Table 1. Conductivity of poly(1-methyl-2-vinylpyridinium TCNQ) films.

Neutral TCNQ (% by wt)	$\sigma_{300°K}$ (ohm^{-1} cm^{-1})
0	$> 10^{10}$
2	$> 10^{10}$
5	3.3×10^{-6}
15	1.0×10^{-4}
22	1.7×10^{-4}
40	1.1×10^{-5}

Table 2. Conductivity of copoly(styrene,1-butyl-2-vinylpyridinium-TCNQ) films.

Neutral TCNQ in films (% by wt)	$\sigma_{300°K}$ (ohm^{-1} cm^{-1})
0	$> 10^{10}$
3	1.0×10^{-10}
6	1.3×10^{-9}
10	6.5×10^{-5}
15	1.1×10^{-3}
20	1.1×10^{-4}
25	1.0×10^{-4}
30	1.3×10^{-5}

cipitated salt was washed repeatedly with ethanol before drying in a vacuum over phosphorus pentoxide. Analysis indicated that seven-eighths of the iodide anions had been replaced by $TCNQ^-$. Films of this product were cast from dimethylformamide. Again conductivity was strongly dependent on the presence of added neutral TCNQ (Table 2).

Results similar to these two have been obtained with quaternized derivatives of other basic polymers, including poly(1-vinylimidazole), poly(4-dimethylaminostyrene), poly(ethyleneimine), poly(4-vinylpyridine), as well as with incompletely quaternized samples of poly(2-vinylpyridine). In all cases the conduction behavior is similar to that just described, ranging from less than 10^{-10} to 10^{-3} ohm^{-1} cm^{-1}, depending on the neutral TCNQ content of the polymer films. The conduction process is electronic, as demonstrated by experiments in which polarization or electrolytic effects have been shown to be absent after the passage of large amounts of charge. The conductivity as a function of temperature can be described by the expression $\sigma = \sigma_0 e^{-E/kT}$. Experimentally determined activation energies in the presence of added TCNQ are of the order of 0.1 to 0.3 ev.

Those TCNQ-containing films having relatively good conductivity, that is, approximately 10^{-5} ohm^{-1} cm^{-1}, have a matte surface and in several cases have been shown by x-ray diffraction to possess a degree of order that does not result from crystallites of neutral TCNQ; in systems having poly(2-vinylpyridine) as the basic unit, this order corresponds to a 6.3-Å spacing. Except at very high concentrations (more than 20 percent), there is no evidence of crystalline neutral TCNQ in the conductive films. The coincidental appearance of regularity with a sharp increase in conductivity over a narrow TCNQ concentration range strongly indicates that ordered structures play an essential part in the conduction process.

The maximum conductivity of the TCNQ-doped films of the various polymers examined lies in the region 10^{-3} to 10^{-5} ohm^{-1} cm^{-1}, regardless of the polymeric cation; the narrowness of this range suggests that the polymer backbone and side chains do not participate directly in charge transport.

In studies of crystalline ammonium and imonium salts of $TCNQ^-$ and $(TCNQ)^-_n$ ions it has been observed that conductivities of the complex salts are usually 10^5 to 10^7 times those of the simple salts (*4*). It is tempting to conclude that conduction in our polymeric TCNQ derivatives is associated with contiguous ordered regions in which the conduction process is related to that in the complex salts. It can be determined from Tables 1 and 2, however, that the overall ratio of neutral TCNQ to its radical anion in our most effectively conductive films is considerably less than unity, the value that would correspond to the $(TCNQ)^-_2$ ion.

Combination of the TCNQ radical anion with the cationic groups of most of the polymers we have prepared to date is apparently not stoichiometric.

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Osmiophilic Reagents: New Cytochemical Principle for Light and Electron Microscopy

Abstract. *Established histochemical procedures have been altered by utilizing reagents which contain a group capable of reacting selectively with OsO_4. This permits the cytochemical demonstration of a particular enzyme or functional group of a macromolecule with the light and electron microscope. The osmiophilic groups used thus far are mercapto, thiocarbamyl, and diazothioether. The "osmium black" produced at the site of reaction is electron opaque, is insoluble in lipids, and is not altered when the issue is embedded in acrylic and epoxy resins. Its fine amorphous character enables it to be maintained precisely in the electron beam.*

To use the electron microscope in cytochemistry, it is important to find reagents that will not only react selectively with the specimen to be viewed but increase the electron opacity at the site of a particular enzyme or functional group of the specimen and hence improve the contrast of the image. Reagents which contain elements of high mass density as well as high atomic number (*1, 2*), or have the ability to combine after the tissue reaction with

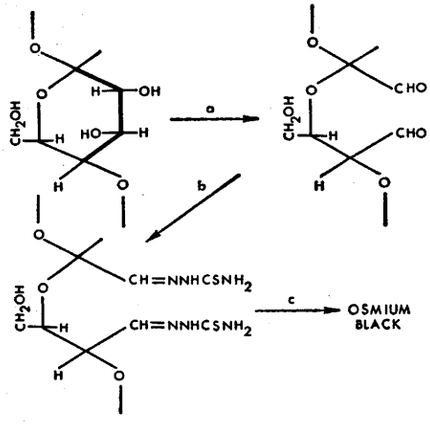


Fig. 1. Formulation for electron microscopic cytochemical demonstration of macromolecular diglycols. (*a*) Periodic acid. (*b*) Thiosemicarbazide, $H_2NNHCSNH_2$. (*c*) Osmium tetroxide.

such elements, would be suitable for this purpose. For tissue to be examined under the electron microscope, a number of individual methods for visualizing enzymes (*3*) or functional groups (*4*) have been developed in recent years. However, none of general applicability has appeared that offers the histochemist a general procedure for the design of reagents for the electron microscopic demonstration of various enzymes or functional groups.

Even though the stability of the iodine-carbon bond in the 50-kv electron beam has been demonstrated (*5*), reagents containing three iodine atoms were surprisingly ineffective in improving image contrast for the electron microscopic demonstration of histochemical methods (*6*). Our initial attempts to develop enzymatic methods with reagents having the ability to chelate with metals of high atomic number, such as mercury and lead, were unsuccessful because of the solubility of the chelates in organic solvents.

Although the effect of osmium tetroxide on image contrast in the electron microscope has been questioned (*7*), OsO_4 has long been the best fixative and stain for biological material in electron microscopy (*8*). Moreover, osmium is the densest element known, and calculations (*2*) have shown that maximum contrast is given by the material with greatest density based on equal thickness. Although oxides and mercaptides of osmium do not have the high density of the metal, the higher the atomic number, the greater the contrast should be, everything else being equal.

We, therefore, decided to incorporate moieties which strongly reduce OsO_4 (*9*) in our reagents for the cytochemical demonstration of enzymes and functional groups. A preliminary survey of reagents containing osmiophilic groups indicated that thiols reduced OsO_4 more readily than alkenes, alkynes, arylamines, or phenols. Reduction of OsO_4 was accomplished with other sulfur-containing groups such as some diazothioethers and the thiocarbamyl group, which has a mercapto group in the tautomeric imidthiol form (*10*).

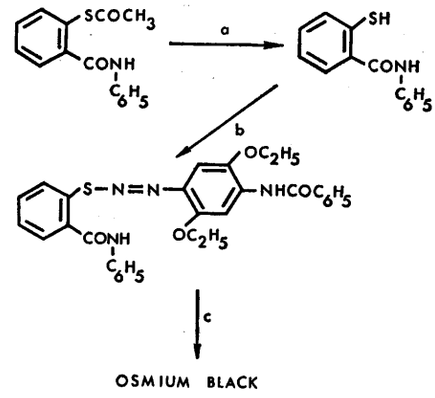


Fig. 3. Formulation for electron microscopic cytochemical demonstration of esterase. (*a*) Esterase. (*b*) Fast blue BBN salt. (*c*) Osmium tetroxide.

We have utilized this new principle in the demonstration of macromolecular diglycols in tissue by means of thiosemicarbazide. Thin sections (0.2 μ) embedded in acrylic or epoxy resin are placed on gold grids. The aldehyde groups, formed by periodic acid oxidation, condense with the hydrazino group of thiosemicarbazide. After rinsing to remove any unreacted thiosemicarbazide, the sections are exposed to OsO_4 vapor, whereupon the thiocarbamyl moiety of the aldehyde-thiosemicarbazone reacts with OsO_4 to yield "osmium black" (Fig. 1). To illustrate the new method histochemically we have demonstrated glycogen in the cytoplasm of liver, the mucopolysaccharide of Descemet's membrane of the cornea, mucus in goblet cells, and mucoprotein in the cuticular border of gut. An electron photomicrograph of a portion of a polymorphonuclear heterophil of spleen stained for glycogen by this method is shown in Fig. 2.

We first demonstrated esterases and phosphatases with phenylthiolacetate or 2-naphthylthiolacetate, and phenylthiolphosphate or 2-naphthylthiolphosphate, respectively. In preliminary experiments with 2-naphthalenethiol, a variety of diazonium salts were found to couple very rapidly, at both acid and alkaline *p*H, to yield insoluble yellow diazothioethers. Their reactivity with OsO_4 was sufficiently rapid when fast blue BBN salt and fast black B salt were used.

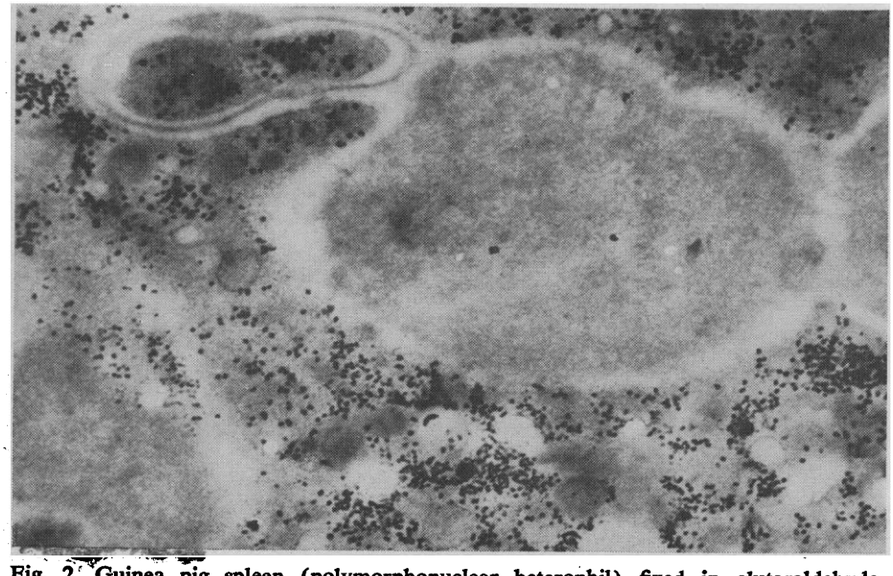
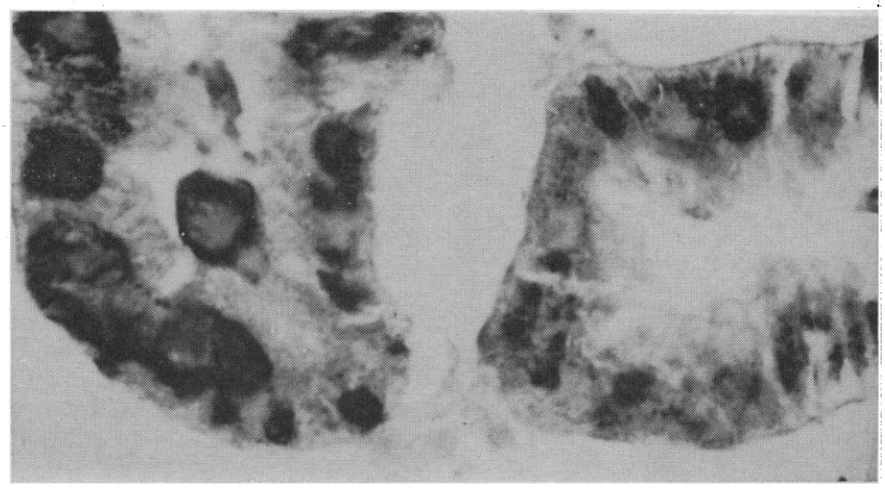


Fig. 2. Guinea pig spleen (polymorphonuclear heterophil) fixed in glutaraldehyde, embedded in araldite, cut 0.2 μ thick, mounted on gold grids, and stained for glycogen by treatment with periodic acid for 1 hour, thiosemicarbazide for 1 hour, and OsO_4 vapor for 4 hours. Glycogen appears in the electron microscope as discrete black granules scattered in the cytoplasm around the multiform nucleus. Glycogen was absent from controls in which either periodic acid or thiosemicarbazide was omitted.

These thiolacetates demonstrated esterase activity in the general cytoplasm of renal tubular cells upon incubation at room temperature for 20 minutes. When incubated at 0°C for 1 hour, the stained droplets corresponded exactly with the distribution noted with 1-thiolacetoxy-2-benzanilide. The M-band of fresh frozen sections of cardiac muscle, which is demonstrated with thiolacetic acid and lead ion, failed to hydrolyze the thio-ester substrates. This negative result with our method establishes the fact that M-band staining is not due to acetylcholinesterase activity. This observation was confirmed by Karnovsky (*11*) with our reagents.

We synthesized the following thiolacetates and used them as substrates for esterase: octadecyl thiolacetate, trityl thiolacetate, 1-thiolacetoxy-2-benzanilide, and 3-thiolacetoxy-2-naphthanilide. When used on sections of rat kidney, all of these substrates were split exclusively by esterase contained in the droplets of the tubular cells, and the thiols coupled with fast blue BBN to give diazothioethers which subsequently reduced OsO_4. The formulation of the reaction is shown in Fig. 3 for 1-thiolacetoxy-2-benzanilide and fast blue BBN. By selecting an appropriate substrate, the two major types of esterase in the renal tubular cells (*12*) could be distinguished, indicating that there are no special thioesterases. Droplets of esterase in glutaraldehyde-fixed kidney epithelium incubated with 1-thiolacetoxy-2-benzanilide are shown in Fig. 4. An electron micrograph showing the acetylcholinesterase activity in the synaptic clefts of a motor endplate is shown in Fig. 5. The advantage of 1-thiolacetoxy-2-benzanilide and 2-naphthylthiolacetate over phenylthiolacetate is that the former two substrates give good results with very thick sections needed for preparation for the electron microscope, whereas the last give good results only with very thin sections.

Phenylthiolphosphate or 2-naphthyl-



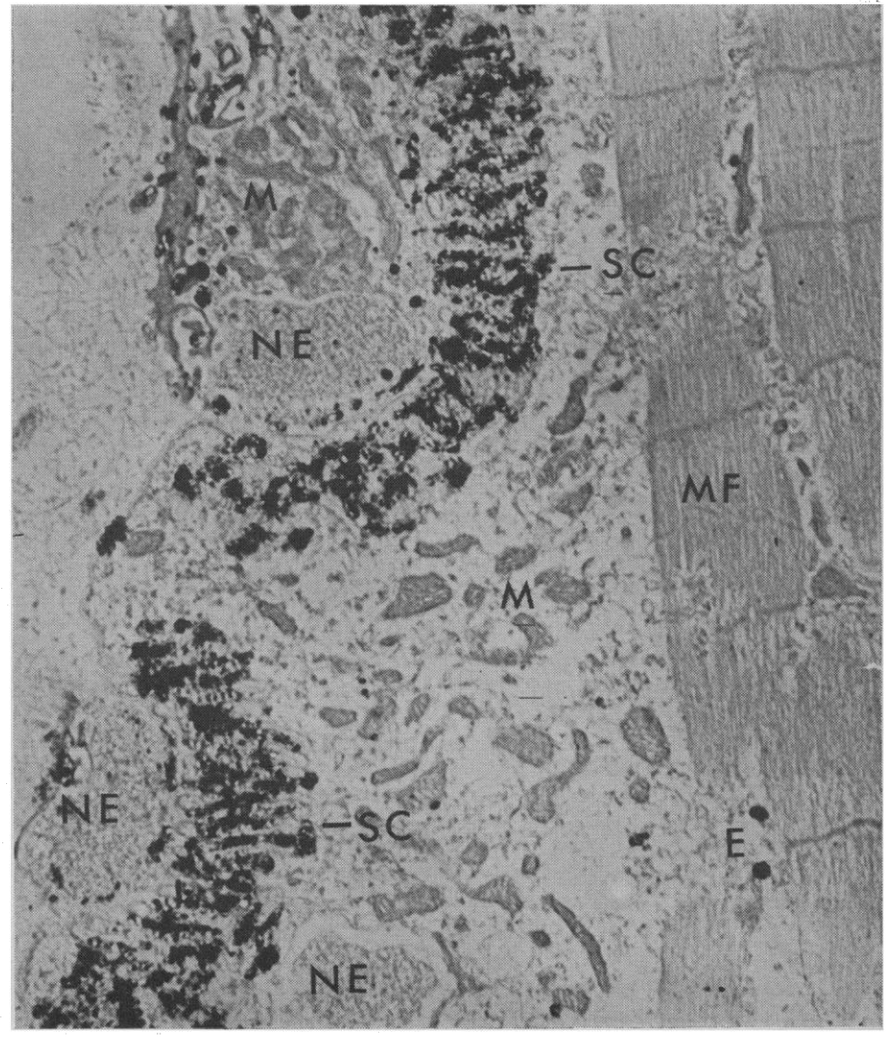
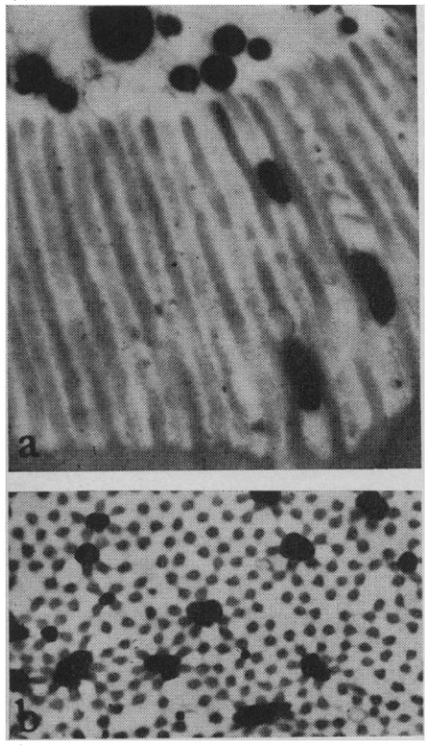


Fig. 4 (top right). Rat kidney fixed in glutaraldehyde, cut in frozen sections 2 μ thick, and incubated with 1-thiolacetoxy-2-benzanilide and fast blue BBN for 20 minutes at 37°C followed by OsO_4 vapor for 8 minutes. The most intense reaction is on the periphery of the esterase active droplets.

Fig. 5 (bottom right). Rat intercostal muscle fixed in formalin. The reaction was carried out on sections 40 μ thick; incubation was for 1 hour and osmication for 20 minutes. The thick sections were dehydrated, embedded in epon, cut in 0.2 μ sections, stained with lead hydroxide for fibrillar contrast, and photographed with an RCA EMU_3 model C electron microscope. The acetylcholinesterase reaction is intense in the synaptic clefts (*SC*). Other symbols: nerve cell endings (*NE*), mitochondria (*M*), myofibrils (*MF*), esterase activity (*E*). These structures may be compared with plate 22 in the atlas by K. R. Porter and M. A. Bonneville. [*An Introduction to the Fine Structures of Cells and Tissues* (Lea & Febiger, Philadelphia, 1963)]



thiolphosphate was used to demonstrate alkaline phosphatase in the brush border of cells of the proximal convoluted tubule and in the cuticular layer of intestine. Electron micrographs of intestine incubated with the latter substrate are shown in Fig. 6. Acid phosphatase was demonstrated exclusively in droplets in formalin-fixed sections

Fig. 6 (top left). The reaction for alkaline phosphatase was carried out on 40-μ sections at *p*H 9.0 for 15 minutes each of two changes of medium at 0°C with naphthylthiolphosphate and fast blue BBN, followed by osmication for 15 minutes. The thick sections were dehydrated, embedded in Vestopal-W, cut in thin sections (pale yellow interference color), and photographed with an RCA EMU$_3$ model E electron microscope. Alkaline phosphatase activity is evident in ovoid deposits on the microvilli of the cuticular border of rat intestine and in round deposits in the lumen of the gut; (*a*) in longitudinal section, (*b*) in cross section.

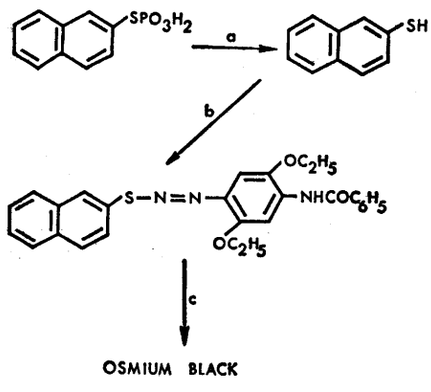
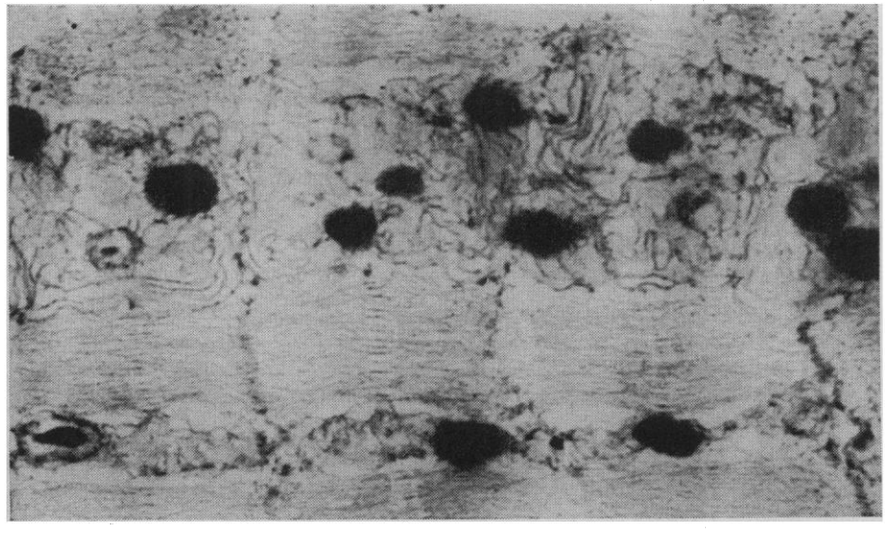


Fig. 7 (middle left). Formation for electron microscope demonstration of the phosphatases. (*a*) Alkaline or acid phosphatase. (*b*) Fast blue BBN salt. (*c*) Osmium tetroxide.

Fig. 8 (below). Rat heart, cut in fresh frozen sections 40 μ thick, stained for cytochrome oxidase by incubating with 1-hydroxy-4′-mercapto-2-naphthanilide and *p*-amino-diphenylamine for 20 minutes and exposed to OsC_4 vapor for 40 minutes. The thick sections were washed, dehydrated, and embedded in Vestopal-W, cut in thin sections (pale yellow interference color), and photographed with an RCA EMU$_3$ model E electron microscope. Cytochrome oxidase is shown in round and ovoid deposits within the mitochondria. The significance of the peripheral lighter zone in some of these deposits is not yet clear.



(4 μ) of renal tubular cells. When very thick sections (40 μ) were used the results were poor with phenylthiolphosphate but good with 2-naphthylthiolphosphate. The formation of the reaction for the phosphatases is given in Fig. 7.

Cytochrome oxidase was demonstrated in thick sections of freshly frozen heart after incorporating the thiol group into the naphtholic component of the reagents of the Nadi reaction (*13*) by the preparation of the osmiophilic reagent, 1-hydroxy-4′-mercapto-2-naphthanalide. This reagent was used with the improved reagents 4-amino-1-*N*,*N*-dimethylnaphthylamine (*14*) or *p*-aminodiphenylamine (*15*). The sections were exposed to OsO_4 vapor, washed, dehydrated, and embedded in Vestopal-W, Maraglas, or Araldite for sectioning. The discrete localization of ovoid deposits of osmium black in the mitochondria of rat myocardial cells is shown in Fig. 9. This electron micrograph may be compared with that shown by Sabatini *et al.* (*16*), prepared with reagents for cytochrome oxidase that apparently do not have a moiety that readily reduces OsO_4.

Osmiophilic groups can also be incorporated into diazonium salts, tetrazolium salts, and substrates for other enzymes such as the dehydrogenases, proteolytic enzymes, peroxidase, glucuronidase, sulfatase, monoamine oxidases, and aldolase. Diazonium salts with an osmiophilic moiety could also be coupled into antibodies or antigens for the demonstration of the antigen-antibody reaction in cells.

The chemical nature of "osmium black" is still under investigation. Although the reduction of osmium tetroxide by organic compounds is generally believed to result in hydrated osmium dioxide, $OsO_2 \cdot nH_2O$ (*17*), there is also indication that reduction to the metal occurs (*18*). Preliminary experiments indicate that the reduction of OsO_4 by osmiophilic groups in organic compounds occurs less rapidly in 2 percent buffered solution, *p*H 7.4, than in the absence of buffer. However, at any given temperature, the reduction of OsO_4 vapor is apparently more rapid than the reduction of a 2-percent unbuffered aqueous solution. Different groups reduce OsO_4 at different rates, for example, mercapto >> thiocarbamyl > diazothioether. In the search for reducing groups it was noted that some compounds produced various hues of

gray or brown, suggesting incomplete reduction of OsO_4. For example, reduction of 2-percent OsO_4 solution or OsO_4 vapor by thiourea gave a brown precipitate whereas thiosemicarbazide gave a black precipitate.

The properties of the reagents which are required for the various histochemical methods impose certain limitations on this new technique. If, for example, the products of the histochemical reaction before osmication have too great an affinity for lipid, the localization of the "osmium black" will reflect this shortcoming. In the continual redesign of reagents for perfecting histochemical methods for electron microscopy, it may become necessary to incorporate other groups for selective osmication in particular instances.

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19. Supported by research grant (CA-02478-10) from the National Cancer Institute, NIH. We acknowledge the valuable assistance of Lionel Katzoff in synthesis and characterization of the reagents, Hermine Dmochowski in histochemistry, Harriet Storm, Hannah Wasserkrug, Julia Silhan, and Richard Shuger. Intermediates for substrates were obtained from Cyclo Chemical Corporation, 1922 East 64 Street, Los Angeles 1, Calif. This work was presented in part at the Fifteenth Annual Meeting of the Histochemical Society in Chicago, Ill., on 11 April 1964, and in part, at the Second International Congress for Histochemistry and Cytochemistry in Frankfurt, Germany, on 17 August 1964.

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27 August 1964

Radium-226 and Polonium-210 in Leaf Tobacco and Tobacco Soil

Abstract. *Contents of radium-226 and polonium-210 in leaf tobacco and tobacco-growing soils vary with the source. The differences may result from production locality, culture, and curing. The polonium seems to be not entirely derived from the radium; plants probably take it up from the soil or air.*

Naturally occurring radioelements in manufactured tobacco and in smoke have been investigated many times. Earlier reports concern mostly beta activity of K^{40} (*1*), while recent studies mainly refer to alpha activity, especially of the radium and thorium series (*2*). The report on Po^{210} (*3*) is of special interest, for this element is volatile at the combustion temperature of manufactured tobacco.

Polonium-210 in tobacco plants is derived from either the soil or the air. It may be taken up directly from the soil or may result from radioactive decay of lead-210 or radium-226 taken up from the soil. It may also result from radioactive decay of the daughters of radon-222 deposited on the leaves. In re-examining the Po^{210} content of tobacco and in attempting to establish its origin, we studied the natural radioactivity in different types of leaf tobacco produced in various years in various localities. All samples had been stored in sealed glass containers or in hogsheads, without prolonged exposure to fresh air. Soils producing tobaccos in 1963 were sampled and tested for natural radioactivity in February 1964.

For determination of Po^{210}, 10-g samples of air-dried, finely cut tobacco were wet ashed in nitric acid with lead carrier. A sample was precipitated as the sulfate and chelated with ethylenediamine tetraacetic acid. The lead sulfide precipitated was dissolved in 3*N* HCl, and the Po^{210} was plated onto a nickel disc. The sample was mounted with ZnS phosphor, and the alpha particles, a measure of Po^{210} activity, were counted. With this technique the background is 0.01 count/min and the detection efficiency is 52 percent. The activity of the blank (0.04 count/min) has been subtracted from all Po^{210} data.

Quantitative measurements of Pb^{210} made by counting the beta activity of its Bi^{210} daughter failed because of the low activity, but beta activity data were qualitatively consistent with the Po^{210} analysis.

For Ra^{226} determination, 5 to 10 g of tobacco ash were fused with sodium carbonate along with barium carrier and Ba^{133} tracer. Barium and radium were separated from the calcium, and the barium was precipitated as the chromate and dissolved in perchloric acid. Recovery was determined by counting the gamma activity of Ba^{133} in the final solution, and radium was determined by counting the alpha activity of its gaseous daughter, Rn^{222}.

Natural activities found in the tobacco samples are shown in Table 1. Although lower than the others in Ra^{226} content, the 1938 tobacco samples also differ in type, growth locality, and culture and curing methods. There is a difference in Ra^{226} activity among flue-cured tobaccos from different areas. Tobacco from eastern North Carolina generally has lower Ra^{226} activity than that from Georgia. The same tendency is shown in Table 2 in the natural activity in tobacco-producing soils. The Ra^{226} content of soil samples from Tifton, Georgia, is higher than that of samples from Oxford, North Carolina. Fertilizer added to the soil may contribute in part to this difference. Sample No. 4, from a field continuously planted to burley tobacco, is high in available phosphorus and contains 2 to 3 times more Ra^{226} than the others. Similarly, sample No. 8, from a field continuously planted to tobacco and fertilized with 225 kg of P_2O_5 per hectare each year (200 pounds per acre), also has a higher Ra^{226} content.

Certain phosphate rocks contain