# A ROUTINE TECHNIQUE FOR DOUBLE-STAINING ULTRATHIN SECTIONS USING URANYL AND LEAD SALTS

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For more than 2 years, a double-staining of sections of $OsO_4$-fixed and Epon-embedded tissues with uranyl acetate followed by lead hydroxide has been routine technique in this laboratory. It was found to give substantially higher contrast, thus permitting the use of thinner sections. It facilitated focusing and routinely provided clearer pictures of cell fine structure than could be obtained with either stain alone. However, the formation of a fine precipitate frequently marred the sections, and this difficulty could never be satisfactorily controlled. Apparently, it is caused by the uranyl acetate solution which is not stable at the pH and concentration used. Decreasing the concentration and/or the pH of the solution caused an undesirable loss of staining intensity. Therefore, a number of different uranyl salts were tested as a substitute for uranyl acetate in the procedure, and one of them, uranyl magnesium acetate, has been found to give consistently good results.

## MATERIAL AND METHODS

Mammalian tissues of lung, heart, kidney, gingiva, and liver were fixed for 1 hour at 0°C in 1 per cent phosphate-buffered osmium tetroxide at pH 7.3 (7), then dehydrated with increasing concentrations of ethanol in water at 0°C, and embedded in small, uncovered plastic cups. The embedding medium was Epon 812, according to Luft (5), with the following modification: after incubation overnight at 26°C in mixture B, the tissue blocks were polymerized under reduced pressure (200 mm Hg) at 40°C for 3 days and then transferred to a 60°C oven for another 3 days. This modification gave blocks of uniformly good cutting quality. Sections exhibiting silver to gray interference colors were cut on the LKB microtome with a duPont diamond knife and mounted on grids with lightly carbon-coated Formvar grids.

Originally, a 3.8 per cent aqueous solution of uranyl acetate was used for staining the sections. Later, a 7.5 per cent solution of uranyl magnesium acetate[1] in triple-distilled water (pH 5.2) was substituted. The solution is filtered into the wells of a nine-hole glass spot plate,[2] through a Millipore filter (pore size, 0.45 $\mu$), from a 5-ml

[1] K & K Laboratories, Inc., Jamaica, New York.
[2] Fisher Scientific Company, New York.

syringe fitted with a Swinny adapter. The wells should be a little more than half full. Not more than four grids are placed in a single well, and any unused wells are filled with distilled water to reduce evaporation from the staining solution. Grids are introduced vertically and then placed tissue-side down at the bottom of the well. During staining, the spot plate is covered with a flat glass plate and kept for 3 hours at 40°C under a light-tight cover. At the end of the staining period the grids are removed and washed in three changes of 50 per cent ethyl alcohol in three separate jars. The first jar is removed after every twelve grids, filled with fresh alcohol and used as the third wash. The position of the grids at both insertion into and removal from the stain, as well as during washing, seems to be important in reducing surface contamination. With forceps, the grids are held perpendicular to the liquid surface and dipped vigorously and repeatedly into each wash for 10 seconds. Drops adhering to the forceps are blotted dry with lens paper which must be inserted between the jaws and carried as far toward the tips as possible. Excess liquid is removed from the grids by touching to the edge of a piece of lens paper, and the grids are left on filter paper for 30 minutes or longer until ready for exposure to the lead solutions.

Several modifications of Watson's lead hydroxide stain (9) have been introduced in order to eliminate the contamination on the surface of sections. Most frequently we used Millonig's technique (6), but Karnovsky's (3) and Reynold's (8) modifications also have given satisfactory results in combination with the uranyl magnesium acetate. The lead staining is carried out exactly as described by these authors and the grids are handled and dried in the same manner as described above following the uranyl staining.

Micrographs were taken at initial magnifications of 1,300 to 40,000, employing a Siemens Elmiskop I with a double condenser lens system operating at 80 kv and with a 50 $\mu$ objective aperture. Micrographs were recorded on Ilford N-60 plates and developed with Ilford ID 2. Routinely prints were made on Agfa paper, grade 2 and 3, and, occasionally, on grade 4.

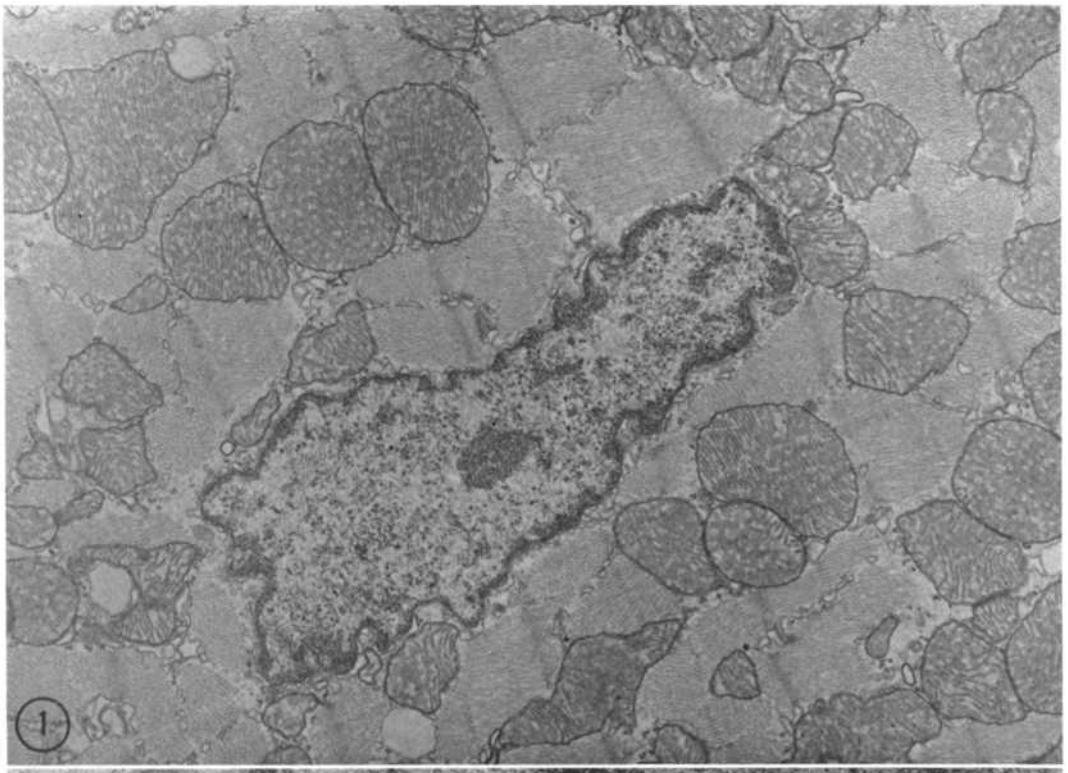
## DISCUSSION

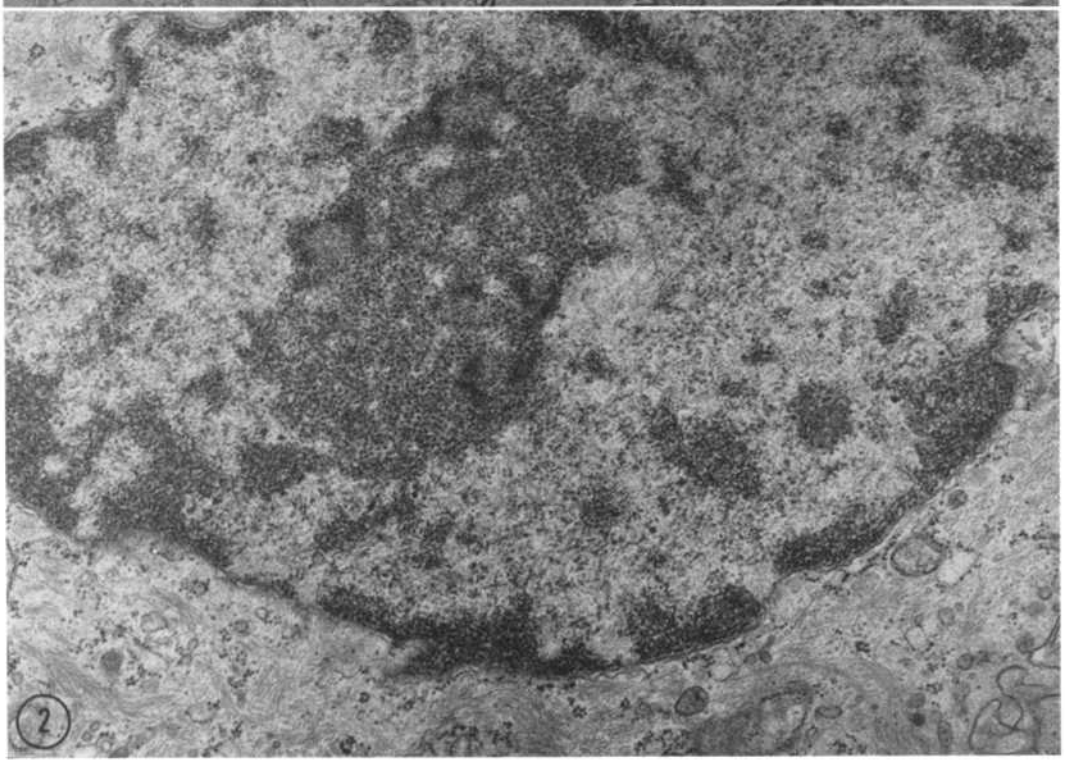
The use of uranyl or lead salts to enhance contrast in sections has been repeatedly described (1, 3, 4, 7–9) and is routinely used today by most electron microscopists working with biological materials. Huxley and Zubay (2) seem to have been the first to combine uranyl acetate and lead hydroxide in a double-staining technique. They noted that it caused a strong increase in staining intensity. However, they were mainly concerned with the specificity of the stain for nucleic acids and used extremely short fixation times. In our experience, fixation extended to 1 hour has no appreciable effect on the over-all contrast, but with this longer fixation the preferential staining of nucleic acid-containing structures is not so marked as described by Huxley and Zubay. Instead, other cell structures, especially membranes, stain strongly, which clearly is an advantage in a routine stain for general purposes.

The main advantage of uranyl magnesium acetate is the reduction of the amount of fine precipitate very often found on the sections when uranyl acetate is used in the double-staining. It also seems to introduce less diffuse staining of the background and therefore the cell structures stand out more clearly. It is also useful as a single stain and gives approximately the same results as uranyl acetate but with much less contamination of the sections. Uranyl magnesium acetate solutions are stable for at least several months. Our oldest solution has been kept in the dark at room temperature for more than 1 year and still gives satisfactory results.

FIGURE 1 Section of mouse cardiac muscle illustrating the contrast obtained by the double-staining technique and the relative absence of stain contamination. (7.5 per cent uranyl magnesium acetate and Millonig's lead stain). × 12,000.

FIGURE 2 This portion of a basal cell from hyperplastic human bronchial epithelium demonstrates the marked staining of the nucleolus and the chromatin aggregate along the nuclear membrane. Cytoplasmic filaments are clearly defined. (7.5 per cent uranyl magnesium acetate and Millonig's lead stain). × 30,000.





The advantages of the double-staining technique were noticed especially in work on squamous epithelium which is notoriously difficult to stain. Here and in the human bronchial epithelium, the chromatin structure, the nucleoli, and dense nuclear bodies first described by Watson (10) were very conspicuous. Also the striated rootlets of the cilia and fibrils in the cytoplasm stood out much more clearly than in sections stained with uranyl acetate or lead hydroxide alone. In general, double-staining appears to increase the density of all cell structures beyond the degree that can be obtained with either stain alone.

No staining procedure with uranyl or lead salts is always completely free of contamination, as evidenced by the number of modifications published since Watson (9) first introduced these techniques. However in our hands, uranyl magnesium acetate in combination with lead hydroxide consistently gives preparations with high contrast and considerably less contamination than any other technique tried.

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FIGURE 3 Apical portion of the cytoplasm of a ciliated epithelial cell from human bronchus in which double-staining renders rootlets of cilia, cytoplasmic filaments, and cell membranes readily apparent. (3.8 per cent uranyl acetate and Karnovsky's lead stain B). × 60,000.

FIGURE 4 Section of human bronchial epithelium showing portions of columnar cells. Membranous structures are clearly visualized by the double stain. A chromatin granule can be seen at the upper left. (7.5 per cent uranyl magnesium acetate and Millonig's lead stain). × 60,000.