phenol. The investigation for the presence of the xylenol in urine was carried out using gas chromatography. Phenols were separated from the urine of a dog that had received 18.7 g of metaxalone by continuous ether extraction of acidified urine. The acids were removed from the extract with $NaHCO_3$. Injection of an aliquot onto a 10% Apiezon on Chromosorb W column at 185° using a thermoconductivity detector showed the presence of 3,5-xylenol which was not present in control urine. Peak-height comparison with known amounts of the xylenol indicated that 0.6% of the dose was present in the urine. Enzymatic hydrolysis of a sample of the urine with glucuronidase and sulfatase did not increase the amount found. The urine was also investigated for the presence of 3-methyl-5-carboxyphenol by thin layer chromatography. The presence of this acid could not be definitely confirmed, although a spot corresponding to the known acid was found using toluene–ethyl formate–formic acid (5:4:1) and benzene–dioxane–acetic acid (18:5:0.8) on silica gel, from an extract of enzymatically hydrolyzed urine.

Since urinary excretion of metaxalone accounted for the major portion of the dose and only small amounts of unchanged drug appeared in the feces, the drug must be well absorbed in these species. These results indicate that in man and dog the metabolite changes shown in Scheme I take place.

The excretion of metaxalone-^{14}C was studied in the rat, rabbit, and dog. The rabbit appeared to excrete the major portion of the radioactivity in the urine very rapidly; 96% appeared within the first 48 hr. Within the same period, 11% appeared in the urine of the dog. The pattern of metabolites, by chromatography, appeared to be similar in these two species. The rat excreted 71% in the urine during a corresponding time. The pattern of metabolites in the rat, however, was different. At least five separate, radioactive spots could be distinguished on chromatography of the urine. These were not identified.

Design and Synthesis of Thiolesters for the Histochemical Demonstration of Esterase and Lipase *via* the Formation of Osmiophilic Diazo Thioethers[1]

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The design, preparation, and properties of thiolesters as substrates for the cytochemical demonstration of esterolytic enzymes is described. The relative reactivity with osmium tetroxide of several diazo thioethers formed by S coupling with diazonium salts of the thiophenols resulting from enzymatic hydrolysis of the thiolesters was studied. This information has resulted in the development of a new technique for the demonstration of esterases and lipase by the selective deposition at the enzyme sites of osmium black, an ideal end product for light and electron microscopy. Examples of results with light and electron microscopy are included.

This investigation was prompted by the need for developing methods for esterase and lipase in electron microscopy that, in addition to utilizing well-established histochemical reactions, would yield electron-opaque end products.[2a-d] Thiolesters appeared to be worthy of synthesis as substrates to fulfill this need for several reasons. Fatty acid esterases have been known for some time to hydrolyze thiolesters.[3a-c] The thiophenols and thionaphthols produced on hydrolysis of aryl thiolesters are unable to undergo nuclear coupling with diazonium salts[4a] but do couple very readily on the sulfur (Figure 1) at both acid and alkaline pH to form insoluble yellow diazo thioethers (S-azo compounds[4a-c]). This would permit a rapid capture reaction, upon enzymatic hydrolysis of the thiolester, which would greatly improve localization. Moreover, aryl diazo thioethers are quite unstable and are readily decomposed to yield thiophenols.[5]

Since thiophenols as well as thiols readily reduce OsO_4 and react with it to form mercaptides, it was expected that conditions would be found where diazo thioethers would react with OsO_4 to yield osmium black, an ideal end product for light and electron microscopy.[2a-c] A formulation for the over-all reaction is shown in Figure 1.

Once it was shown[2a,b] that phenyl thiolacetate (I) gave satisfactory histochemical localization of esterase in thin, formalin-fixed sections of rat kidney and rat liver, other thiolesters were prepared in three categories. 2-Naphthyl thiolacetate (II) was prepared to study the effects of increasing the size of the molecule, and two large aliphatic thiolesters were prepared to compare esterase localization with that of the aromatic esters. These two substrates were the octadecyl thiolacetate (III) and the triphenylmethyl (trityl) thiolacetate (IV).

(1) This investigation was supported by a research grant (CA-02478) from the National Cancer Institute, U. S. Public Health Service, Washington, D. C.

(2) (a) J. S. Hanker, A. R. Seaman, L. P. Weiss, H. Ueno, R. A. Bergman, and A. M. Seligman, *Science*, **146**, 1039 (1964); (b) A. M. Seligman in Proceedings of the Second International Congress of Histo- and Cytochemistry, Springer-Verlag, 1964, p 9; (c) J. Hanker, A. Seaman, L. Weiss, H. Ueno, H. Dmochowski, L. Katzuff, and A. M. Seligman, *J. Histochem. Cytochem.*, **13**, 3 (1965); (d) L. A. Sternberger, E. J. Donati, J. S. Hanker, and A. M. Seligman, *Exptl. Mol. Pathol.*, in press.

(3) (a) J. Suzuoki and T. Suzuoki, *J. Biochem.* (Tokyo), **40**, 599 (1953); (b) J. Suzuoki and T. Suzuoki, *Nature*, **173**, 83 (1954); (c) G. B. Koelle and J. S. Friedenwald, *Proc. Soc. Exptl. Biol. Med.*, **70**, 617 (1949).

(4) (a) A. Hantzsch and H. Freese, *Ber.*, **28**, 3237 (1895); (b) P. Friedlaender and A. Chwala, *Monatsh.*, **28**, 247 (1907); (c) J. S. Hanker, L. Katzoff, L. D. Aronson, M. L. Seligman, H. R. Rosen, and A. M. Seligman, *J. Org. Chem.*, **30**, 1779 (1965).

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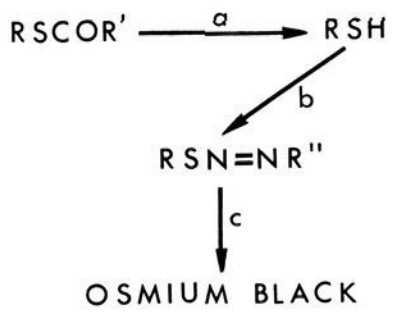

Figure 1.—Formulation of reactions for cytochemical demonstration of esterolytic enzymes. a, esterolytic enzyme; b, diazonium salt; c, OsO_4 vapor.

In order to emulate the good results and distribution of esterase noted[6] with the acetate of naphthol AS (3-hydroxy-2-naphthanilide), 2-thiolacetoxybenzanilide (V), 3-thiolacetoxy-2-naphthanilide (VI), and 3-thiolacetoxy-2-naphth-*o*-toluide (VII) were prepared. It was anticipated that the solubility of V would be similar to that of naphthol AS acetate due to the effect of the larger sulfur atom.

The propionate (VIII) of 2-mercaptobenzanilide (IX) was prepared in order to compare the electron microscopic localization of propionyl cholinesterase in rat heart with that reported by Karnovsky[7] using propionylthiocholine and copper ferricyanide.

After establishing the usefulness of the substrates for esterase[8] we turned our attention to substrates for pancreatic lipase, an enzyme that is uniquely activated by bile salts thus enabling measurement of its activity in the presence of considerable esterase activity. The nonanoate esters of 2- and 1-naphthol are preferentially hydrolyzed by pancreatic lipase in the presence of bile salts.[9] The nonanoate ester of naphthol AS (3-hydroxy-2-naphthanilide) has been used as a substrate for the histochemical demonstration of pancreatic lipase.[10] We therefore prepared the nonanoates (X and XI, respectively) of 2-naphthalenethiol and 2-mercaptobenzanilide (IX) for testing[11] as substrates for the electron microscopic demonstration of pancreatic lipase. The use of thiolphosphates for the demonstration of acid and alkaline phosphatase by virtue of conversion to osmiophilic diazo thioethers has also been reported.[2a,b]

The esterases of rat kidney hydrolyzed I or II more readily than V or III, IV, VI, and VII. The distribution of esterase activity with I or II as substrates was diffusely scattered in small granules within the cytoplasm of the renal tubular epithelial cells, whereas the esterase activity with V as substrate (as well as with III, IV, VI, and VII) was largely confined to large droplets within the tubular epithelium, especially so when the more generally distributed esterase was inhibited by conducting the histochemical reaction at 0° rather than at 37°.[8a] These two localizations of esterase activity corresponded to inhibitor-sensitive and inhibitor-resistant esterase, respectively.[6] An advantage of II over V was its ability to penetrate deeper into the tissue slice in histochemical preparations of unfrozen tissue for the electron microscope.[8] Substrate I gave good results only in very thin preparations for light microscopy, but resulted in diffusion when thick preparations were tried for electron microscopy. With pancreatic lipase,[11] on the other hand, XI was a more suitable substrate than X. It was hydrolyzed better in the presence of the enzyme activator, taurocholate, and localization of the deposits was more precise. Figures 2–5 show the precise localization of esterase achieved with sub-

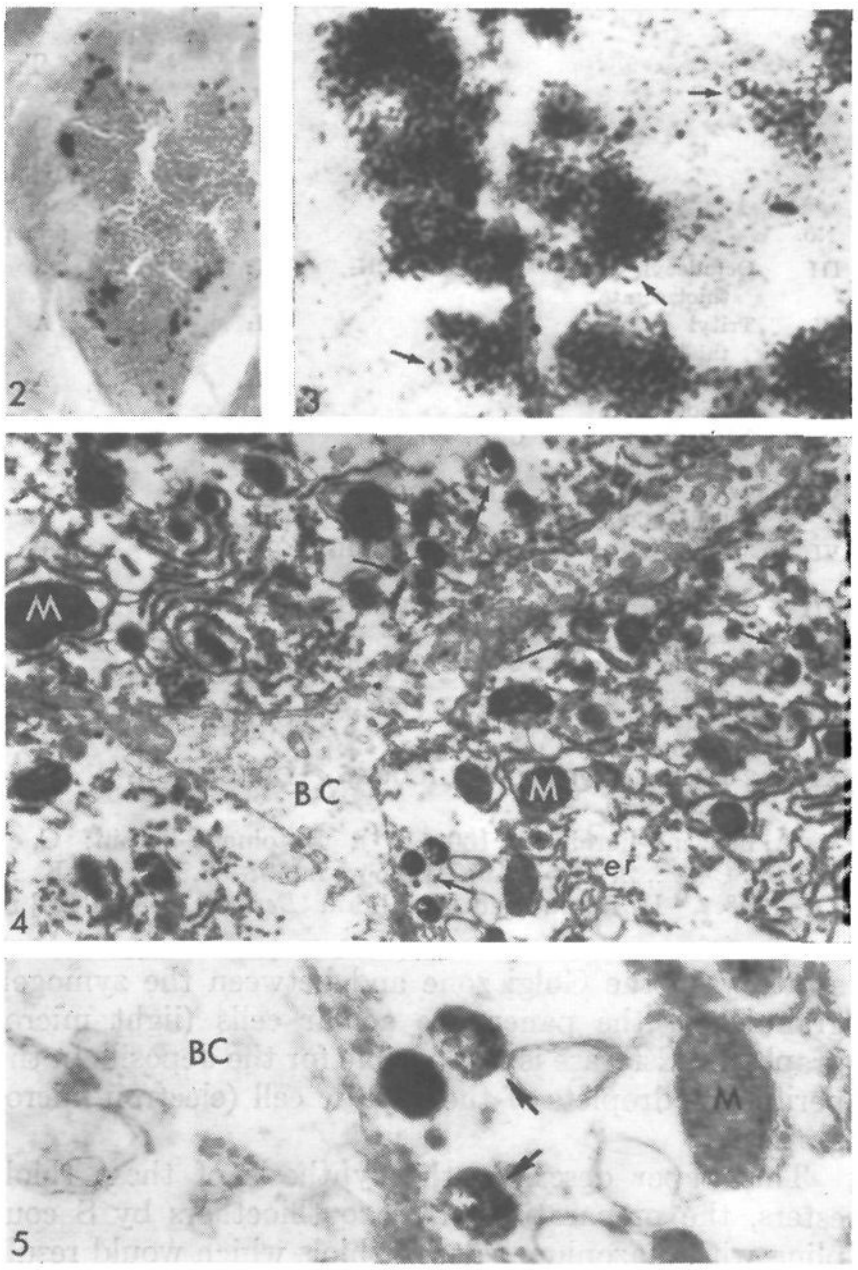

Figure 2.—Pancreas from fasted rat was fixed in calcium–formol for 24 hr and 4-μ frozen sections were stained for esterase using the substrate, 2-thiolacetoxybenzanilide (V). Osmium black is deposited in the Golgi region of the acinar cells.

Figure 3.—Pancreas from fasted dog was fixed in calcium–formol for 24 hr and 4-μ frozen sections were stained for esterase using the substrate V. Osmium black, denoting sites of esterase activity, is deposited in the apical region of the acinar cells, surrounding and outlining the closely packed zymogen granules (arrows).

Figure 4.—Electron micrograph of rat liver fixed in calcium–formol for 24 hr. Unfrozen thick slice was treated for esterase with substrate V, osmicated for 60 min in vapor and embedded in Araldite. In making ultrathin sections, care was taken to include one surface of the slice where reaction would be maximal. Note small dense deposits in droplets (arrows) near biliary capillary (BC). Some droplets (long arrows) have a surrounding membrane. Mitochondria (M) and endoplasmic reticulum (er) were readily identified.

Figure 5.—Higher magnification of Figure 4 reveals the pattern of deposition of osmium black within the two incompletely filled peribiliary droplets (arrows).

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TABLE I
THIOLESTERS
RSCOR'

No.	Compd	R	R'	Method of prepn	Recrystn solvent[a]	Mp or bp (mm), °C	Yield, %	Formula	Calcd, % C	Calcd, % H	Calcd, % S	Found, % C	Found, % H	Found, % S
III	Octadecyl thiolacetate	$CH_3(CH_2)_{16}CH_2$	CH_3	A		38[b]	78							
IV	Trityl thiolacetate	$(C_6H_5)_3C$	CH_3	A	A	139–140[c]	64							
I	Phenyl thiolacetate	C_6H_5	CH_3	A		107–108 (10)[d]	80							
II	2-Naphthyl thiolacetate	$C_{10}H_7$	CH_3	C		50–51[e]	83							
V	2-Thiolacetoxybenzanilide	$C_6H_5NHCOC_6H_4$	CH_3	B	B	126–127	68	$C_{15}H_{13}NO_2S$	66.4	4.8	11.8	66.0	4.5	11.3
VI	3-Thiolacetoxy-2-naphthanilide	$C_6H_5NHCOC_{10}H_5$	CH_3	B	B	162–164	33	$C_{19}H_{15}NO_2S$	71.0	4.7	10.0	70.7	4.6	9.8
VII	3-Thiolacetoxy-2-naphth-*o*-toluide	$C_7H_7NHCOC_{10}H_5$	CH_3	B	C	151–152 dec	50	$C_{20}H_{17}NO_2S$	71.6	5.1	9.6	72.0	5.2	9.2
VIII	2-Thiolpropionoxybenzanilide	$C_6H_5NHCOC_6H_4$	CH_3CH_2	B	B	103–105	52	$C_{16}H_{15}NO_2S$	67.3	5.3	11.2	67.5	5.3	11.2
X	2-Naphthyl thiolnonanoate	$C_{10}H_7$	$CH_3(CH_2)_7CH_2$	A		223–226 (1)	40	$C_{19}H_{24}OS$	76.0	8.1	10.7	76.1	7.8	10.6
XI	2-Thiolnonanoylbenzanilide	$C_6H_5NHCOC_6H_4$	$CH_3(CH_2)_7CH_2$	A	D	81	32	$C_{22}H_{27}NO_2S$	71.5	7.4	8.7	71.3	7.5	8.9

[a] A, benzene–ligroin (bp 100–115°); B, toluene–ligroin; C, ethanol–water; D, ligroin. [b] J. S. Showell, J. R. Russell, and D. Swern [*J. Org. Chem.*, **27**, 2853 (1962)] report mp 38–38.5°. [c] B. K. Morse and D. S. Tarbell [*J. Am. Chem. Soc.*, **74**, 416 (1952)] report mp 137–139°. [d] H. Böhme and H. Schram [*Ber.*, **82**, 453 (1949)] report bp 110° (11 mm). [e] Lit.[14] mp 53.5°.

strate V in the Golgi zone and between the zymogen granules of the pancreatic acinar cells (light micrographs). Esterase is responsible for the deposits in the peribiliary droplets of the hepatic cell (electron micrographs).

This paper describes the synthesis of these thiolesters, the preparation of diazo thioethers by S coupling with diazonium salts of thiols which would result from enzymatic hydrolysis of the thiolesters and the relative reactivity of the diazo thioethers with OsO_4.

Experimental Section[12]

2-Mercaptobenzanilide (IX).[13]—To a stirred solution of aniline (18.6 g, 0.2 mole), *o*-mercaptobenzoic acid (15.4 g, 0.1 mole), and pyridine (9.8 g, 0.13 mole) heated to 70° on an oil bath was gradually added PCl_3 (9.5 g, 0.065 mole). At no time was the pot temperature allowed to rise over 85°. After the addition was completed, the oil bath was heated to 115° for 5 hr. Stirring was continued until the golden yellow mass which formed stopped the magnetic stirring bar. At this point no further attempt was made at stirring. The mixture was cooled to room temperature and dissolved in a minimum amount of ethanol. The ethanol solution was placed in the refrigerator overnight. Water was then added until cloudiness occurred after which it was allowed to stand in the refrigerator several hours until a precipitate formed. The first precipitate which was mainly disulfide (mp 235–237°) was discarded, water was again added, and the mixture was allowed to stand, several days, if necessary, until precipitation occurred. The crude product (10 g, 40%) was collected by filtration, mp 94–96°, and could be used for the acetylation. An analytical sample melting at 109–110° was obtained upon recrystallization from ethanol and treatment with Norit A; λ_{max}^{EtOH} 227 and 255 mμ (ε 11,800 and 11,500).

Anal. Calcd for $C_{13}H_{11}NOS$: C, 68.11; H, 4.84; S, 13.96. Found: C, 67.67; H, 4.86; S, 13.45.

It is of interest to note that when we prepared IX by a modification of the procedure of Hopper, *et al.*,[14] it melted at 109–110° as compared with the reported melting point of 236–237°. The lower melting material had an infrared spectrum practically identical with that of the higher melting material but had an SH stretching absorption at 2550 cm^{-1} which was absent in the higher melting material. Further evidence that the higher melting compound was a disulfide is provided below.

The 60-Mc nmr proton spectrum of the lower melting material in dimethyl sulfoxide with tetramethylsilane as an internal standard consists of a singlet at 2.05 ppm due to SH and multiplets centered at 7.5 and 7.9 ppm due to aromatic protons. The ratio of the area of the singlet to the multiplets is 1:9 which is consistent with the structure. No chemical shift for SH was observed in the nmr proton spectrum of the higher melting material.

The ultraviolet spectrum in ethanol of the lower melting material has a λ_{max} at 227 mμ for thiol which is absent in the higher melting material.

The lower melting material gave a positive nitroprusside test, reduced OsO_4, and coupled with diazonium ions to give yellow diazo thioethers. These properties were absent in the higher melting material which was always obtained as a by-product in the synthesis of the thiol, especially when the temperature was allowed to rise over 85° for a short period. Moreover, on acetylation, the lower melting material gives a product (V) which has an infrared spectrum with two strong carbonyl stretching frequencies. The first appears at 1690 cm^{-1} and is consistent with the thiolester band which is lower than the normal ester band due to increased resonance. The second appears at 1645 cm^{-1} and is consistent with the amide I band. The higher melting material on acetylation gave a product having only one carbonyl frequency in the region of the amide I band, showing broader absorption and shifted to lower wavelength than the amide I band in IX. We presumed that the acetylation had occurred in this case on the anilido nitrogen. On the basis of these observavations, it is certain that the lower melting compound is 2-mercaptobenzanilide and it appears that the compound melting at 236–237° is the corresponding disulfide.

Thiolesters (Table I).—Three procedures were used for acylation of the thiols: method A, refluxing with the acyl chloride; method B, refluxing with the acyl chloride and the corresponding fatty acid; and method C (for acetylation), treatment with acetic anhydride and H_2SO_4.

Compounds I, III, IV, X, and XI were prepared by method A. The appropriate thiol was refluxed over a steam bath in a large excess of acyl chloride for 2 hr. The unreacted acyl chloride was then removed under vacuum and the product was distilled under

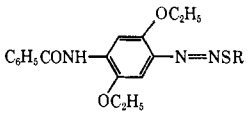
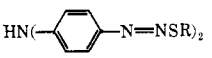
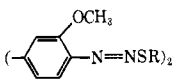
(12) All melting points are uncorrected; analyses were performed by Clark Microanalytical Laboratories. Infrared spectra were recorded on a Perkin-Elmer Model 237 spectrophotometer in KBr disks, electronic spectra on a Perkin-Elmer Model 202 spectrophotometer, and nmr spectra on a Varian Associates A-60A spectrometer.

(13) This material is now commercially available from the Cyclo Chemical Corp., Los Angeles 1, Calif., or Polysciences, Inc., Rydal, Pa. 19046.

(14) The arylamides of the thiolcarboxylic acids were prepared by the procedure outlined by J. V. Hopper, J. H. MacGregor, and F. J. Wilson, *J. Soc. Dyers Colourists*, **57**, 6 (1941). The temperature must not be allowed to rise over 85° during the addition of the PCl_3 because this increases the proportion of disulfide formed.

TABLE II

PROPERTIES OF DIAZO THIOETHERS

R	Mp, °C	Formula	Calcd, % C	H	N	S	Found, % C	H	N	S	λ_{max}^{EtOH}, mμ (ε)	Time for reaction with OsO_4, min[a]
												
Phenyl	86–88	$C_{23}H_{23}N_3O_3S$	65.5	5.6	9.0	7.6	65.6	5.3	9.7	7.4	260, 420 (8500, 19,000)	4
2-Naphthyl	97–98	$C_{27}H_{25}N_3O_3S$	68.9	5.2	8.9	6.8	68.8	5.3	8.8	6.7	264, 425 (12,000, 18,000)	4
												
Phenyl	66–67	$C_{24}H_{19}N_5S_2$	65.3	4.3	15.9	14.5	35.6	4.8	15.6	14.3	265, 452 (13,300, 60,800)	7
2-Naphthyl	89–92	$C_{32}H_{23}N_5S_2$	70.9	4.3	12.9	11.8	71.3	4.7	13.2	12.0	264, 451 (30,000, 16,000)	11
												
Phenyl	95–97	$C_{26}H_{22}N_4O_2S_2$	64.2	4.5	11.5	13.2	64.4	4.6	11.1	13.1	262, 405 (7500, 34,000)	19
2-Naphthyl	103–105	$C_{34}H_{26}N_4O_2S_2$	69.7	4.4	9.6	10.9	69.7	4.8	9.1	11.2	264, 419 (17,500, 26,000)	20

[a] ±0.5 min.

vacuum if a liquid, or crystallized and recrystallized from a suitable solvent if a solid (Table I).

Compounds V–VIII were prepared by method B. The appropriate arylamide[14] of a thiolcarboxylic acid was refluxed in an excess of 1:1 (v/v) mixture of acyl chloride and the corresponding fatty acid for 20 min. After cooling, the mixture was slowly poured on ice with stirring, whereupon the crude product precipitated. The product was collected on a filter and washed with water. It was then taken up in a minimum amount of ethanol, treated with Norit A, and filtered, and the product precipitated by the addition of water (Table I).

Compound II was prepared by method C inasmuch as attempts to prepare it by the reported procedure[15] resulted in a product which melted much too low and contained a considerable amount of free thiol. The 2-naphthalenethiol was dissolved in a 20% excess of acetic anhydride. Three drops of concentrated H_2SO_4 were added, and the yellow solution became purple. The solution was heated on a steam bath for 10 min and then poured on ice. The resulting precipitate upon one recrystallization from ethanol yielded a product (83%) melting at 50–51°; lit.[15] mp 53.5°.

Preparation of Diazo Thioethers (Table II).—To a filtered cold aqueous solution (1 g/10 ml) of the commercially available, stabilized diazonium salt was added 20 ml of ethanol. The solution was chilled in an ice bath and the precipitated salts were removed by filtration. This solution was then added to a solution of an equimolar amount[16] of thiophenol or 2-naphthalenethiol in 10 ml of ethanol. The yellow diazo thioether which precipitated immediately or after the addition of a little water, was filtered, washed with water, and dried in a vacuum at room temperature for a few hours. Although several of these diazo thioethers were stable for several months when stored at room temperature, attempts at recrystallization generally resulted in decomposition with the evolution of nitrogen. They were more stable than the zinc or fluoroborate salt of a diazotized aminodiazo thioether reported earlier,[4c] or the azoprotein prepared by coupling it into immune globulin.[2d]

Relative Reactivity of Diazo Thioethers with OsO_4 (Table II).—To test the reactivity of diazo thioethers with OsO_4, a number of diazo thioethers were prepared as described in the preceding paragraph by coupling several diazonium salts commonly used in histochemical methods with thiophenol and 2-naphthalenethiol. The most reactive diazo thioethers, in decreasing order (Table II), were obtained from diazotized N-(4-amino-2,5-diethoxyphenyl)benzamide (fast blue BN), tetrazotized 4,4′-diaminodiphenylamine (fast black B), and tetrazotized *o*-dianisidine (fast blue B).

Diazo thioethers at room temperature did not reduce 2% OsO_4 solution whether buffered to pH 7.4 or unbuffered. When the solutions were heated on a steam bath the reduction occurred very readily and more rapidly in the unbuffered solution. The reduction of OsO_4 occurred most rapidly near room temperature when moist solid diazo thioethers were exposed to OsO_4 vapor. The relative reactivity of the various diazo thioethers to OsO_4 vapor was tested in the following manner. Approximately 1 mg of diazo thioether was rubbed into the center of a small disk of Whatman No. 1 filter paper. The filter paper disks were moistened with water and placed in the grooves of a Chen staining rack (type A)[17] and placed in OsO_4 vapor in a tightly covered staining dish containing 0.5 g of OsO_4 and a few drops of water to keep the chamber moist. The staining dish was supported by a piece of wire gauze on a flask ring[18] in a water bath at 45–47° so that only the bottom of the dish was in contact with the water.[19] The filter papers were kept in the chamber until the color of the yellow diazo thioether was completely replaced by osmium black and the time was recorded (Table II).

Osmium black prepared in 0.0025 *M* quantities with 1 equiv of the appropriate thiol, washed thoroughly with organic solvents and dried, was found to contain considerable amounts of mercaptide of osmium. Analytical data[20] suggested that at least 75% of the material is mercaptide and the osmium content is 53–70%. Since the carbon content is too low and the osmium content too high for a 1:1 or 2:1 ratio of thiol:osmium, OsO_2 must be present. The presence of metallic osmium was ruled out by its failure to conduct electricity.[20] The analytical data and interpretation will be reported in detail elsewhere.

Acknowledgment.—We wish to thank Hannah L. Wasserkrug and Jay S. Copeland for valuable technical assistance.

(15) F. Krafft and R. Schönherr, *Ber.*, **22**, 821 (1889).

(16) The quantity of stabilized commercial diazonium salt containing an equimolar amount of diazonium component as the zinc chloride salt was calculated from the composition of the commercial salt supplied by the manufacturer. In the case of fast blue B and fast black B, a 2:1 molar ratio of thionaphthol to diazonium salt was used since they are tetrazonium salts.

(17) Purchased from Arthur H. Thomas Co., Philadelphia, Pa.

(18) Purchased from Penn-Chem Corp., Lancaster, Pa.

(19) A photograph of this apparatus has appeared in the paper by A. M. Seligman, J. S. Hanker, H. Wasserkrug, H. Dmochowski, and L. Katzoff, *J. Histochem. Cytochem.*, **13**, 629 (1966).

(20) Analyses and conductivity measurements were performed by Dr. F. Kasler, Department of Chemistry, University of Maryland, College Park, Md.