A NEW STAINING METHOD (OTO) FOR ENHANCING CONTRAST OF LIPID-CONTAINING MEMBRANES AND DROPLETS IN OSMIUM TETROXIDE-FIXED TISSUE WITH OSMIOPHILIC THIOCARBOHYDRAZIDE (TCH)

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Although the introduction of fixation of tissue with osmium tetroxide by Palade (8) ushered in the modern era of electron microscopy of biological materials, a need for greater contrast and greater resolution in visualizing membranous structures has stimulated the introduction of methods for staining with heavy metal salts, in order to enhance the delineation of the fine architecture of cells. This is especially necessary because of the poor contrast of osmium tetroxide–fixed tissue when the epoxy resins are used as embedding materials (9). These methods have relied upon specific affinities of heavy metal salts for various macromolecular components of the cell such as proteins, polysaccharides, nucleoproteins, or lipoproteins (3, 9, 17, 18). Such staining techniques do not necessarily enhance the staining of the same components of the cell originally stained by the osmium tetroxide, although interesting and important staining effects have been produced thereby.

In the course of developing cytochemical methods for electron microscopy, based upon the principle of designing histochemical reagents that yield osmiophilic end products (6, 10, 11), we have discovered a new way to enhance especially the contrast of the lipid components of the cells stained by osmium tetroxide during the initial fixation. On treatment with an excess of thiocarbohydrazide ($H_2NNHCSNHNH_2$, TCH), one end of the molecule attaches to the osmium in the tissue and, when this is followed by exposure to osmium tetroxide, more osmium is bound to these sites. The new method (OTO) utilizes this bridging phenomenon (5) and, although contrast is increased thereby in all osmiophilic components of tissue, greatest enhancement of contrast is produced in tissue components holding the most osmium, i.e., lipid.

The OTO method consists of exposing ultrathin sections of Araldite-embedded, osmium tetroxide–fixed tissue (8) on gold, stainless steel, or nickel grids,[1] without a supporting membrane, to a 1% hot aqueous solution of TCH[2] for 1 hr at 50°C,[3] followed by several washes with hot water (initial temperature 50°C) for 10 to 15 min to remove unbound TCH. The sections are then exposed to osmium tetroxide again, resulting in further deposition of osmium. The best results are obtained by exposing the sections to osmium tetroxide vapor[4] for 1 hr in a closed vessel suspended in a water bath at 60°C as described earlier (11), or very good results may be obtained with perhaps a little less contrast and considerably less cost by treating the grids with a 2% solution of osmium tetroxide

[1] Any inert metal may be used. When copper grids are used, TCH binds to surface oxides resulting, on exposure to osmium tetroxide, in blackening of the grid and in a tendency for the black deposits to scale and contaminate the specimens. This could be only partly eliminated by prior washing of copper grids with acetic acid. Gold, stainless steel, or nickel grids were most useful.

[2] Thiocarbohydrazide may be purchased from Distillation Products Industries, Rochester, New York, or Polysciences, Inc., Rydal, Pennsylvania.

[3] This procedure and the washing procedure were conducted in a porcelain spot plate. Each depression had a capacity of 1 ml and held 1 to 2 grids. During the incubation at 50°C, the spot plate on a tray was covered with a staining dish to cut down evaporation.

[4] It was found convenient to place the grids in an open LKB grid box which was placed on a rack in the vessel containing osmium tetroxide vapor. The plastic of the grid box blackened but no deterioration or scaling occurred on repeated use. After exposure to osmium tetroxide, the grids were transferred to a fresh grid box and exposed to air for several minutes to rid them of excess osmium tetroxide. They were examined with an RCA EMU-3H at 50 kv with a 45 μ objective aperture. Electron micrographs were taken on medium contrast plates with 2 sec' exposure and a reading of 20 on the photometer. Prints were prepared by Mr. Michael Friedman on No. 4 contrast paper at constant background density.

(unbuffered) at 50°C for 1 hr followed by a brief wash. Although silver ion will bridge to tissue-bound osmium through TCH, the deposits at high magnification were not amorphous as with osmium tetroxide, but were granular. Lead citrate (0.5%), however, could be used in place of osmium tetroxide in the T-O procedure. Membranes were well stained although the contrast was not as good as with osmium tetroxide.

Hypothetical formulation of the reaction of excess TCH with lipid fixed in osmium tetroxide, based upon Criegee's first reaction product (unstable) in the oxidation of a double bond by OsO_4 (2, 16), is shown in Fig. 1. Since sections at this stage have no more density than the original osmium tetroxide–fixed sections, it is evident that subsequent treatment with osmium tetroxide results in attachment of more osmium to the unreacted end of bound TCH to yield an increase in contrast (Fig. 2). The OTO method results in striking increase in density of the lipid component of membranous structures such as cristae and outer limiting membranes of mitochondria, plasma membranes, nuclear envelopes, endoplasmic reticulum, and lipid inclusion bodies in mitochondria, droplets, or vesicles. Examples of these are shown in Figs. 3 to 10 from kidney, heart, brain, and small intestine of the rat. In each plate both osmium tetroxide–fixed tissue (top) and OTO-treated tissue (bottom) are shown. The observation of increased density and increased resolution of membranes by making them more distinctly visible is very significant. The enhanced staining makes the membranes appear thinner as well as show greater contrast (Fig. 4). These results could be due to reaction of the TCH with osmium tetroxide that is bound to lipid and is still capable of reaction with TCH (Fig. 1, formula I) to give hypothetical formula II, which could be reduced by excess TCH to hypothetical formulas III or IV. TCH could also react similarly with diesters and polymeric esters of lipid-bound osmium cited by Stoeckenius and Mahr (16). Furthermore, we can assume that all the reactive groups of TCH (which is used in excess) are not consumed in this first reaction because the TCH bound to osmium tetroxide-fixed tissue (formulas II, III, or IV) is capable of reducing and binding additional osmium tetroxide, the last step in producing the OTO phenomenon of contrast enhancement. The formulation of the last step in the OTO procedure is not possible at present, but results of analyses of reaction products of TCH and OsO_4 will be published later. It is possible that osmium tetroxide that is reduced to insoluble lower Os oxides by strong reducing groups of some components of tissue may not react with TCH. Although the precise structures of these reaction products are unknown, we can probably safely assume that osmium tetroxide may have a different structure when attached to lipid (Fig. 1) from that produced when osmium tetroxide has reacted with protein, nucleoprotein, or polysaccharide. Since fixation of the latter macromolecules in osmium tetroxide is much less in degree than that of lipid (1, 4, 16), apparent intensification by the OTO method of the osmium tetroxide stain that is attached to lipid structures suggests that predominantly quantitative factors are involved. This was shown in model experiments with filter paper impregnated with samples of unsaturated lipid, protein, nucleic acids, and carbohydrate (5). Fig. 2 diagrammatically shows how a greater amount of osmium tetroxide bound to lipid than to protein could result in an increase of the total amount of osmium on lipid by TCH bridging (5) and make a poorly delineated thick double membrane appear as two distinct thin membranes or enhance and sharpen the appearance of this double membrane. There is an apparent disagreement concerning the precise localization of osmium in the membranes of osmium tetroxide–fixed tissue. It has been assumed (15) that the hydrophilic end of the lipid component of the cell membrane is the site of the accumulation of osmium, as opposed to the older concept that accumulation is in the unsaturated fatty chain of the lipid (1). Recent work by Stoeckenius and Mahr (4), which attempts to reconcile this controversy, suggests that primary reaction of osmium tetroxide does in fact occur with unsaturated lipid and, after reduction, shifts to the polar end of the fatty chain. Whether this shift will be established as true or not is of no importance in understanding the increase in contrast produced in membranes with the OTO method and studied at the magnifications presented here. Subsequent study with our method at higher magnification may help resolve the controversy. Inasmuch as the evidence for accumulation of osmium at the polar end of the lipid is indirect (15), we prefer at present not to specify the exact site of attachment to lipid as in Fig. 2.

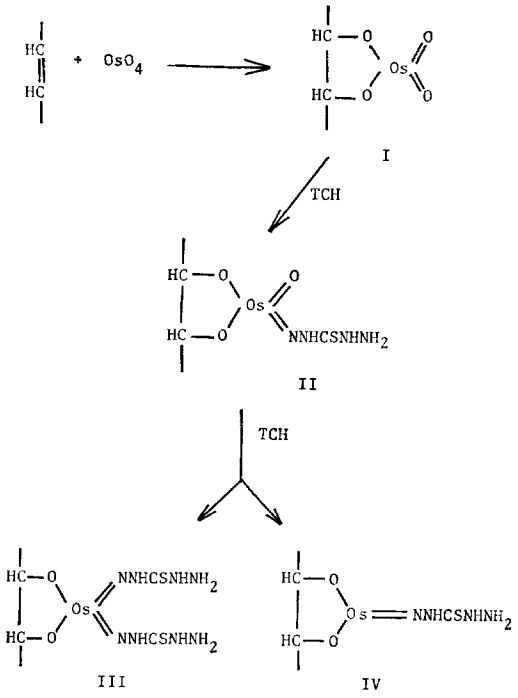


FIGURE 1 Hypothetical formulation of the reaction of excess TCH with lipid fixed in osmium tetroxide, based upon Criegee's first reaction product in the oxidation of a double bond by OsO_4 (2, 16). The hypothetical reaction products II, III, and IV are formulated to reveal their capability of reacting and binding further OsO_4, the last step in the OTO method.

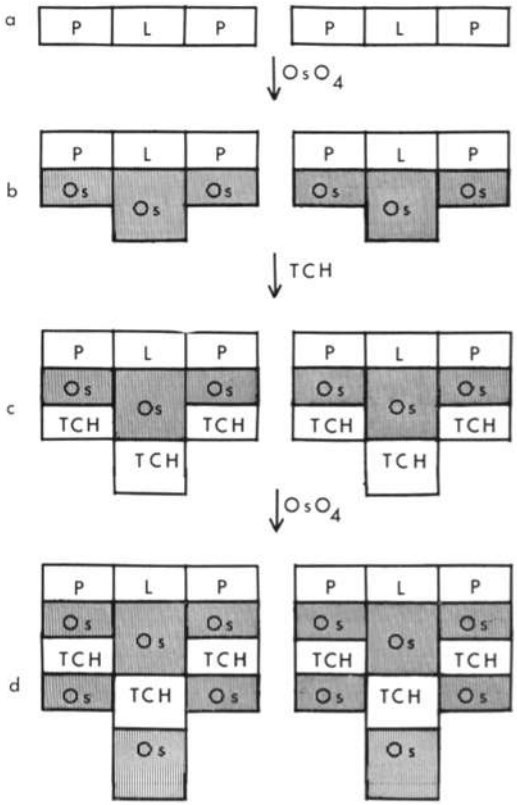
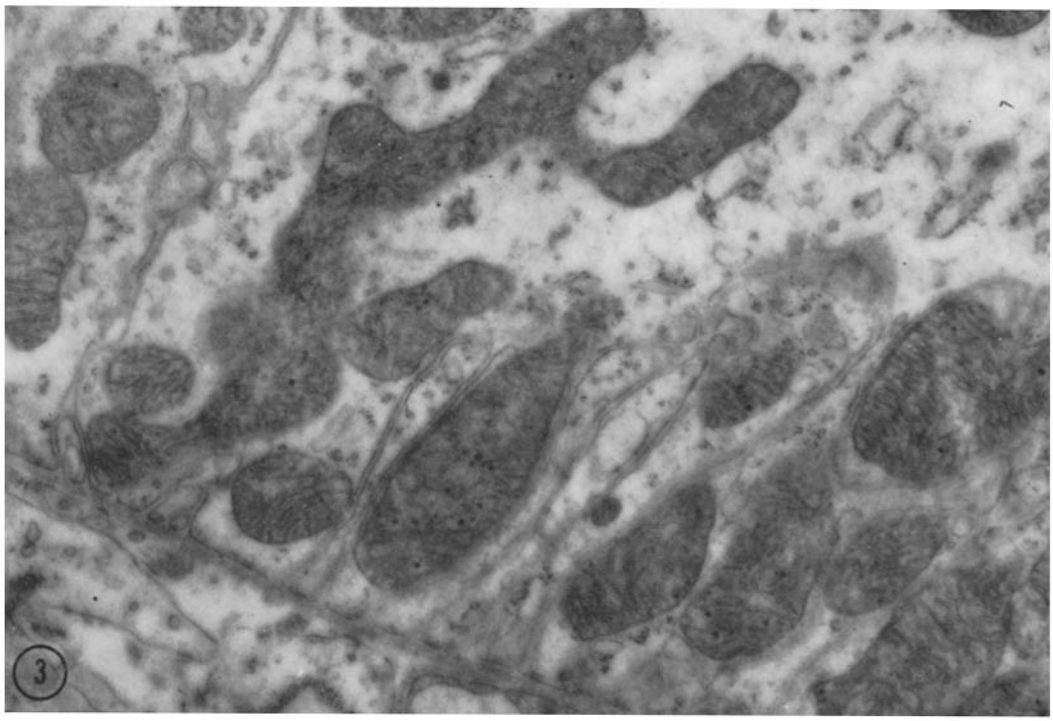


FIGURE 2 Diagrammatic representation of the OTO method used on a double membrane (Fig. 2 *a*) composed of protein (P) and lipid (L). In Fig. 2 *b*, the difference in the degree of osmication of the two components (P and L) in osmium tetroxide is represented by a factor of 1:2, although the actual ratio would be nearer to 1:10. In Fig. 2 *c*, the attachment of TCH to components of the membrane fixed in osmium tetroxide is not quantitated. In Fig. 2 *d*, the enhancement, most marked with lipid, of bridging osmium through TCH to the tissue-bond osmium, is demonstrated.

FIGURES 3 to 10 Rat organs were fixed in Palade's osmium tetroxide (8), embedded in Araldite, cut in ultrathin sections, and mounted on copper grids (top figure of each plate) for comparison with sections mounted on gold grids and treated with TCH followed by wash and exposure to osmium tetroxide (bottom figure of each plate).

FIGURES 3 and 4 Kidney. Control, Fig. 3; OTO method, Fig. 4. Note enhancement of contrast of mitochondrial cristae, mitochondrial dense granules, plasma membrane, and nuclear envelope. Also, note some lightening of the mitochondrial matrix.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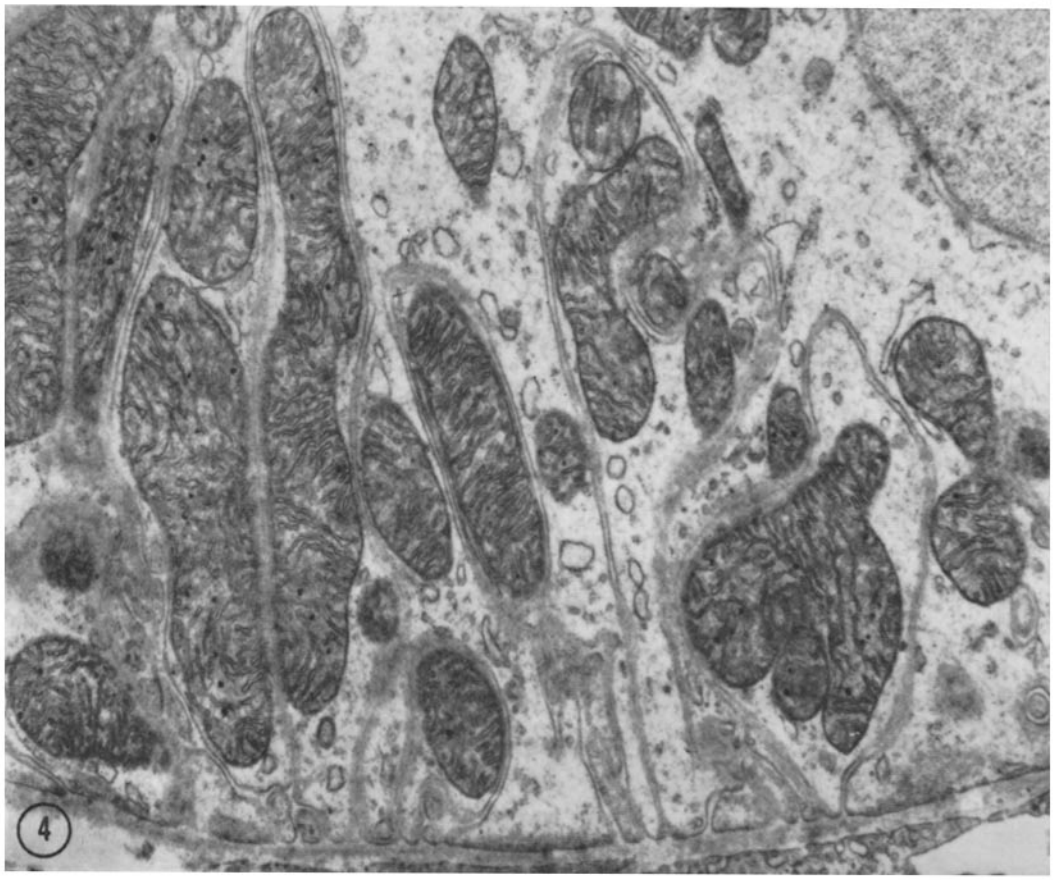
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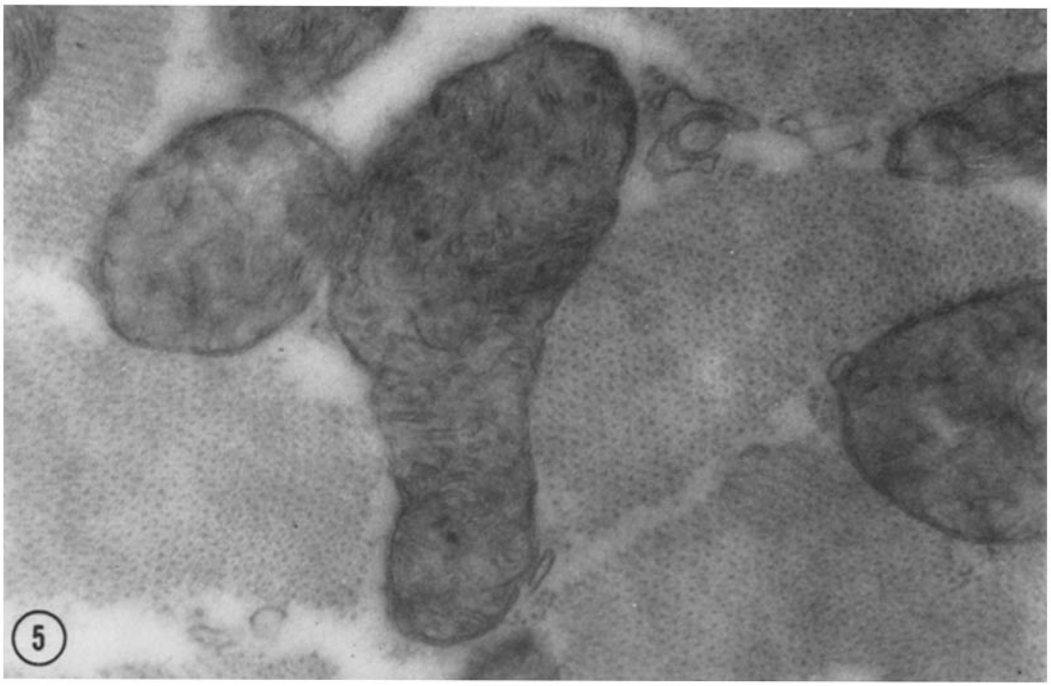
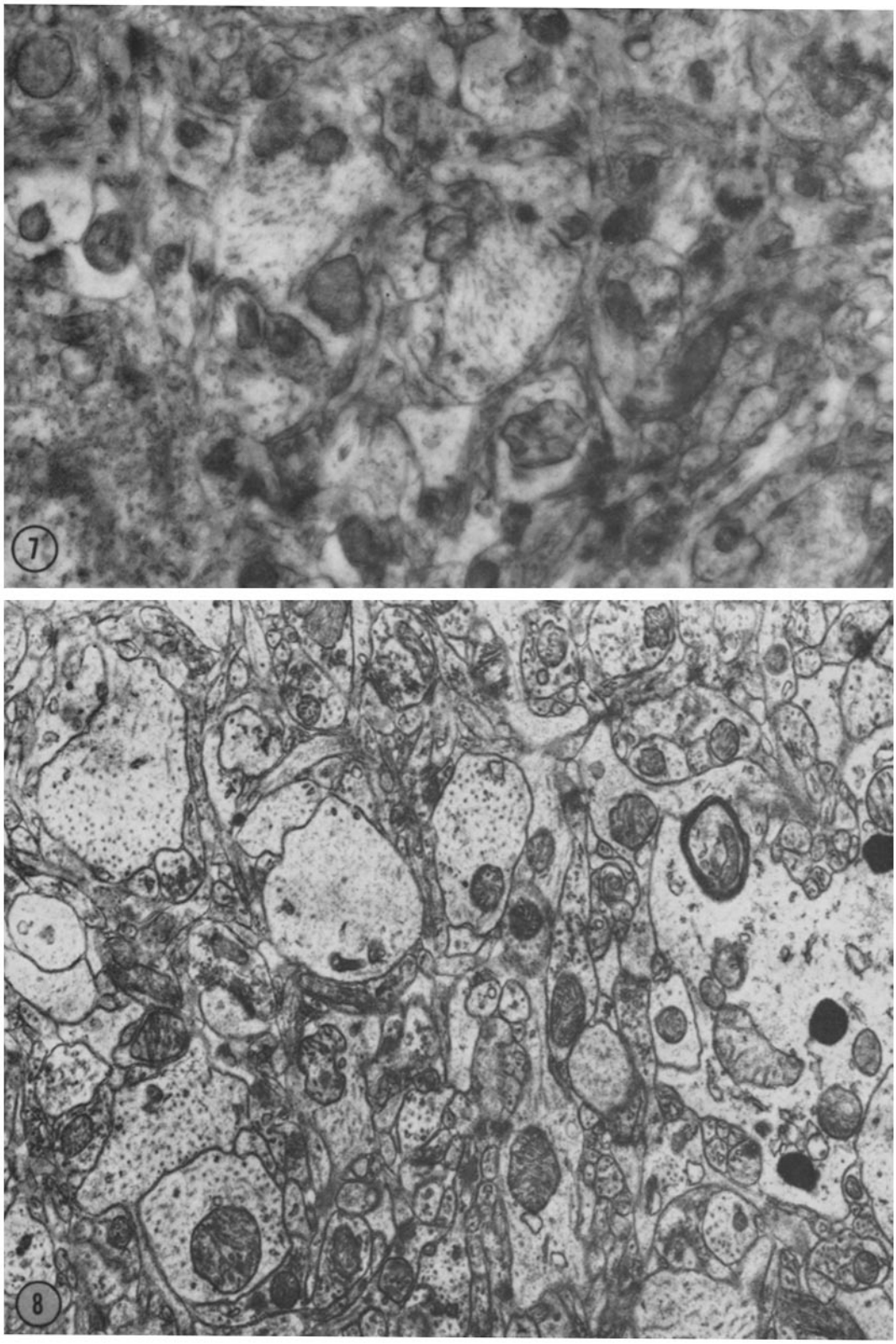
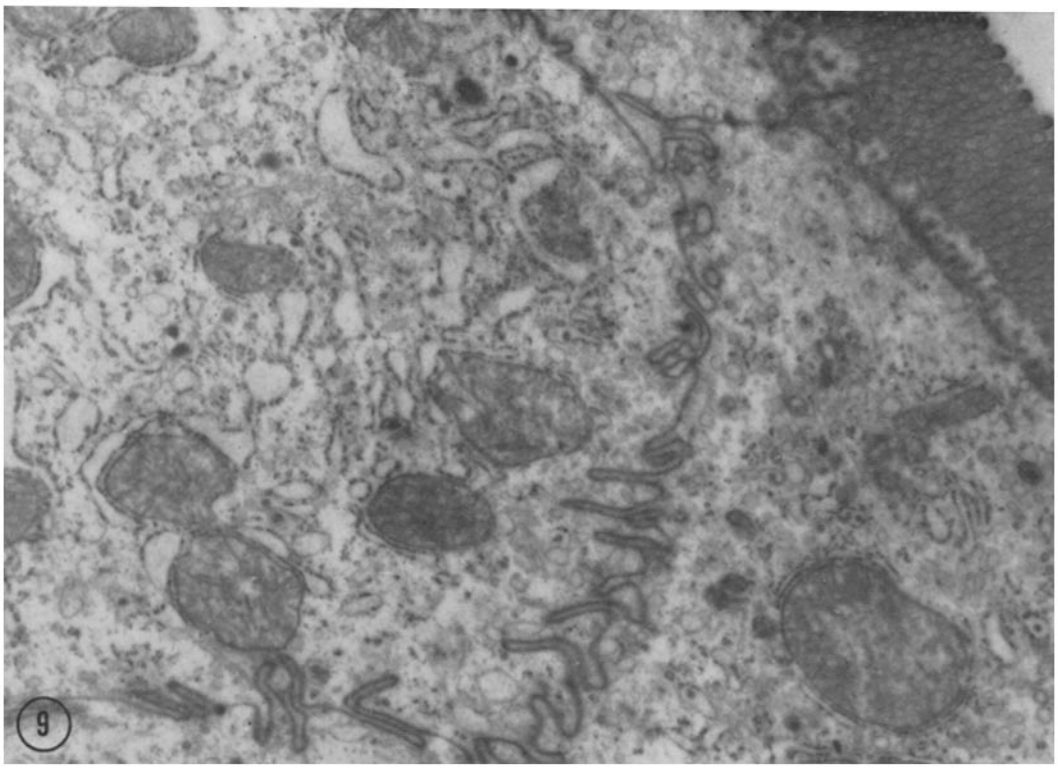
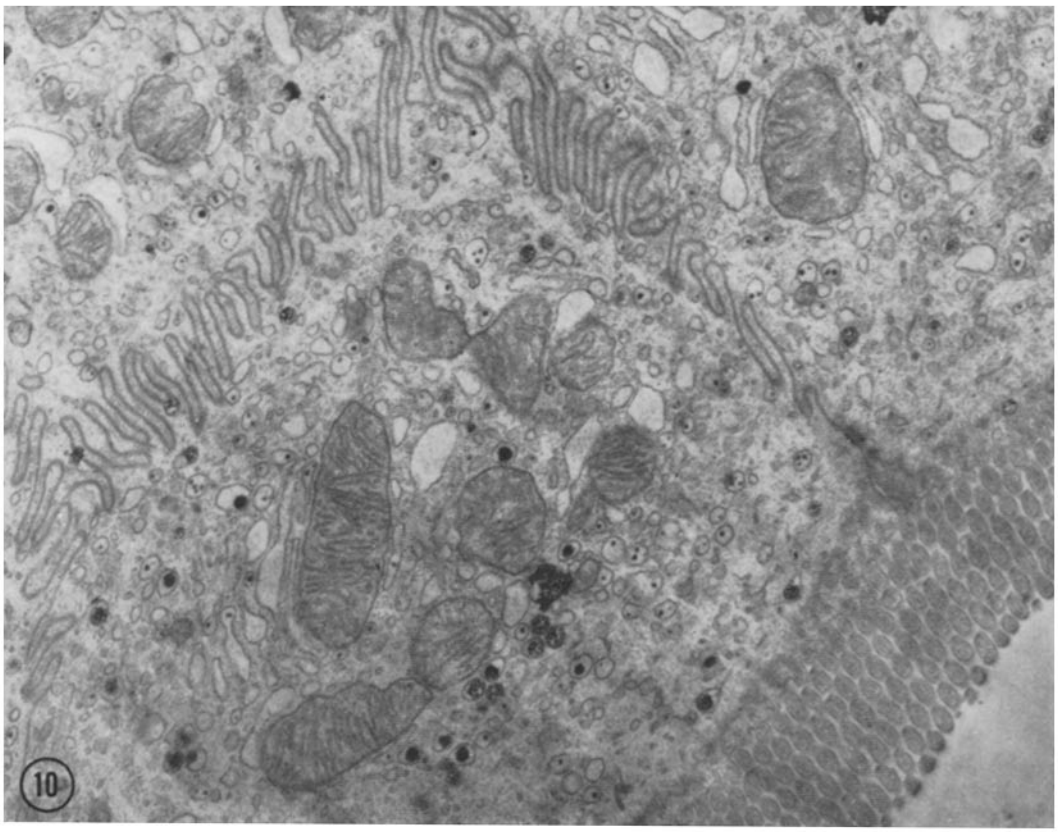


FIGURES 5 and 6 Heart in cross-section. Control, Fig. 5; OTO method, Fig. 6. Note enhancement of contrast of mitochondrial membranes, and lightening of the mitochondrial matrix. Note increased density of the myofilaments and of the sarcotubules.



FIGURES 7 and 8 Cerebral cortex. Control, Fig. 7; OTO method, Fig. 8. Note enhan trast of all membranous structures and of lipid droplets and myelin.





FIGURES 9 and 10 Small intestine. Control, Fig. 9; OTO method, Fig. 10. Note enhancement of contrast of all membranous structures, of internal structure of the microvilli, and especially of lipid droplets in vesicles.

When we substituted for TCH other sulfur-containing osmiophilic reagents which were capable of bridging metals, we noted that some of these agents were without effect, such as thiosemicarbazide ($H_2NNHCSNH_2$), or increased density generally but not membrane contrast, such as *p*-chlorothiophenol, or decreased the density of the mitochondrial matrix resulting in some increase in membrane contrast, such as hydrazine (H_2NNH_2), 1,4-dithiothreitol ($HSCH_2CHOHCHOHCH_2SH$) and 1,2-dithioglycerol ($HSCH_2CHSHCH_2OH$), or decreased the density of the matrix as well as increased the density of membranes producing maximum membrane contrast, such as carbohydrazide, ($H_2NNHCONHNH_2$, CH) and hydrogen sulfide (H_2S). None of the reagents was as striking as TCH. It was our impression that some of the reagents were capable of decreasing the density of the mitochondrial matrix. These were TCH, CH, hydrazine, and hydrogen sulfide. These reagents seemed to us capable of removing osmium, from osmium tetroxide–fixed tissue, selectively from components other than lipid, such as protein, thus lightening the matrix of mitochondria around membranes, which contributed to their sharpness. This way of increasing contrast was much less effective than the increased density of the bridging reaction. That this occurs with TCH may be seen by comparing the matrix of the mitochondria in Figs. 3 and 4. The fact that good contrast can be obtained with TCH, CH, and H_2S, suggests that, in addition to osmiophilic sulfur, a bifunctional hydrazide or coordination to two metal atoms (bridging) is also required and may be the most important feature, as revealed by the quite good contrast at low density seen with CH. However, neither TCH, CH, or H_2S increases contrast when used alone without the final step of exposure to osmium tetroxide vapor. This means that selective removal of osmium by these agents must play a minor role, if any, in producing the striking features of the OTO method.

Not only was thiosemicarbazide (TSC) unable to yield the OTO phenomenon when substituted for TCH, but it proved to be a fairly good blocking agent in preventing TCH from producing the OTO reaction when osmium tetroxide–fixed tissue was exposed to TSC prior to performing the T-O procedure. This indicates that although TSC is a bidentate ligand, both the hydrazino group and the thiocarbonyl group are involved in the original attachment to tissue-bound osmium. This type of attachment also may occur with TCH, but the additional hydrazino group is capable of reaction with osmium tetroxide.

Application of a second T-O procedure upon a first OTO method resulted in further increase in density, without further improvement in contrast or in delineation of structure. However, when viewed in a 100 kv beam, the sections so treated looked similar to the sections treated with the original OTO method at 50 kv.

Since hydrazone formation is facilitated by acid or alkaline pH, the role of acid was tested. The reaction of TCH with osmium tetroxide–fixed sections was unaffected by 1% acetic acid but could be slightly reversed by washing with 50% acetic acid solution. However, after completion of the OTO method, 50% acetic acid wash was without effect. Although more water soluble, TCH mono- or dihydrochloride, or diacetate gave very poor results when compared with those with TCH, in spite of the use of concentrations higher than 1%.

Affinity of TCH for other metal ions bound to tissue was also studied with the electron microscope. Glutaraldehyde-fixed tissue, embedded in Araldite, cut in ultrathin sections, placed on gold grids and stained with 5% solutions of lead, uranyl, mercury, copper, zinc, or chromate salts, or 0.1% K_2PdCl_4, was washed and treated with 1% TCH followed by osmium tetroxide vapor (T-O procedure). Acid was avoided in the washing process, to prevent reversal. Exposure to osmium tetroxide resulted in enhancement of the particular metallic stain as far as contrast was concerned, but increased sharpness of membranes was not produced by any of these metals as compared with the OTO method. Significant contrast was conferred on metal stains (such as copper and zinc ions) which are not by themselves particularly good stains for electron microscopy. The most striking increase in contrast occurred when the T-O procedure was applied to sections stained with basic lead acetate or uranyl acetate. However, greater delineation of membranes or visualization of new structures was not produced by this process. The results were no better when osmium tetroxide–fixed tissue was stained with these metal salts before applying the T-O procedure. It is also fair to point out that the OTO method itself applied to ultrathin sections of glutaraldehyde-fixed tissue did not give results that in any way

compared in excellence with those obtained with tissue initially fixed in osmium tetroxide. However, osmium tetroxide fixation after brief formaldehyde or glutaraldehyde fixation gave good results with the OTO method.

A comparison of the results using the OTO method with those obtained with double heavy metal salt staining methods will be published later, and will show the superiority of the former in delineation of membranes. There is much less contamination of the sections with the OTO method and the results are more reproducible. Furthermore, increased contrast is confined to structures that were osmiophilic to begin with, and not to new components of the tissue unstained by osmium fixation.

The T-O procedure to enhance staining by metals other than osmium tetroxide has found useful application in enhancing contrast, in the electron microscope, of uranium-labeled antibodies (13, 14), and may find use with uranium-azo dyes in studies directed to determination of base sequence in nucleic acids (7). Not only is greater contrast produced by the final step of exposure to osmium tetroxide, but greater stability in the electron beam would be expected than with uranium alone; and this was found to be the case (13). The T-O procedure may also be used on the copper chelate which is the end product of the histochemical reaction for aminopeptidase, to give a permanent pigment useful in electron microscopy as well as in light microscopy (5). Further enhancement in contrast of underlying structure may then be afforded by performing a second T-O procedure (note Fig. 7 of reference 5).

We have recently found the OTO method especially useful in enhancing the visualization of fine structure of tissues stained for various enzymes, where initial exposure to osmium tetroxide must be avoided until after the enzyme reaction is completed. For example, the cristae of mitochondria may be more sharply delineated in fresh heart slices stained for cytochrome oxidase by the production of an osmiophilic pigment (6, 10), when the T-O procedure is subsequently superimposed. The T-O procedure enabled us to demonstrate mitochondria and other membranous structures more clearly than we could obtain by other metal staining methods, in formalin-fixed or glutaraldehyde-fixed tissue stained for hydrolytic enzymes by methods recently introduced and dependent upon the production of osmiophilic products (6, 10). This has been shown to be helpful for esterase (12) and will be demonstrated in subsequent publications dealing with the detailed presentation of other methods.

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