AN IMPROVED STAINING METHOD FOR ELECTRON MICROSCOPY

JEROME G. STEMPAK and ROBERT T. WARD. From the Department of Anatomy, State University of New York Downstate Medical Center, Brooklyn, New York

An aqueous solution of uranyl acetate was first employed as an electron stain by Watson (1958). We have found that this stain, although frequently effective, is capricious. The stain may not increase contrast of a given specimen, and may, in other instances, leave undesirable contamination on a stained grid.

While an experiment on dehydrating agents for electron microscopy was in progress, uranyl acetate was dissolved in methanol in an effort to accomplish *en bloc* staining. It was noted that the uranyl acetate was very soluble in methanol in contrast to its limited solubility in water and ethanol. At the time of this observation, it was felt that an increased concentration of uranyl acetate might aid in staining some tissue components or might stain tissue more consistently than the less concentrated aqueous stain.

METHOD

One to 15 grams of hydrated uranyl acetate ($UO_2(CH_3COO)_2 2H_2O$) are dissolved in 25 ml of absolute, acetone-free methanol with a magnetic stirrer or by constant agitation. A small amount is then pipetted, poured, or filtered into any small vessel which may be securely corked. A few drops of absolute methanol are added to the staining dish if the solution is relatively concentrated, in order to insure that any incipient precipitate will be kept in solution. The grids are dropped into the staining dish, which is then securely stoppered, and stained for 5 to 30 minutes.

After staining, the grid is removed from the solution with forceps and dipped or dropped into the first rinse of 100 per cent methanol or ethanol *immediately*. Any delay at this stage greatly increases the danger of contamination. If the grid is dropped into the wash, it is removed with a second pair of clean forceps, or the first pair of forceps may be used after rinsing in a larger bath of methanol. If the grid is dipped in the first rinse, it should be transferred to a second clean pair of forceps for the second rinse. Dipping should be perpendicular to the surface of the rinse fluid, with no rotary or horizontal motion. The reason for the extreme caution at this point is the tendency of the staining liquid to adhere between the jaws of the forceps and not be removed by several rinses unless the jaws are opened. We have found that if a grid is held in one pair of forceps and rinsed with a gentle stream of fluid or dipped in several baths, the fluid retained between the jaws of the forceps flows onto and around the grid when it is dropped on lens tissue to dry. The contamination after such treatment is most impressive.

Rinsing is continued for five or six baths and the grid is dried on Ross lint-free lens tissue. The first two rinses should be absolute methanol or ethanol; the next two, mixtures of alcohol and water; the last one or two, distilled water. It is also felt desirable to use freshly rinsed forceps for each step. It will be noted after rinsing two or three grids (if the rinsing solutions are in 5- or 10-ml beakers), that the first rinse bath and possibly the second are tinted. These two baths must be frequently changed. Careful adherence to the above schedule has consistently yielded stained grids with no greater contamination than a control grid which was simply rinsed in the various baths and dried. The rinsing should be as rapid as possible to minimize possible extractive effects of the rinse solutions.

The solutions utilized as staining solutions were 4, 20, 40, 60, and about 120 per cent w/v. (One, 5, 10, 15, and between 29 and 30 grams, respectively, were dissolved in 25 ml of methanol by mechanical agitation.)

The staining solutions should be refrigerated to retard the formation of a precipitate which eventually forms in all of the solutions on standing. The precipitate is not harmful, since the solution remains clear, and may be carefully pipetted off and used. The precipitate cannot be redissolved in the same solution,

no matter what the extent of agitation. In order to assure ourselves of a contamination-free stain, we feel it best to make up a fresh batch of solution whenever a noticeable precipitate begins forming.

Tissues selected for staining in this study were rat liver, submaxillary gland, and spinal cord (both embryonic and adult), frog ovary, mouse ovary, and mouse pancreas. They were fixed in osmium tetroxide solutions buffered by various methods and embedded in methacrylate (Ward), Epon, and Araldite (Luft, 1961). The blocks were sectioned with diamond or glass knives on Huxley and Porter-Blum microtomes and picked up on carbon-coated formvar grids or on uncoated athene 400-mesh grids. In experiment No. 1, one hundred and twenty grids with sections of Epon-embedded material were divided into subgroups of three to five each which were constant with respect to tissue type, block, and grid type. One grid was untreated or rinsed and dried, one was stained with saturated aqueous uranyl acetate (according to Watson), and the remaining were stained with a solution of methanolic uranyl acetate for 15 or 30 minutes. In experiment No. 2, 41 thin sections were carefully selected for similar coloration. Individual sections were picked up and treated as above, except that staining time was varied. After staining, a technician scrambled the order of the grids so that the previous treatment of each grid was unknown during estimation of the grade of contrast.

Four brands of fresh reagent grade uranyl acetate were tested. These were Mallinkrodt, Fisher, Shattuck, and Baker. After addition to reagent grade acetone-free methanol, Mallinkrodt and Fisher uranyl acetate dissolved quite completely in high concentrations and left a clear solution. The Shattuck and Baker products dissolved in large amounts, but left a definite insoluble material which slowly settled. Bottles of uranyl acetate known to be 1 year old or older gave rise to large amounts of insoluble material and left the solution quite turbid. As a consequence, fresh lots of Mallinkrodt or Fisher are recommended, but any other uranyl acetate which dissolves completely and leaves the solution clear may be utilized. Several brands of absolute, acetone-free methanol were used. In one case, two different bottles of the same brand gave different solubilities of the same uranyl acetate. The methanol which was the poorer solvent was shaken with Linde Molecular Sieve Type 4A, in an effort to purify and dehydrate it, and it regained its solvent capacity.

RESULTS

The results of both experimental groups may be seen in Table I. Of the 34 grids in the control group, 23 were almost lacking in contrast (grade 0), and 11 were estimated as grade +. In two instances not tabulated, control sections were as high in contrast as a grade ++ staining reaction. There are occasional reports from some laboratories that unstained Epon sections possess high contrast, as for example, Luft (1961), who noted that Epon sections have "good contrast in the electron microscope." In our experience (except for the above case), sections of Epon blocks were found invariably to be so poor in contrast that staining was required. A few grids were stained for 2, 5, and 10 minutes in aqueous and methanolic stains. Two minutes in the 25 per cent w/v methanol stain gave a grade ++ contrast to 3 of 4 grids, while the aqueous stain placed none above control levels. In the 5 and 10 minute stains, the maximum grade +++ is achieved by grids stained by the methanolic stain. The maximum level of stain is not seen in aqueous stained grids until an hour of staining takes place.

A total of about five-hundred grids of Epon sections in addition to the experimental material have been stained with the methanolic uranyl acetate solution. It was found that all sections on every grid indicated a staining reaction, examples of which may be seen in Figs. 1 and 2. Sections from a few blocks of methacrylate (n butyl 85 per cent, methyl 15 per cent) and Araldite (American Ciba 502 and 6005) also stained well in 10 minutes or less.

DISCUSSION

Initial experiments with carbon stabilized parlodion grids indicate that they may not be used in this procedure because the methanol dissolves the parlodion. The formvar grids used were made according to Pease (1960), (0.2 to 0.5 per cent solution in ethylene dichloride, usually 0.4 per cent) and carbon-coated. The formvar may be solubilized by excessive staining time or by exceedingly strong solutions. In an approximately 120 per cent w/v solution, all the formvar was dissolved off the immersed grids within 15 minutes. In a 60 per cent w/v solution, 15 minutes' staining finds most of the formvar intact, but tending toward fragility in the beam. These comments are made with some reservation, since formvar film quality is quite variable from laboratory to laboratory, depending on such factors as concentration of the formvar solution, technique of draining, and drying the slide on removal from the solution, age of solution, thickness of carbon coat, etc.

The 4, 20, and 40 per cent solutions did not harm the immersed grid if stained for 1 hour or less.

TABLE I

Comparison of Grids Stained with Aqueous and Methanolic Uranyl Acetate

Time stained (*min.*)	0	2		5		10		15		30		60+
Treatment	C	M	W	M	W	M	W	M	W	M	W	W
Contrast grade												
+++	—	—	—	2	—	3	—	14	—	17	—	6
++	—	3	—	1	2	1	1	15	—	14	2	13
+	11	1	2	1	1	—	3	3	1	2	2	14
0	23	—	1	—	1	—	—	—	1	—	—	—
No. of grids in each group	34	4	3	4	4	4	4	32	2	33	4	33

Experiments Nos. 1 and 2 were combined in Table I because the results were similar. Unstained control sections (C) are compared with the staining reactions of Epon sections in 25 per cent methanolic uranyl acetate (M) at various times (2, 5, 10, 15, and 30 min., respectively) with similar sections in aqueous uranyl acetate (W). The figures in the body of the table refer to the number of grids which attained the various levels of contrast. Grade zero, the most frequently observed grade of contrast in the control group may be defined as a section in which cell cytoplasmic components are obscure at low magnification (about × 2000) and only red blood cells are clearly discernible. Grades + to +++ indicate levels of increasing contrast from the base grade zero.

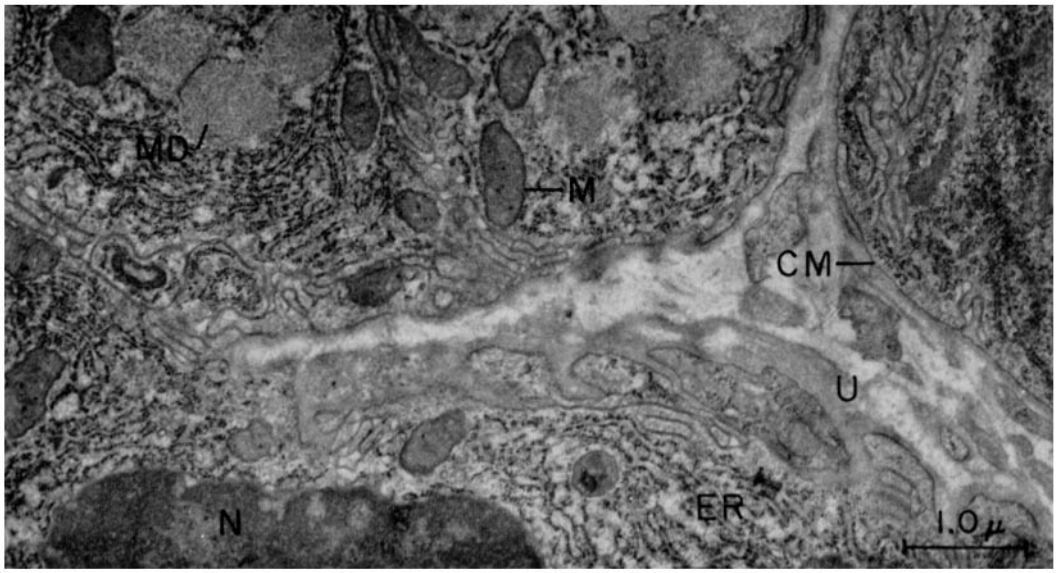

FIGURE 1 Acinar secretory cells of rat submaxillary gland. Fixation, OsO_4, 1 hour. Embedding, Epon 812. Stain, Methanolic uranyl acetate. Grade, +++. Negative, Kodak High Contrast Copying Film. Print, Kodabromide F-5. Magnification, × 16,000. Kv, 80. Obj. aperture, 20 microns. Microscope, Phillips 100B.

CM, cell membrane. *ER*, rough endoplasmic reticulum. *M*, mitochondrion. *MD*, mucin droplet. *N*, nucleus. *U*, unknown amorphous material (secretion?).

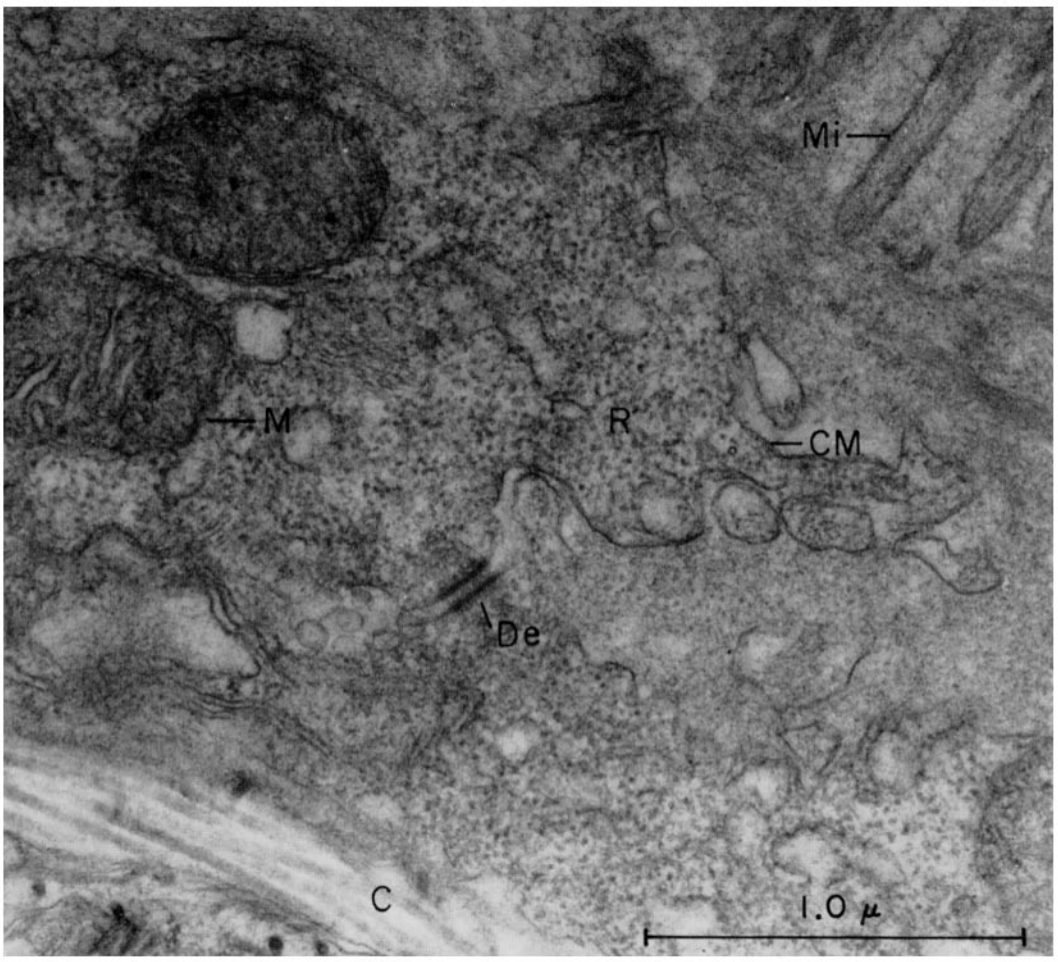

FIGURE 2 Follicle cell-oocyte junction in *Rana pipiens*. Fixation, OsO_4, 1 hour. Embedding, Epon 812. Stain, Methanolic uranyl acetate. Grade, +++. Negative, Kodak High Contrast Copying Film. Print, Kodabromide F-5. Magnification, × 50,000. Kv, 80. Obj. aperture, 20 microns. Microscope, Phillips 100B.

De, desmosome. *CM*, cell membrane. *C*, collagen. *Mi*, microvillus. *M*, mitochondrion. *R*, ribosomes.

Because contrast was not increased by the additional time, staining time was limited to half an hour, although 15 minutes was most frequently used. Generally, increased concentration of stain has not shown a tendency to increase the staining reaction (at least so far as may be subjectively determined) in the 20 to 60 per cent solutions, but the 4 per cent solution required a slightly longer staining time to yield similar results. Presently, we employ a 25 per cent solution and stain for 10 or 15 minutes. If one wishes to increase the intensity of the stain in very short periods of time, it is possible to do so with highly concentrated solutions (of the order of 100 per cent w/v). It must be remembered that the formvar may be dissolved, however.

En bloc staining was most effective using very dilute solutions (0.1 and 0.01 per cent) of vacuum-dehydrated uranyl acetate in absolute methanol. The only tissue used was rat liver, so more experiments are needed in this area.

The mechanism behind the improved staining with methanol solutions is not known, but a hypothesis is suggested by a simple experiment. If several polymerized Epon blocks are weighed and dropped into absolute methanol and allowed to

remain for 12 hours, rinsed with distilled water, and then dried for several hours, the blocks gain about 4 per cent in weight. This gain is not permanent, but is slowly lost over a period of time. Such a result can be explained as due to imbibition of methanol by the Epon and its gradual loss by evaporation. On thin sections, a swelling of the Epon at the section surface due to fluid imbibition might permit deeper penetration of the uranyl ion into the tissue than is possible in aqueous solution, resulting in more dense staining of certain components.

There is no indication that the methanolic stain is different from the aqueous in regard to specificity of staining.

In conclusion, it may be stated that the stain described has four important advantages. It stains in a shorter time than the aqueous stain, it yields, on the average, an image with greater contrast, and the increased contrast may be obtained more consistently, and stain contamination is extremely small in amount, or lacking altogether.

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