# THE IMPORTANCE OF OSMIOPHILIA IN THE PRODUCTION OF STABLE AZOINDOXYL COMPLEXES OF HIGH CONTRAST FOR COMBINED ENZYME CYTOCHEMISTRY AND ELECTRON MICROSCOPY

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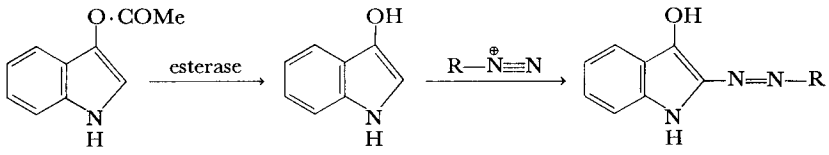
The versatility of dye methods for locating cellular enzymes gives them great potentialities as alternatives to metal salt precipitation techniques for electron microscopy, although their use has many attendant difficulties. For example, gross diffusion of the organic dye occurs in embedding media when indigogenic or certain azonaphthol methods are used in attempts to determine the cellular locations of lysosomal or microsomal esterases (13, 14). Such dyes also have relatively poor contrast in the electron microscope.

In attempts to overcome these limitations, Hanker et al. have exploited their finding that certain sulphur-containing dyes are able to reduce osmium tetroxide to "osmium black," a product that is opaque to both light and electrons, and have described several cytochemical staining methods based upon this new principle (8). The alternative procedure of introducing metal atoms to increase contrast by simple chelation of metal ions with preformed dyes has so far been unsuccessful, as the products are usually so soluble in organic solvents and embedding media that they have no advantage over the free dyes for electron microscopy (8, 13). On the other hand, encouraging results have been obtained by Tice and Barrnett (21), with a variant of this principle in which diazophthalocyanine coupling agents are used in an azo dye method. Here, a metal is already firmly bound in the coupling agent and thus becomes incorporated in the reaction product and provides increased electron scattering.

We now wish to report upon another principle, based upon the formation of azo dyes (III) derived from indoxyl (II) following enzymic hydrolysis of an appropriate substrate (I). This coupling reaction was first described by Baeyer nearly a century ago (1), although it has only recently been applied in enzyme cytochemistry (4, 5, 10). However, the azo dyes derived from indoxyl have unique chelating propensities, because they are theoretically able to form two classes of chelate compounds. One of these involves binding of a metal (*M*) by the azo and enol groups (IV); the other involves binding by the azo and imino groups (V). Evidence is now presented that certain azoindoxyls will form insoluble osmium-containing complexes that are particularly suitable for enzyme

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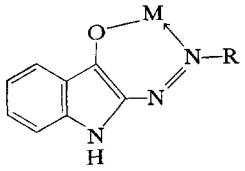

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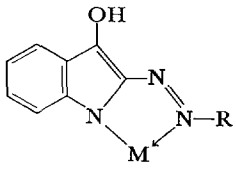
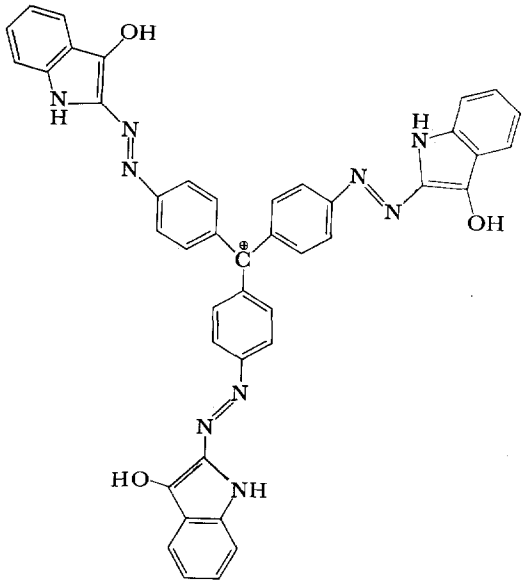


V

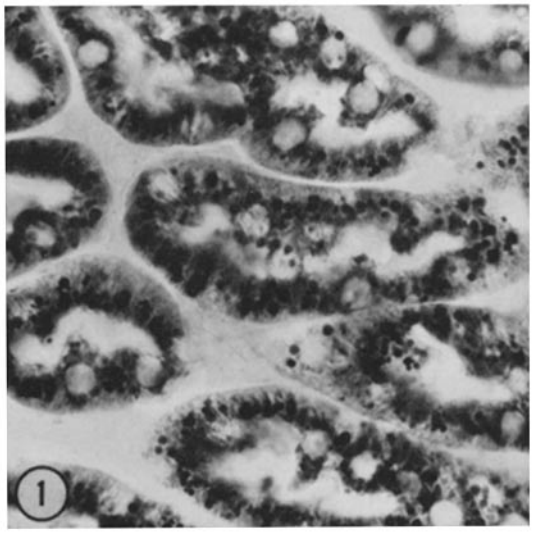
localization by electron microscopy. Extensive comparative studies (11) with the series of indoxyl acetates previously used in developing indigogenic staining methods for esterases (15), and a wide variety of diazonium salts as coupling agents have shown that such complexes are formed. It was also found that the dye (VI) derived from unsubstituted indoxyl (II) and hexazotized pararosaniline (4) possesses the most useful degree of osmiophilia of those dyes studied.

The formation of an osmium complex by this dye was confirmed in three ways. First, the solubility of the dye was altered by postfixation of stained tissues in osmium tetroxide (Figs. 1 to 3). Fig. 1 shows the pattern of esterase activity in a stained section of rat kidney mounted in glycerine jelly, where the azo dye is seen in the droplets and cytoplasm of cells of the proximal convoluted tubules. When such stained tissue is dehydrated and embedded directly in epoxide resins, the dye is almost completely extracted (Fig. 2). In contrast, if the sections are postfixed in osmium tetroxide, no extraction or redistribution of the stain occurs during subsequent embedding (Fig. 3). Second, a



VI

consistent change was observed when the visible absorption spectra of stained smears (esterase) of the microsome fraction of rat liver were recorded before and after treatment of each smear with osmium tetroxide (Fig. 4). A standard type spectrophotometer (Perkin-Elmer Model 137 UV) was used, and, in spite of the flattening of the absorption curves inherent in the use of solid suspensions in this way, the change observed is indicative of complex formation. Stained tissue is not suitable for spectrophotometry with this



Figs. 1, 3, and 5 are of rat kidney fixed for 4 hr in 5% glutaraldehyde (19) buffered with 0.067 M cacodylate at pH 7.3. Frozen sections were cut at 7.5 $\mu$ (Figs. 1 to 3) or at 30 $\mu$ (Fig. 5) and stained at 0°C for 2 hr at pH 6.0 in a substrate medium containing 1 mM each of indoxyl acetate and hexazotized pararosaniline and 0.03 M citrate buffer. FIGURE 1 A stained section mounted directly in glycerine jelly. Note the prominent staining of droplets and cytoplasm of cells of the proximal convoluted tubules by the azoindoxyl dye. Light micrograph. × 600.

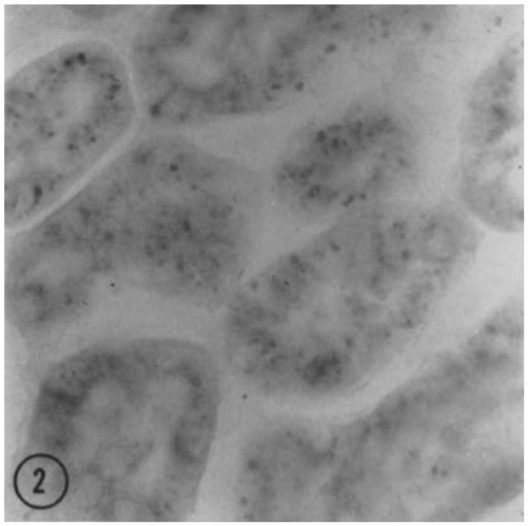


FIGURE 2 The same as Fig. 1, but dehydrated in alcohols, mounted in Epon (17), and heated at 60°C for 24 hr to polymerize the resin. Apart from a few droplets that show weak residual stain, most of the azo dye has been extracted by the Epon. Print was deliberately overexposed to emphasize the remaining dye. × 600.

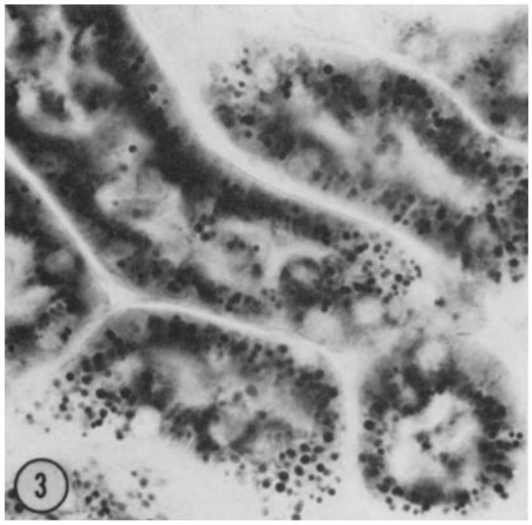


FIGURE 3 The same as Fig. 2, but postfixed in 1% osmium tetroxide for 1 hr before being dehydrated and heated in Epon. The osmium complex of the azo dye has resisted extraction, and the result is almost indistinguishable from Fig. 1. × 600.

instrument, because an even greater degree of flattening of the absorption curves is produced by the localized dye deposits of high optical density. Third, after treatment with osmium tetroxide and embedding, the stabilized dye was clearly visible in the electron microscope. For example, the osmium complex may be observed in the droplets and cytoplasm of the proximal convoluted tubule cells of rat kidney (cf. light micrographs, Figs. 1 and 3), but it is now evident that the cytoplasmic stain is located within the rough-surfaced endoplasmic reticulum and the nuclear envelope (Fig. 5).

The marked staining of the rough-surfaced endoplasmic reticulum (Fig. 5, inset) and nuclear envelope is undoubtedly enzymically produced, and not an adsorption artefact, because, in adjacent distal tubules, these sites are not stained even though droplets in the same cells show the reaction product. Furthermore, deposition of stain in the endoplasmic reticulum of the proximal tubules is abolished by treating the tissue before staining with $10^{-6}$ to $10^{-5}$ M concentrations of the esterase inhibitor E600.

The probable composition of the opaque osmium complex, formed by the azo dye acting as a bidentate ligand as in IV or V, can be inferred with some confidence from the known chelate

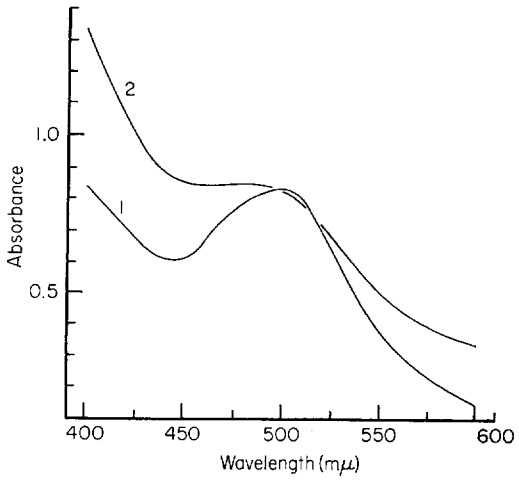



FIGURE 4 Curve 1 shows the visible absorption spectrum of the azoindoxyl dye in a stained film prepared from a suspension of the microsome fraction of rat liver in 2% bovine plasma albumin (microsomes equivalent to 200 mg liver/ml). The film was fixed in glutaraldehyde at 0°C for 30 min before staining as described for the sections and was then mounted in glycerol. A film of the same thickness, treated in exactly the same way except for the absence of indoxyl acetate at the staining stage was inserted in the reference beam of the spectrophotometer to compensate for the effect of the coupling agent on the proteins.
Curve 2 shows the effect of treating the stained and control films with 1% osmium tetroxide before remounting in glycerol and recording the difference spectrum again. The prominent absorption maximum of the azo dye at 500 mμ is replaced by a shallow maximum at about 475 mμ when the osmium complex is formed, and a broad shoulder develops in this region. The general difference between the curves is seen clearly.

chemistry of osmium, although it awaits confirmation by structural analysis. The most stable chelates formed by this metal with bidentate ligands are those in which it possesses a principle valency of two (2, 18). In preparing such chelates from readily available high valence state osmium compounds, it is obligatory to react them with the ligand under reducing conditions (3, 7). It is therefore highly significant that the osmium complex of the azoindoxyl is only formed in tissues, which are known to reduce osmium tetroxide during fixation. The finely divided free dye does not react with osmium tetroxide. This observation also discounts the possibility that the reaction product is osmium black, formed by some reaction similar to that described by Hanker et al. (8). There is little question that in the complex, the osmium is linked to the dye *via* the imino group of the indoxyl moieties as well as to the azo groups in the molecule, since substitution of the hydrogen atom of the imino group in indoxyl acetate by methyl or acetyl, radicals which do not form links with metals, gives enzymically produced azo dyes that remain highly soluble and show no spectral changes when treated with osmium tetroxide. Thus, it appears that structures similar to IV are not present in the chelate, as might be expected from the absence of osmiophilia in the analogous azonaphthols; it further appears that the stable chelate contains the bidentate nitrogen system (V) similar to that present in well known osmium chelates (3, 7).

The most likely structure for the opaque complex is therefore one in which an azoindoxyl group from each of three dye molecules (VI) are linked together by divalent osmium to form an octahedral complex in which the metal exhibits its chief coordination valency of six. Scale models show that a relatively strain-free chelate would be formed in this way. The same type of association could also occur at each of the remaining azoindoxyl centers. The over-all effect would be the formation of an osmium-linked network or polymer containing about 70% by weight of the metal, an amount consistent with the high contrast obtained.

When a standard method for calculating the net charge on chelates (6) is used, it shows that one negative charge is produced as each osmium atom is bound in forming such a polymer. These negative charges would then alternate with the positive charges on the central carbonium ions of each constituent dye molecule (see VI) to produce an ionic lattice analogous in many ways to those occurring in crystals of metallic salts. The unusually low solubility of the osmium chelate is to be expected from this type of structure.

Dyes formed from simpler diazonium and tetrazonium salts and indoxyl are less suitable for electron microscopy, because, even after post-osmication, they are extracted from tissue during the embedding procedure. The osmium complexes of the simpler dyes are theoretically more soluble than those derived from hexazotized pararosaniline, as their potentialities for cross-linking by osmium are less, and they are unlikely to form large polymers with the characteristics described above.

It is relevant to comment on the divergence between the results given by the azoindoxyl

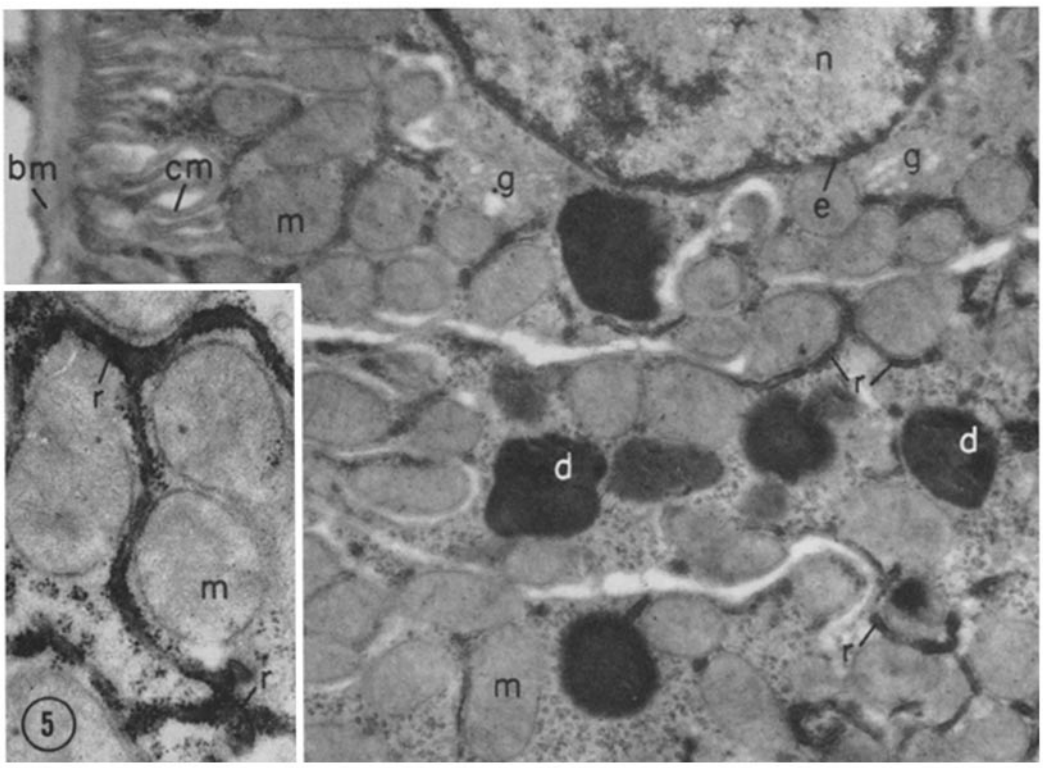



FIGURE 5 A thin section cut from a postosmicated 30 $\mu$ stained section embedded in Epon. The field shows part of the base of a cell from a proximal convoluted tubule. At the top is the nucleus (*n*), and at the left is the infolded cell membrane (*cm*) which rests on the basement membrane (*bm*). The dense osmium complex of the enzymically deposited stain can be seen within the cisternae of the rough-surfaced endoplasmic reticulum (*r*), within the nuclear envelope (*e*), and in the protein absorption droplets or lysosomes (*d*). There is no stain in the mitochondria (*m*) or Golgi zone (*g*). Electron micrograph. × 12,500. The inset shows the dense stain within the cisternae of the rough-surfaced endoplasmic reticulum at higher magnification. × 30,000.

procedure and those given by ferricyanide oxidation of indoxyls to indigoid dyes (9, 16). The pattern of droplet plus cytoplasmic staining seen in Fig. 1 does not change significantly over the pH range 4 to 7, whereas the indigogenic method gives relatively little cytoplasmic stain at pH 8.5, but progressively more down to pH 5 to 6 (5, 16). The result of the oxidation method at high pH has been interpreted by Shnitka and Seligman (20) as being due to ferricyanide inhibition of cytoplasmic esterase. However, biochemical studies have now shown that the oxidant does not inhibit any of the esterases that hydrolyze indoxyl acetate or a variety of other substrates (12). The weak cytoplasmic staining at high pH appears to be wholly due to the alternative pathway of oxidation of indoxyls to colorless products (9, 10) being more extensive in the cytoplasmic areas. This pathway is known to be influenced greatly by the tissue environment in which it occurs (10).

Thus, the azoindoxyl method for esterases has much to commend it for correlated cytochemical and biochemical studies. Moreover, the osmiophilia of the dye opens up the prospect of high resolution studies of the intracellular distribution of other hydrolases with the electron microscope by using appropriate substrates derived from indoxyl.

A more extensive description of various aspects of this work is in preparation.

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## REFERENCES


1. BAEYER, A., Ueber die Verbindungen der Indigogruppe, *Ber. deut. chem. Ges.*, 1883, **16**, 2188.
2. BRANDT, W. W., DWYER, F. P., and GYARFAS, E. C., Chelate compounds of 1,10-phenanthroline and related compounds, *Chem. Rev.*, 1954, **54**, 959.
3. BURSTALL, F. H., DWYER, F. P., and GYARFAS, E. C., Optical activity dependent on a six-covalent bivalent osmium complex, *J. Chem. Soc.*, 1950, 953.
4. DAVIS, B. J., and ORNSTEIN, L. E., High resolution with a new diazo reagent "Hexazonium Pararosaniline," *J. Histochem. and Cytochem.*, 1959, **7**, 297.
5. DELELLIS, R., and FISHMAN, W. H., The variable of pH in the bromindoxyl acetate method for the demonstration of esterases, *J. Histochem. and Cytochem.*, 1965, **13**, 297.
6. DIEHL, H., The chelate ring, *Chem. Rev.*, 1937, **21**, 39.
7. DWYER, F. P., GIBSON, N. A., and GYARFAS, E. C., The chemistry of osmium. Part IV. The preparation and resolution of the tris *o*, phenanthroline osmium II ion, *J. and Proc. Roy. Soc., N. S. Wales*, 1950, **84**, 68.
8. HANKER, J. S., SEAMAN, A. R., WEISS, L. P., UENO, H., BERGMANN, R. A., and SELIGMAN, A. M., Osmiophilic reagents: New cytochemical principle for light and electron microscopy, *Science*, 1964, **146**, 1039.
9. HOLT, S. J., Indigogenic staining methods for esterases, *in* General Cytochemical Methods, (J. F. Danielli, editor), New York, Academic Press Inc., 1958, **1**, 375.
10. HOLT, S. J., Some observations on the occurrence and nature of esterases in lysosomes *in* Ciba Foundation Symposium on Lysosomes, (A. V. S. de Reuck and Margaret P. Cameron, editors), London, J. & A. Churchill Ltd., 1963, 114.
11. HOLT, S. J., in preparation.
12. HOLT, S. J., and BARROW, P. C., in preparation.
13. HOLT, S. J., and HICKS, R. M., Combination of cytochemical staining methods for enzyme localization with electron microscopy, *in* The Interpretation of Ultrastructure, (R. J. C. Harris, editor), New York, Academic Press Inc., 1962, 193.
14. HOLT, S. J., and HICKS, R. M., Specific staining methods for enzyme localization at the subcellular level, *Brit. Med. Bull.*, 1962, **18**, 214.
15. HOLT, S. J., and SADLER, P. W., Studies in enzyme cytochemistry. II. Synthesis of indigogenic substrates for esterases, *Proc. Roy. Soc. London, Series B*, 1958, **148**, 481.
16. HOLT, S. J., and WITHERS, R. F. J., Studies in enzyme cytochemistry. V. An appraisal of indigogenic reactions for esterase localization, *Proc. Roy. Soc. London, Series B*, 1958, **148**, 520.
17. LUFT, J. H., Improvements in epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
18. NYHOLM, R. S., Recent stereochemistry of the Group VIII elements, *Quart. Rev. (London)*, 1949, **3**, 321.
19. SABATINI, D. D., BENSCH, K. G., and BARRNETT, R. J., Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation, *J. Cell Biol.*, 1963, **17**, 19.
20. SHNITKA, T. K., and SELIGMAN, A. M., Role of esteratic inhibition on localization of esterase and the simultaneous cytochemical demonstration of inhibitor sensitive and resistant enzyme species, *J. Histochem. and Cytochem.*, 1961, **9**, 504.
21. TICE, L. W., and BARRNETT, R. J., Diazophthalocyanins as reagents for fine structural cytochemistry, *J. Cell Biol.*, 1965, **25**, 23.