THE USE OF VANADIUM AS A STAIN FOR ELECTRON MICROSCOPY

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Utilization of heavy metals in electron microscopy to obtain image contrast of a degree greater than that resulting from fixation with osmium tetroxide or potassium permanganate is now an accepted practice. Staining is virtually essential when an epoxy resin is used as embedding medium, but only a small number of compounds have been suggested and few of these are in routine use. Lead hydroxide (21), one of the more effective stains, is probably the most widely employed in laboratories working with biological material. A number of modifications of this stain have been introduced, however, attesting to the difficulties encountered in obtaining preparations free of lead carbonate precipitate. Several mechanical devices have been proposed (6, 9, 15, 16), and chemical alterations

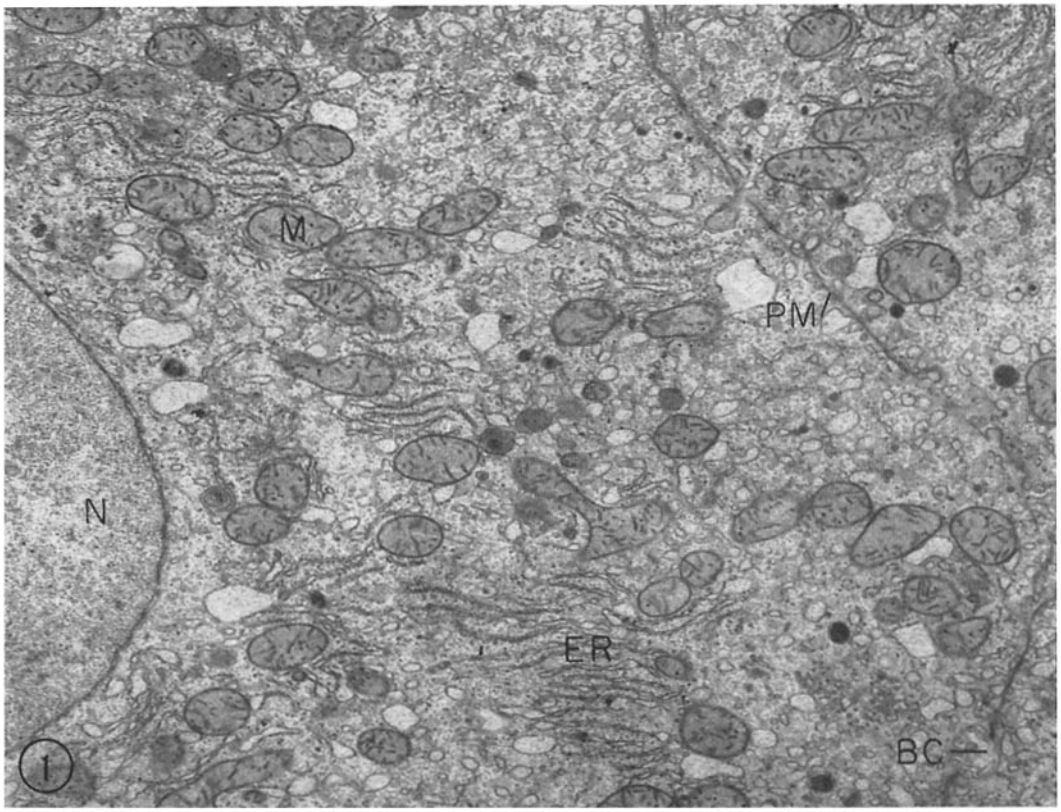
All figures are of rat tissue fixed in osmium tetroxide, embedded in Araldite, with sections placed on uncoated grids.

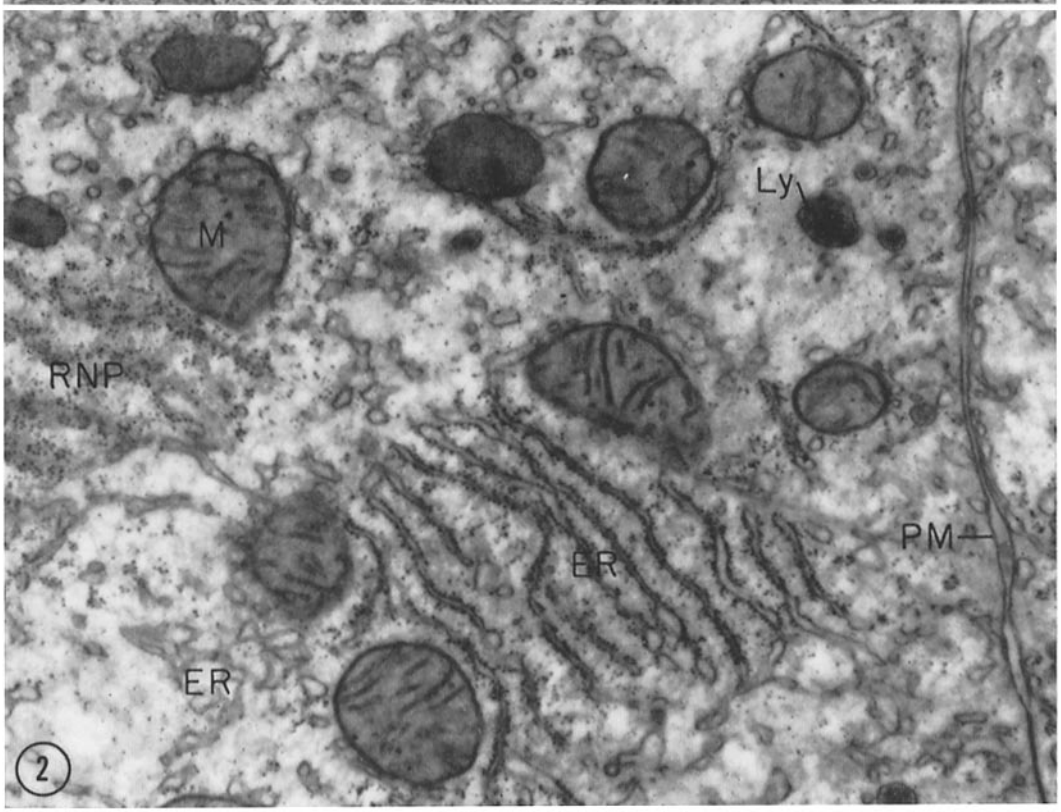
Key to Labeling

N, nucleus	*BM*, basement membrane
M, mitochondrion	*PM*, plasma membrane
ER, endoplasmic reticulum	*BC*, bile canaliculus
RNP, ribosomes	*SG*, secretion granules
S, blood sinusoid	*Ly*, lysosomes

FIGURE 1 Section of rat liver parenchyma treated with vanadatomolybdate pH 7 for 30 minutes. A portion of a nucleus is seen at the left and plasma membrane at the right. Mitochondria, endoplasmic reticulum (both granular and agranular), and vacuoles are clearly visualized. Elements of the Golgi apparatus are seen near the bile canaliculus at lower right. Scattered dense bodies are lysosomes. × 10,500.

FIGURE 2 Section of rat liver treated with vanadyl sulfate for 15 minutes. Mitochondrial membranes and intramitochondrial granules, both classes of endoplasmic reticulum, and plasma membranes are well stained. Ribosomes are seen clearly associated with membranes of the endoplasmic reticulum in the center, and in separate clusters due to tangential sectioning at the left. A lysosome is seen at upper right. × 23,500.





suggested (5, 10, 12, 14, 18), in attempts to prevent the formation of this stain artefact, but none have proven entirely reliable. Other electron-opaque materials, such as phosphotungstic acid (8), phosphomolybdic acid (19), uranyl acetate (3, 7, 9, 20), potassium permanganate (11) and chromyl chloride (1), have been used, but, in many cases, these are either weakly effective, difficult to prepare, or present other disagreeable properties.

Because of the difficulties in obtaining adequately stained, artefact-free preparations, a further search for a simple and effective stain was undertaken. Preliminary observations made in this laboratory (2) on two compounds containing vanadium appeared to satisfy these requirements and have been evaluated more extensively.

PROCEDURE

Small blocks of liver, anterior pituitary, and cardiac muscle of the rat were fixed for 30 minutes at 4°C in 1 per cent osmium tetroxide, buffered at pH 7.4 with veronal-acetate, containing sucrose (4). Tissues were dehydrated rapidly in the cold and routinely embedded in Araldite. Epon-embedded tissues were also prepared as a further test of stain effectiveness. Thin sections were cut with glass knives on a Porter-Blum microtome and mounted on uncoated grids.

Evaluation of a compound being tested was facilitated by applying it to a grid and, for comparison, treating grids bearing adjacent sections with either lead hydroxide (5, 21), uranyl acetate (7, 9), or chromyl chloride (1). Thus, minor variations in tissue field, degree of fixation and dehydration, or section thickness, which could influence the evaluation of stain quality, were held to a minimum.

Effective staining was obtained by floating the grids, tissue surface down, on a drop of stain in a porcelain spot plate. This method facilitated staining a number of grids at one time and, by covering the preparation with a large glass slide, dust particles were excluded and evaporation avoided. Filtration of solutions through a 0.01 μ Millipore filter in a Swinny-type adaptor immediately prior to use was found effective as a precautionary measure against contamination by undissolved particulate material or formed precipitate. After staining, rinsing was accomplished by holding the grid in a stream of distilled water from a wash bottle. The grid was then dried between sheets of bibulous paper and was ready for viewing.

Examination of tissue was made with an RCA EMU-3C operated at 50 kv employing a 37 μ objective aperture. Photographs were taken on Kodak medium-contrast lantern slide plates or DuPont "Cronar" sheet film. The quality of the negatives was such that prints on Kodabromide F-2 or F-3 paper provided adequate contrast.

Of the many compounds evaluated,[1] only two were found to satisfy the requirements of simplicity in preparation and application, as well as providing an effective non-granular, precipitate-free stain. Both of these were vanadium compounds for which preparation details are presented.

STAIN PREPARATION

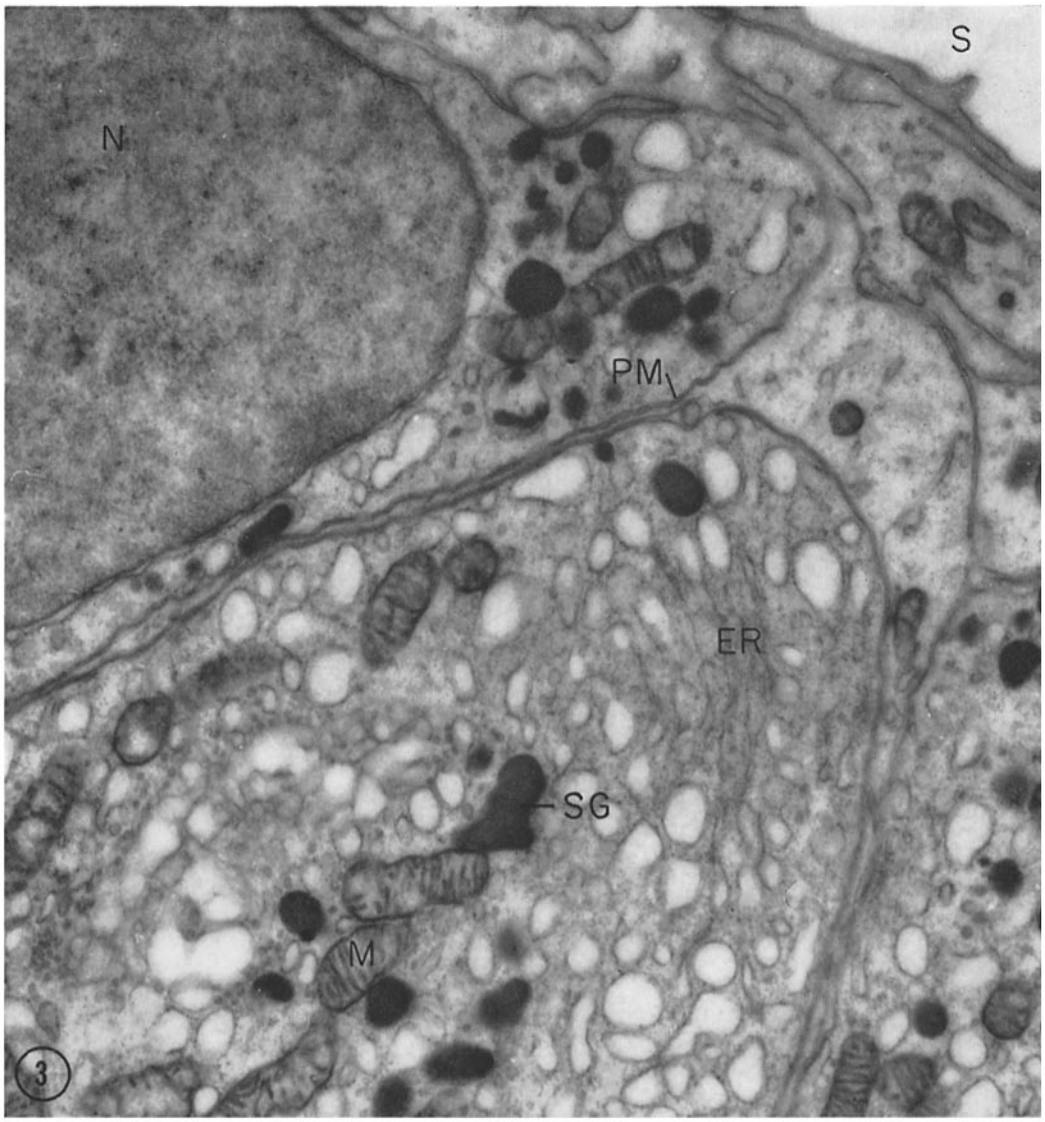
VANADYL SULFATE: A 1 per cent solution of vanadyl sulfate ($VOSO_4 \cdot 2H_2O$)[2], which has a pH of 3.6 when freshly prepared, is a satisfactory stain and remains effective for about 2 weeks. After

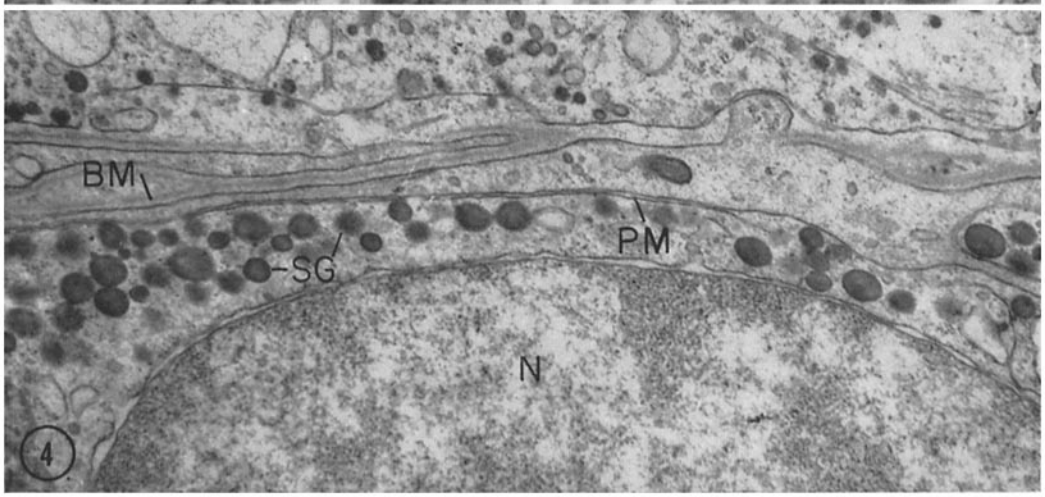
[1] A number of compounds having relatively high electron-scattering properties were tested, but most of these revealed one or more undesirable qualities such as inadequate specificity, instability under the electron beam, precipitate formation, or excessive granularity. Compounds tested, but rejected as ineffective were: cobalt sulfate, nickelous sulfide, ferric chloride vapor, cerium oxalate, rubidium chloride, silver nitrate, lead iodide, potassium tri-iodide, and iodine vapor.

[2] Fisher Scientific Company, Pittsburgh.

FIGURE 3 Section of rat pituitary stained with vanadyl sulfate for 20 minutes showing portions of several cells. Nucleus and nuclear envelope of one cell is seen at upper left. Plasma membranes are easily visualized throughout their length. The double nature of mitochondrial membranes is readily apparent, as are the scattered portions of the endoplasmic reticulum. Dark bodies are secretion granules. × 21,500.

FIGURE 4 Section of rat pituitary stained with vanadatomolybdate pH 7 for 20 minutes. Junction between several parenchymal cells illustrates the sharp quality of plasma membrane staining and distinct basement membrane visualization. Area of cytoplasm in the somatotroph, above nucleus in lower portion of field, contains secretion granules revealing varying densities, while secretion granules of the thyrotroph in upper half of field stain most intensely. × 18,000.





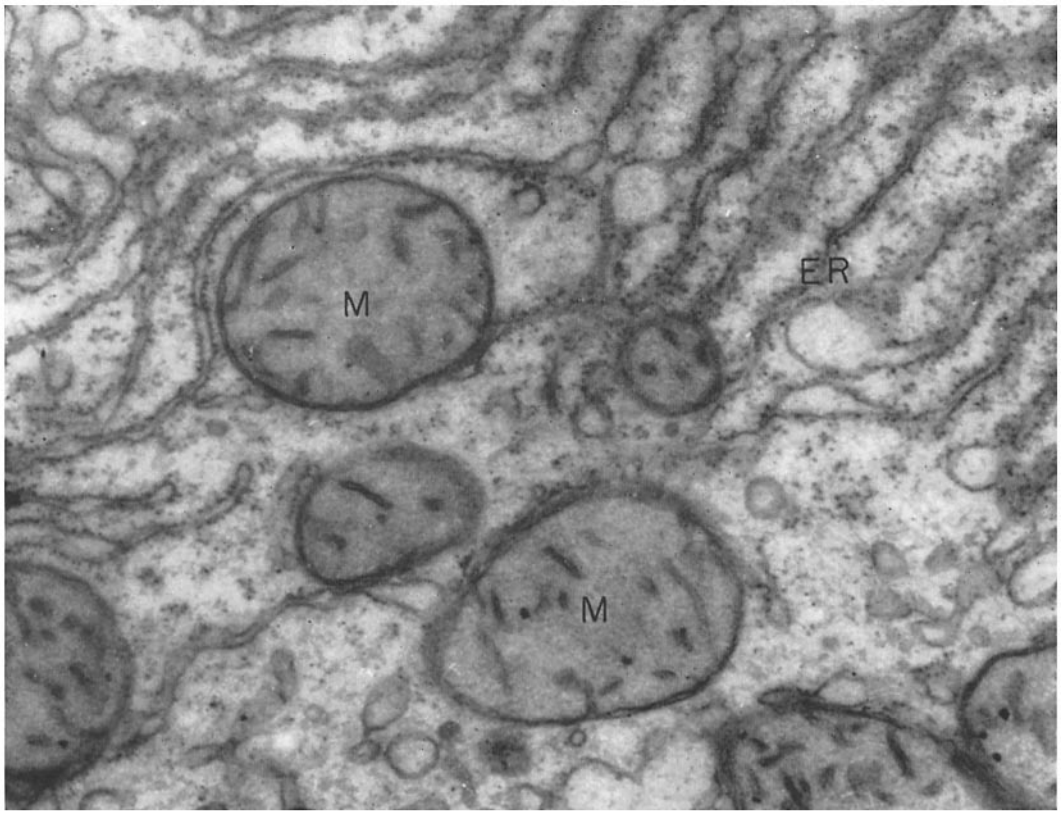


FIGURE 5 Section of rat liver parenchyma treated with vanadatomolybdate at pH 7 for 30 minutes. Mitochondria, agranular endoplasmic reticulum, and reticulum containing well stained ribonucleoprotein granules are clearly visualized. × 35,000.

this period of time, a significant hydrolytic reaction will have taken place as indicated by the formation of vanadium dioxide crystals (17), and, as a consequence, the stain effectiveness decreases.

VANADATOMOLYBDATE: The staining solution is prepared by mixing 20 ml of 1 per cent vanadyl sulfate ($VOSO_4 \cdot 2H_2O$) with 80 ml of 1 per cent ammonium hepta-molybdate $[(NH_4)_6Mo_7O_{24}]$.[3] A dark purple solution forms initially which, upon oxidation, undergoes a further reaction and color change to clear yellow.[4] This resultant solution which has a pH of 3.2 is the stain and is quite stable, producing satisfactory staining for a period of 6 to 12 months. The complex chemical reactions involved are not thoroughly understood, but according to Mellor (13) the formula for vanadatomolybdate is $3(NH_4)_2O \cdot 2V_2O_5 \cdot 4MoO_3 \cdot nH_2O$.

OBSERVATIONS AND COMMENTS

The criteria established initially for a desirable stain were: (1) uniform and effective contrast enhancement; (2) simplicity of preparation; (3) short and uncomplicated application; and (4) freedom from precipitate. It is felt that the vanadium stains satisfy these requirements ade-

[3] This procedure represents a weight ratio and concentration found empirically to yield an effective stain. The ratio is based on the stoichiometry of the reaction. The precise concentration is not critical since it has been observed that there is a narrow range within which staining effectiveness is comparable. It should be noted that if the two compounds are mixed dry and water added, the resultant solution requires a considerably longer period of time to undergo the color change and may never reach this end point. One possible explanation of this is the formation of a chemical complex between the undissolved salts and the solution initially formed.

[4] Time required for this color change is less than 30 minutes if oxygen is bubbled through the solution.

quately and, therefore, would be useful additions to the stains in routine use.

The quality of staining achieved with either compound is quite good and resembles the appearance obtained with lead hydroxide. All membranes, including cytoplasmic, reticular, mitochondrial, nuclear, and those of the Golgi apparatus, stain uniformly (Figs. 1 to 5). Ribosomes are well stained (Figs. 2 and 5) although perhaps not so intensely as with lead hydroxide, and glycogen granules stain quite darkly. Secretion granules of pituitary cells show increased density which appears to vary with the stage in the secretory process. Granules which tend to stain uniformly black with other stains reveal densities varying from gray to black when treated with these stains (Fig. 4). Collagen fibrils stain with either compound, but particularly well with vanadatomolybdate. In general, it is observed that, for comparable periods of staining, vanadatomolybdate provides somewhat higher contrast than vanadyl sulfate. Tissue embedded in Epon appears adequately stained after 5 to 10 minutes, but increased contrast is gained by longer periods of treatment. Further study is needed, however, before stain effectiveness with this embedding medium is fully evaluated.

Background cytoplasmic material is not densely stained with vanadium, in contrast to our observations of tissue stained with uranyl acetate. This lack of staining perhaps contributes to the apparent selective contrast enhancement of cytoplasmic organelles, effected by vanadium or lead treatment. Furthermore, the vanadium-stained preparations have a sharp or crisp, non-granular quality which is not easily obtained with the uranyl stains, particularly the alcoholic procedure.

The extreme simplicity in preparation of the vanadyl sulfate stain is an attractive feature, particularly when contrasted with the elaborate procedures involved with some of the lead stains. This feature eliminates the possible objection arising from the relatively short period of usefulness of the compound in solution. Vanadatomolybdate is admittedly somewhat more time consuming to prepare, but it is not complicated, and the solution maintains its staining properties for months.

Variations in the period of staining from 5 to 90 minutes were tested and it was found that adequate staining was obtained within 5 to 30 minutes with either compound, depending upon the tissue. Intensity of staining was proportional to the period of treatment, and no leaching effect was noted.

In view of the fact that vanadyl sulfate is readily soluble in water and does not react with carbon dioxide to form an insoluble precipitate, no distracting artefact at natural pH is expected. This allows large areas to be photographed without the necessity of having to select fields between precipitate granules (Fig. 1). Attempts to adjust the pH of vanadyl sulfate resulted in the formation of a heavy precipitate and reduction of staining qualities. Vanadatomolybdate was studied over the range pH 3 to 11. The mild tendency to form precipitate at low pH values was absent at pH 7.0, and no diminution of stain effectiveness was noted. At pH values greater than 8, precipitate occurred and there was a definite reduction in staining.

As mentioned earlier, the precise composition of the solution containing vanadatomolybdate is not known. It is the product of ammonium molybdate, which was reported by Watson (20) to have certain staining qualities, and vanadyl sulfate, found here to be effective for electron microscopy. The possibility exists that the stain referred to as "vanadatomolybdate" is actually a mixture of three or more compounds, at least two of which are effective stains.

These two vanadium stains have been used routinely in this laboratory for over a year and have been found effective on all tissue studied. Thus, these advantages are here summarized: (1) contrast is adequate, uniform, and comparable to that obtained with lead hydroxide; (2) preparation is simple in comparison to the process involved in many of the lead methods; (3) application is both simple and brief, in contrast to the precautions often required with lead, or the lengthy stain period recommended with aqueous uranyl acetate; and (4) a non-granular, precipitate-free stain is obtained which allows relatively large artefact-free areas as well as small high-magnification areas to be photographed.

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