzyme activity is rendered visible with light microscopy or electron opaque with electron microscopy.

This approach involves shorter incubation periods than do other methods in that only the deposition of a catalytic quantity of transition metal is required. This provides significant advantages over other approaches to ultrastructural cytochemistry. Inhibition of enzymes by capture reagents (*7*), for example, does not appear to be a problem. Less random precipitation (*8*) of metal capture reagent occurs and problems associated with nonenzymatic substrate hydrolysis, such as those induced by metal ions (*9*), are diminished.

The generality of the approach is evident in that we have been able to use it to demonstrate enzymes such as acetylcholinesterase, nonspecific cholinesterase, esterase, phosphatases, arylsulfatase B, β-glucuronidase, monoamine oxidase, and succinic, lactic, and NADH dehydrogenases. In most of these enzyme procedures (Fig. 1), cupric ferrocyanide, $Cu_2Fe(CN)_6 \cdot 7H_2O$ (Hatchett's brown), may be used as the catalyst for DAB polymerization. This was introduced as a suitable end product in itself for electron microscopic cytochemistry (*10*). We have demonstrated sites of acid mucopolysaccharides and of basic protein by selective deposition of a transition metal compound, treatment with DAB, and subsequent treatment with osmium tetroxide, or osmication.

The application of our approach to the localization of a particular enzyme can most readily be seen with acetylcholinesterase. The conditions used to deposit catalytic quantities of cupric ferrocyanide at the sites of acetylcholinesterase activity were essentially similar to those given by Karnovsky and Roots (*10*). The shorter incubation times resulted in a reduction in deposition of nonspecific metal ions as well as diminished tendency toward crystal formation or gel aggregation (*11*). Our approach permits longer fixation and results in decreased enzyme diffusion artifacts (*12*). It also facilitates light microscopic study.

Using catalytic osmiophilic polymer generation, we also obtained ultrastructural localization of hydrolases with substrates which were previously useful only for light microscopy; for example, esterase activity with the substrate 2-thiolacetoxybenzanilide (TAB) (*13*) and acid phosphatase activity with the substrate didicyclohexylammonium-2-naphthylthiolphosphate (DDNTP) (*14*). A light micrograph showing esterase-containing lysosomes in kidney tubules is seen in Fig. 2. Reaction product, showing extralysosomal acid phosphatase activity, is seen within the endoplasmic reticulum of a Sertoli cell (Fig. 3). The overall reaction sequence for the demonstration of acid phosphatase is shown in Fig. 4.

In addition, we have extended this approach to the localization of nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenases such as lactic dehydrogenase (LDH), to NADH dehydrogenase, and to monoamine oxidase by using *N*-thiazolyl formazans chelated with cobalt (*15*) as catalysts for DAB polymerization.

The ability of transition metal compounds deposited in tissues to catalyze the oxidative polymerization of DAB was also seen by our demonstration of phosphomolybdic acid-bound acid mucopolysaccharides which catalyze the oxidation of benzidine (*16*). Phosphotungstic acid (PTA) bound to basic proteins (*17*) also was effective in catalyzing DAB oxidation. This is consistent with the known ability of transition metals "stabilized" in higher oxidation states to catalyze the oxidation of benzidine-type compounds (*18*). It is also an example of the ability (*19*) of metal ions in transient valency states to initiate polymerization.

With the exception of the demonstration of PTA bound to basic proteins (*20*), the catalytic generation of the osmiophilic DAB polymer and its subsequent treatment with osmium tetroxide were performed in an identical manner. Cryostat sections on cover slips or 1-mm^3 blocks of tissues in which a transition metal compound was selectively localized as a result of one of the above cytochemical reactions (*21*), were passed through three washes, of 5 minutes duration each, in distilled water. They were then treated for ½ hour (*22*) with a freshly prepared solution of 0.05 percent DAB in 0.05*M* phosphate buffer, *p*H 6.8. In catalytic osmiophilic polymer generation by deposition of transition metal compounds, the oxidation of DAB appears to occur stepwise, going through an initial "benzidine-blue" type stage (*23*) before the final reddish brown product is at-

tained. This is not seen in the oxidation of DAB by cytochrome oxidase.

Attempts to isolate a sample of the "benzidine-blue" type product by methods which had previously been used (*6*) to prepare the characteristic reddish brown osmiophilic polymer were unsuccessful. More successful was an approach in which Whatman No. 1 filter papers were impregnated with very small quantities of cupric ferrocyanide by successive treatment with dilute solutions of cupric sulfate and potassium ferrocyanide. Papers so impregnated were dried and immersed in the DAB solution described above for the cytochemical studies. Polymer generation occurred immediately on the papers and they were withdrawn from the monomer solution at various times, and were thoroughly rinsed and dried. Studies performed with a derivative recording, double wavelength, ultraviolet-visible spectrophotometer showed a difference between the 2-minute (blue) species and the (reddish brown) species obtained at later time intervals (*24*). The reddish brown form of the polymer generated catalytically by cupric ferrocyanide held 1.3 atoms of osmium per mole of DAB monomer (*25*).

In addition to using transition metals as catalysts for osmiophilic polymer generation, we were able to intensify the sites of the transition metal compounds, such as Hatchett's brown, by bridging (*3*, unsaturated lipid) them to electron-opaque osmium through thiocarbohydrazide (TCH). Although the existence of insoluble unreactive complexes, many of which have infinite chain structures, has been shown (*26*) for the reaction of excess hydrazine with certain transition metal complexes, we have no evidence of polymerization in these studies with TCH. However, TCH bound to Hatchett's brown held considerably less osmium than the 1.3 atoms held per mole of DAB monomer.

The oxidative polymerization of monomers other than DAB, such as *p*-phenylenediamine (PPD) and many biogenic amines, to form osmiophilic melanin-type polymers was also catalyzed by Hatchett's brown. This could be blocked by *o*-phenylenediamine which is also consistent with the suggestion of initial complexing between cupric ferrocyanide and DAB (*24*).

An advantage of catalytic osmiophilic polymer generation over bridging with TCH was demonstrated (*21*) in the amplification of *N*-thiazolyl formazan chelated with transition metal which was deposited at the sites of dehydrogenases (*15*) and monoamine oxi-

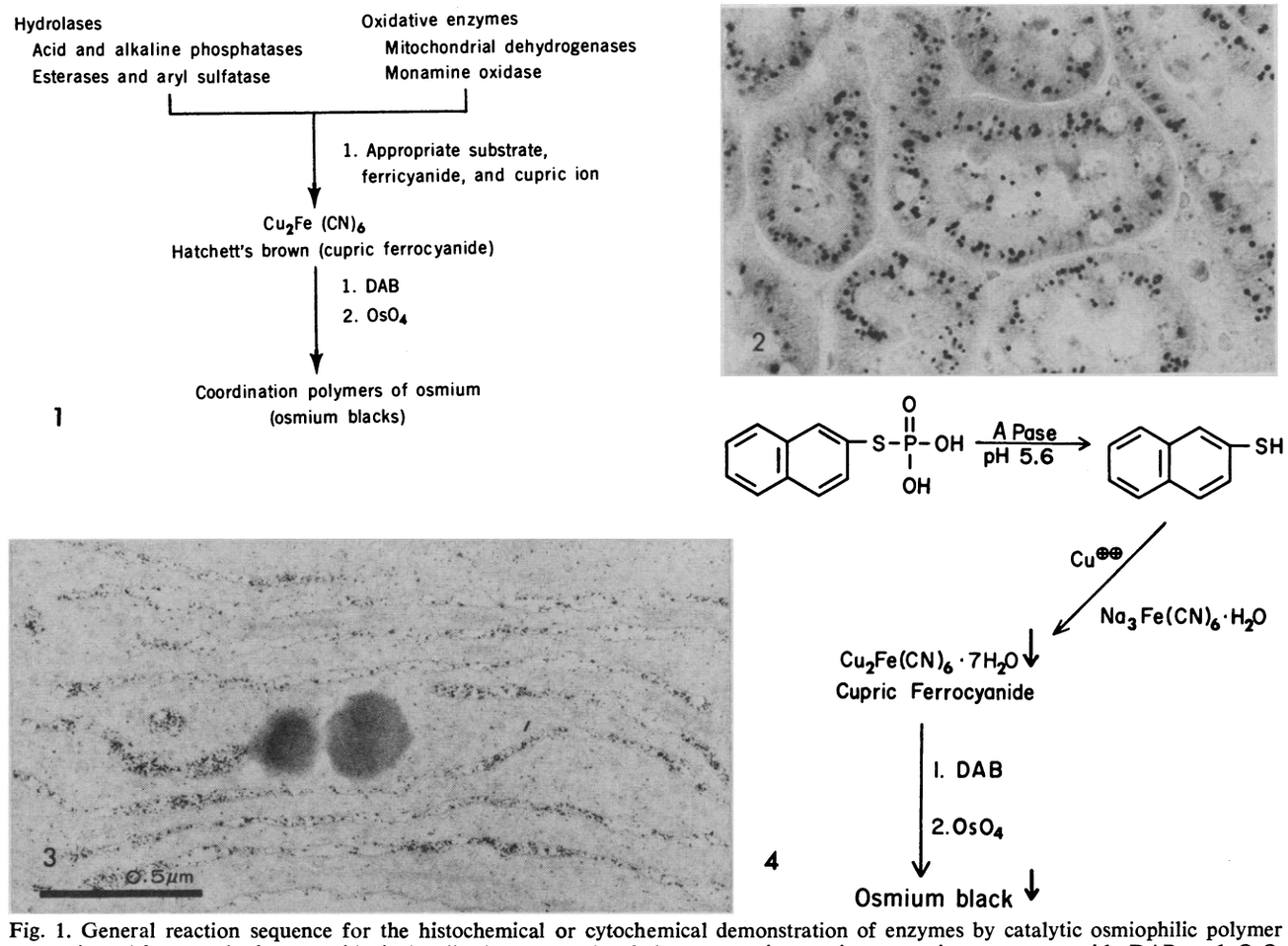

Fig. 1. General reaction sequence for the histochemical or cytochemical demonstration of enzymes by catalytic osmiophilic polymer generation. After cupric ferrocyanide is localized as a result of the enzymatic reaction, stepwise treatment with DAB and OsO_4 results in the deposition of an osmium *black* at the sites of the enzyme. Fig. 2. Light micrograph showing esterase in lysosomes in rat renal tubules. Frozen section of calcium-formalin fixed kidney treated (*21*) according to reaction sequence shown in Fig 4. No counterstain (×560). Fig. 3. Electron micrograph showing extra-lysosomal acid phosphatase associated with endoplasmic reticulum of rat testicular Sertoli cell. Short fixation at *p*H 5.6 in formalin (freshly prepared by depolymerization of paraformaldehyde) and treated (*21*) according to reaction sequence shown in Fig. 4. No counterstain (calibration bar = 0.5 μm). Fig. 4. Reaction sequence for acid phosphatase (APase) demonstration. The didicyclohexylammonium salt (DDNTP, not shown) of the substrate 2-naphthylthiolphosphoric acid (NTP, shown) is used in an incubation medium containing cupric ion and alkali ferricyanide. The 2-naphthalenethiol liberated by phosphatase activity forms cupric ferrocyanide at the sites of enzyme activity. Polymer generation and osmium treatment as above.

dase (*27*). Although the cobalt-chelated formazan conforms rather well to the sites of mitochondria in the cell, it is soluble in organic solvents and therefore does not withstand dehydration. Although we were able to bridge osmium to the cobalt-chelated formazan by means of TCH, the product resulting from treatment with osmium tetroxide was likewise soluble in organic solvents. The products resulting from osmium treatment after catalysis of DAB polymerization by the transition metal-chelated formazan, on the other hand, withstood dehydration in organic solvents. This is contrary to the suggestion that some translocation of marker could occur in procedures, such as ours, that depend upon several reactions until osmium *blacks* are formed. In some cases our procedure actually prevents or inhibits translocation.

Osmiophilic melanin-type polymers (*28*) formed by oxidation of mixtures of PPD or catechol and biogenic amines were especially intense stains. It would be expected that these polymers would complex well with proteins and nucleic acids and conform well to ultrastructure. It must be stressed, however, that the localization that is obtained is dependent upon and limited by the accuracy of localization of the initial transition metal compound.

That the transition metal compounds are behaving as catalysts is supported by several considerations. First, our spectral studies (*24*) suggest that DAB initially complexes with cupric ferrocyanide. This, in turn, suggests that the cupric ferrocyanide is a catalyst in this reaction since one theory of catalytic activity of transition metal ions and compounds requires the formation of unstable complexes with the organic substrate (*29*). In addition, although our reaction was initially very rapid, distinct increases in absorbency on papers were noted with time. This could only occur if the transition metals were behaving as catalysts. Incubations for enzymes, such as acetylcholinesterase in peripheral nerve, which previously require incubations of 18 to 24 hours could now be performed with incubations of less than an hour. The amount of Hatchett's brown formed was barely visible with our procedures but the polymers or osmicated polymers were intense stains. The special ability of transition metal ions and compounds to act as catalysts in the oxidation of organic substrates such as the aromatic diamines is related to the variable valency of these metals (*29*). This variable valency results from the small difference in energy levels between orbitals in the outer and penultimate shells of the metals, which, in turn, facilitates the promotion of electrons, complexing ability, and catalytic activity in oxidative reactions.

The principle of selectively localizing a small amount of a transition metal compound for its catalysis of osmiophilic polymer generation may well have other general applications to cell biology.

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20. The PTA is weakly bound to basic proteins and is readily removed if treated with an aqueous solution of DAB. In this case a solution of 0.05 percent DAB (tetrahydrochloride) in 95 percent alcohol was prepared and titrated to *p*H 6.8.
21. J. S. Hanker *et al.*, unpublished experiments.
22. Staining occurred immediately upon treatment of the sections (containing deposited transition metal compound) with DAB. However, sections treated for 1 hour had considerably greater accumulations of polymer than sections incubated for shorter periods of time. The DAB (3,3′-diaminobenzidine tetrahydrochloride) was purchased from Sigma Chemical Co., St. Louis, Mo. Since DAB may be carcinogenic and the powder scatters readily due to the accumulation of excessive electrostatic charge it should be handled with appropriate safety equipment. Polysciences, Inc., Warrington, Pa., is now packaging DAB in preweighed vials to which buffer can be added for immediate use.
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24. Papers were withdrawn at 2, 5, 10, 30, and 60 minutes. The papers appeared blue when withdrawn at 2 minutes, and the succeeding papers were reddish brown. No essential difference in color was seen from 10 to 30 minutes although the papers at 30 minutes were appreciably darker. Attempts to study the different polymeric species on the filter papers by internal reflectance infrared spectroscopy were unsuccessful. Studies performed in the derivative mode with a double wavelength ultraviolet-visible spectrophotometer (Perkin-Elmer model 356) showed a distinctive difference between the 2-minute species and the species obtained at later time intervals; those obtained at later time intervals were essentially alike. However, present interpretation of the full significance of the spectral shifts is complicated by the similarity in the ultraviolet absorption maxima of cupric ferrocyanide on paper (λ_{max} = 280, 305, 335 nm), DAB monomer in solution (λ_{max} = 222, 280, 305 nm), and DAB polymeric species on paper ($\lambda_{max,\ 2\ min}$ = 275, 305, 340 nm; $\lambda_{max,\ 5\text{-}30\ min}$ = 260, 305, 340 nm). These results would not be inconsistent with and perhaps are even suggestive of initial complexing between the cupric ferrocyanide and DAB. This has been borne out (*21*).
25. This is comparable with the value of 0.8 atom of osmium otained previously (*6*). The present value was obtained by averaging results of different experiments, one on filter papers and the other on fritted glass disks. A greater surface area of polymer was exposed to OsO_4 in these new experiments.
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