A Simplified Method of Staining Thin Sections of Biological Material with Lead Hydroxide for Electron Microscopy.

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A method of staining thin sections with lead hydroxide which enhances both resolution and contrast was described by M. L. Watson (1958) and is now undoubtedly in routine use in many laboratories.

It is the purpose of this report to present a simplification of this method which eliminates the lengthy procedure of preparing the lead hydroxide reagent and the necessity of maintaining a CO_2-free atmosphere during the staining interval. On exposure to air, lead hydroxide forms lead carbonate, which may contaminate the specimen grid. In the procedure presented here it is believed that the reaction leading from lead acetate to lead hydroxide occurs in the specimen section. In any case it was only on rare occasions that a minimal amount of fine precipitate, presumably of lead carbonate, could be observed in the tissue.

The tissue represented in the accompanying plates was prepared in the following manner. The pancreas of an 8 day old posthatched chick was diced and fixed for 1 hour in chrom-osmium solution (Dalton, 1955; Zeigel, in press). The small blocks of tissue were then immersed in 10 per cent formalin for 1 hour and dehydrated in a graded series of ethanols. They were infiltrated with, and embedded in, a 3:1 mixture of *n*-butyl to ethyl methacrylate which was catalyzed by 0.25 per cent benzoyl peroxide at 80°C. Sections were cut with a Porter-Blum microtome using a diamond knife and picked up from a 30 per cent acetone-in-water solution on formvar-coated Athene type grids.

The sections are stained in the following manner. A saturated solution of lead acetate in boiled distilled water is maintained in a glass stoppered bottle so that a visible number of undissolved crystals remain at the bottom of the bottle. After several drops of the solution have been removed, the reagent bottle should be refilled to the top with boiled distilled water, thus eliminating any air pocket at the top of the bottle. It is thought that this prevents crystals of lead carbonate from forming on the surface of the reagent and thus decreases the possibility of contaminating the grids. With a glass dropper, a few drops of the saturated lead acetate solution are placed on a clean polished glass slide in a covered, dust-free Petri dish. The grids are inverted section side down on the drops of lead acetate solution from 5 to 30 minutes. After staining, the grids are touched to absorbent paper to drain off the excess lead acetate. The grids are immediately washed vigorously in a series of 3 or 4 changes of boiled distilled water in separate beakers. It is believed that swirling and breaking through the meniscus of the wash water helps remove contamination. For best results it is mandatory that the glassware and boiled distilled water be immaculately clean and dust-free.

The washed grids are placed on absorbent paper in a clean Petri dish and allowed to *dry thoroughly* (approximately 10 to 15 minutes).

The grids are then held in forceps and waved (section side down) through the vapors of 1 to 5 per cent ammonium hydroxide. The grids are held a minimum of 5 cm. from the surface. Several passes or a total exposure of about 5 seconds is usually sufficient. Overexposure may result in damage to the formvar or methacrylate and consequent distortion of the sections. It is suggested that grids treated with 5 per cent NH_4OH not be placed in the same Petri dish with grids previously stained with lead acetate (which are not to receive the NH_4OH vapors) if they are to serve as comparison studies. It seems important to repeat the words of caution by Watson (1958): dried lead acetate or lead hydroxide residue may be dangerous if inhaled. It is suggested that used glassware be washed immediately.

The sections may be examined immediately in the electron microscope or sandwiched with a thin carbon layer for stability.

The electron micrographs were taken with an RCA-EMU-2C using a 15 mil condenser aperture and 30 μ platinum objective apertures.

Figs. 1 through 4 compare the results obtained: (1) without staining, (2) staining with uranyl nitrate, (3) staining with lead acetate, and (4) staining with lead acetate followed by treatment with ammonium hydroxide. It is our impression that Fig. 4 illustrates results at least equal to those obtained by the more involved procedure of staining with a solution of lead hydroxide (Fig. 5).

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The reason for the greater contrast in sections stained with lead hydroxide compared with those stained with lead acetate is not clearly understood but it may be related to the fact that phosphate, sulphydryl, and carboxyl groups of proteins become more completely ionized at increasing pH levels. Thus, assuming that ionization of these groups may occur after fixation with osmium tetroxide, more free radicals would become available for combining with lead in solutions with increasing pH levels. With this as a working hypothesis, sections were stained with a saturated solution of monobasic lead acetate

$$[Pb(C_2H_3O_2)_2 \cdot Pb(OH)_2 \cdot H_2O]$$

(Fig. 6) for comparison with the sections stained with lead acetate (Fig. 3) which gives a light general stain and lead hydroxide (Fig. 5) which gives a heavy general stain. (The pH of a solution of lead acetate is 5.9, that of lead hydroxide 8.15, and that of monobasic lead acetate 7.0).

It can be seen that monobasic lead acetate selectively stains the ribonucleoprotein granules. This result should have been expected perhaps since more carboxyl groups would become available from nucleoprotein than from other proteins at pH 7.0. This hypothesis would also explain why a general increase in contrast is obtained when sections stained with lead acetate are exposed to vapors of NH_4OH, since, under these conditions greater numbers of free radicals would be made available in most proteins for binding the excess lead present in the section.

Bibliography

Dalton, A. J., *Anat. Rec.*, 1955, **121,** 281.

Watson, M. L., *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 727.

Zeigel, R. F., in press.

Explanation of Plates

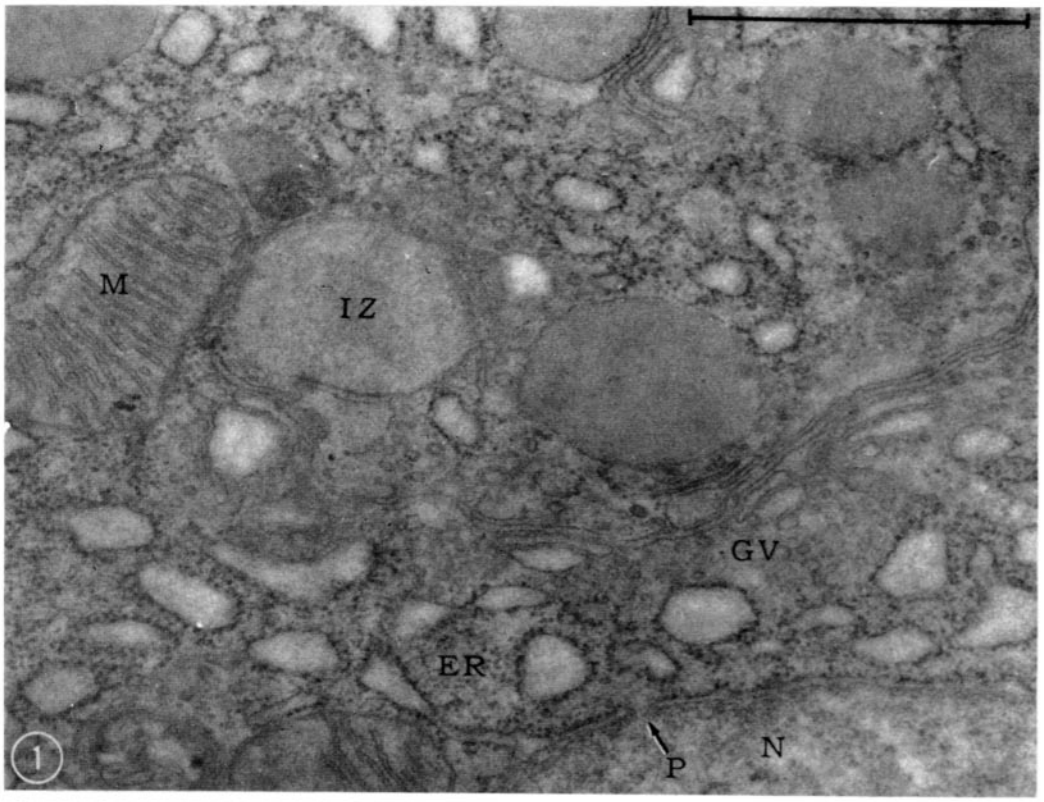
All figures are of portions of 8 day posthatched chicken pancreatic acinar cells. The prints from which the figures were prepared were all made on Kodabromide F_4 paper from negatives taken at × 8390. The final magnification of each figure is × 46,000.

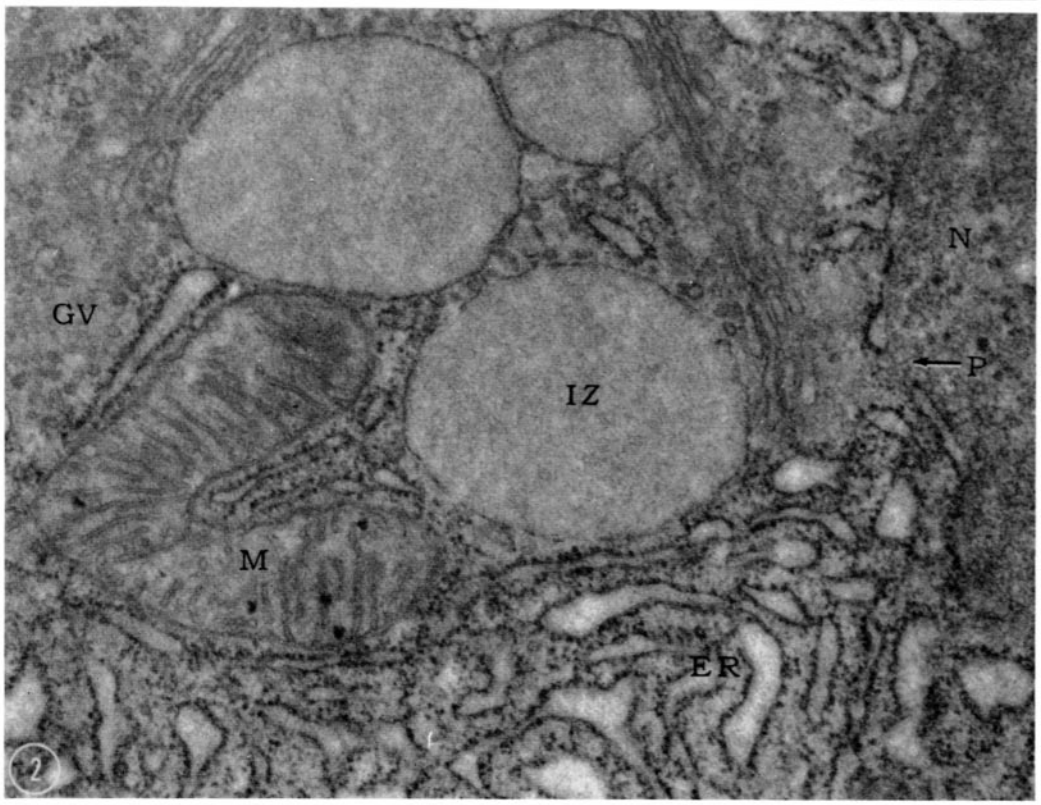
ER = endoplasmic reticulum or ergastoplasm
GV = Golgi vesicles
IZ = immature zymogen granules
M = mitochondrion
N = nucleus
P→= nuclear pore

Plate 224

Fig. 1 is of an unstained section and shows a portion of the nucleus with a nuclear pore at the lower right separated from the Golgi zone by a band of ergastoplasm. Above are several zymogen granules in different stages of maturation.

Fig. 2, of a portion of a cell stained with uranyl nitrate, shows part of the nucleus with a nuclear pore at the right. The Golgi zone with immature zymogen granules occupies the center of the figure. Organized ergastoplasm is present in the lower part of the figure. Contrast is clearly improved over that of Fig. 1.



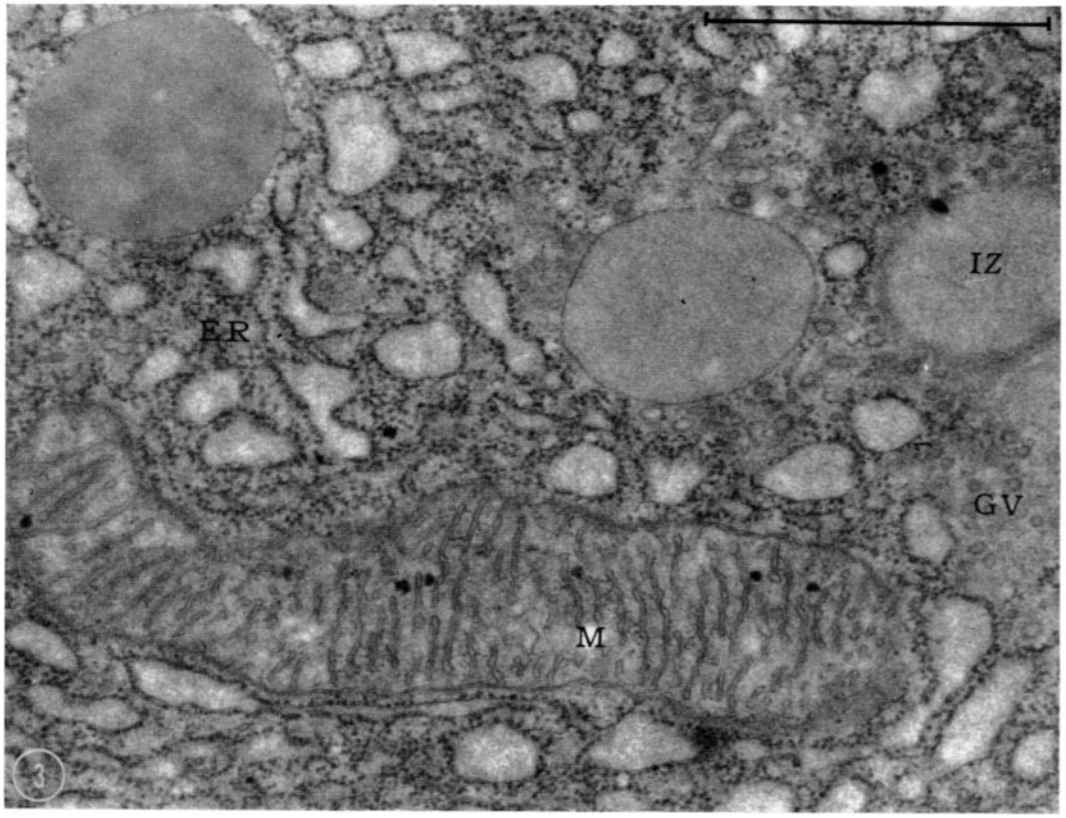


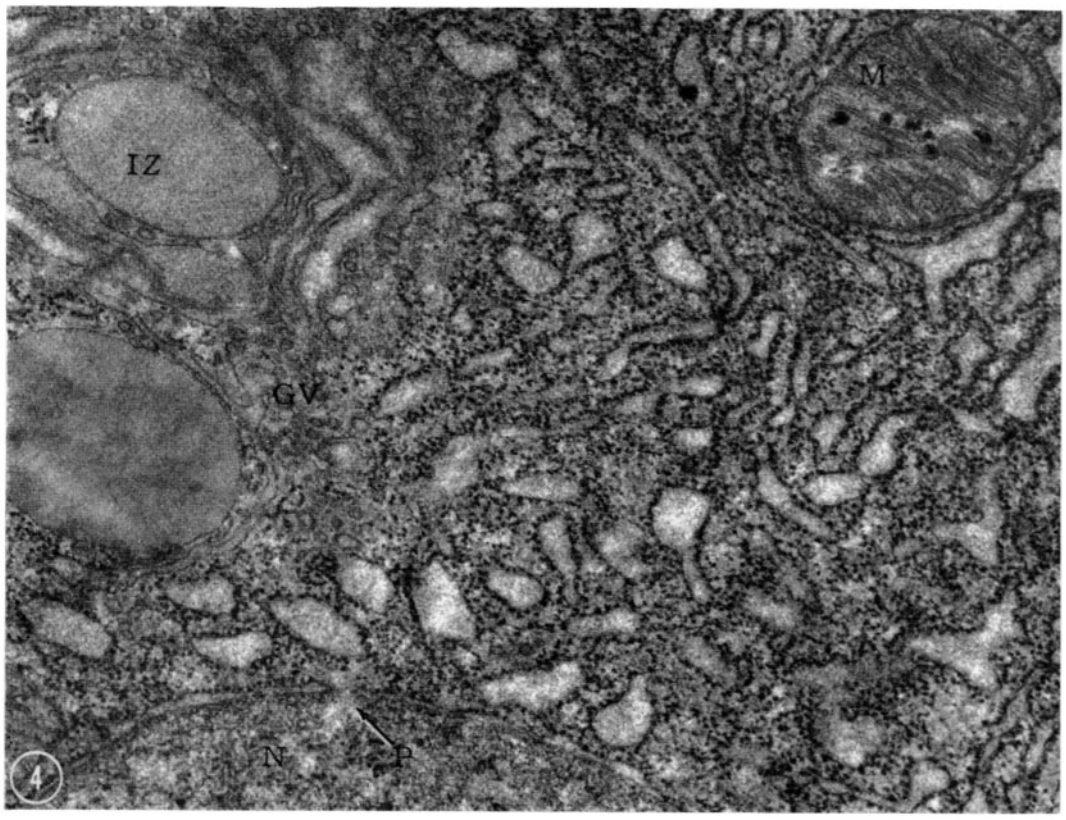
(Dalton and Zeigel: Lead hydroxide staining of biological material)

PLATE 225

FIG. 3, of part of a cell stained with lead acetate, shows a portion of the Golgi zone with immature zymogen granules at the right. Ergastoplasm and a relatively mature zymogen granule are present at the upper left. A large mitochondrion occupies much of the lower portion of the figure. Contrast is only slightly improved over that in Fig. 2.

FIG. 4 is of a portion of a cell stained with lead acetate followed by treatment with ammonium hydroxide vapor. A part of the nucleus with a nuclear pore is present at the lower left. The center of the figure is occupied by ergastoplasm while a portion of the Golgi zone appears at the upper left and a mitochondrion at the upper right. The contrast apparent in all cell components in this figure is definitely superior to that in any of the other three.



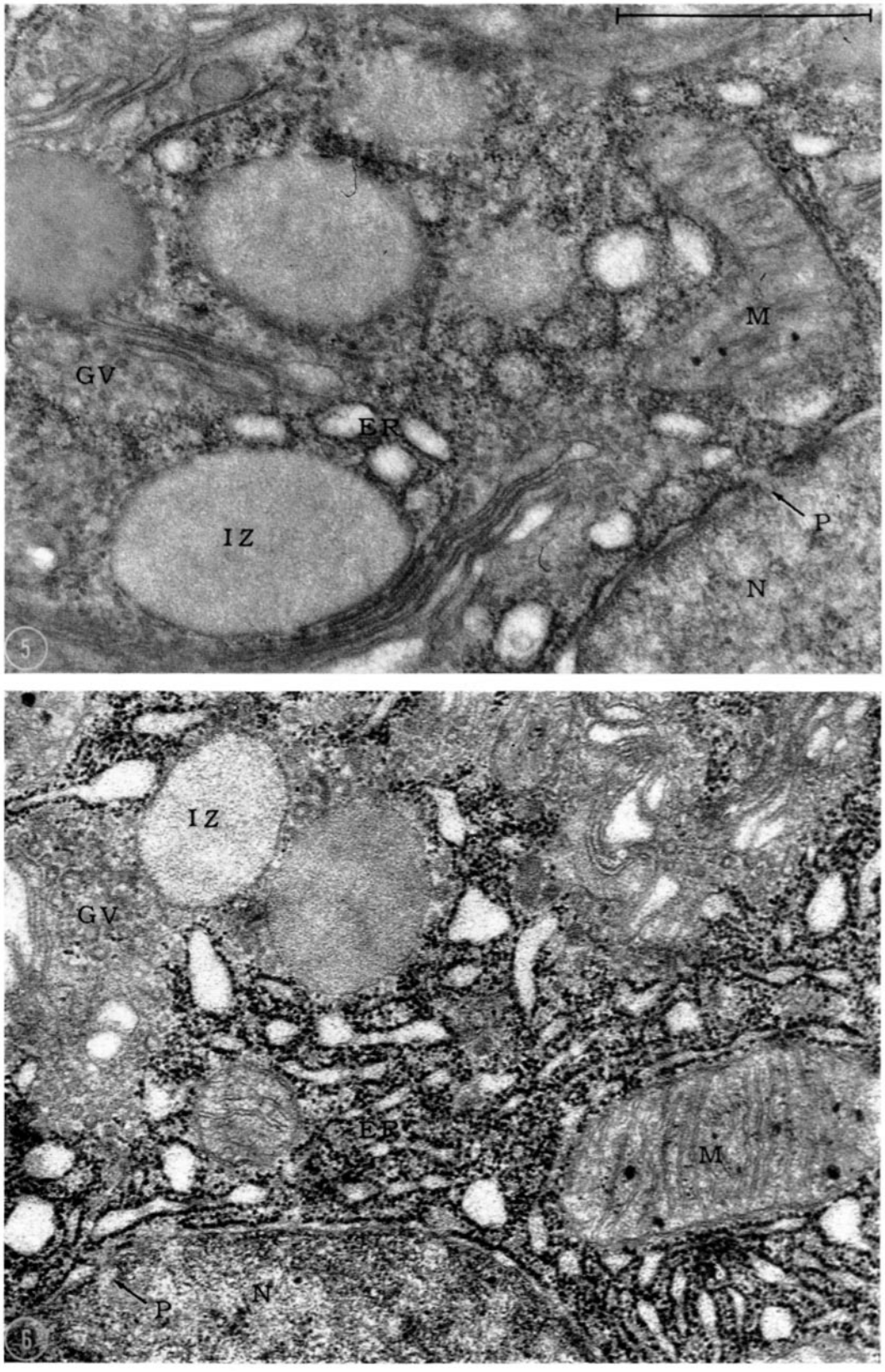


(Dalton and Zeigel: Lead hydroxide staining of biological material)

PLATE 226

FIG. 5 is of a portion of a cell in a section stained with a saturated solution of lead hydroxide for 20 minutes. A portion of the nucleus with a nuclear pore is at the lower right, a mitochondrion at the upper right and toward the left are ergastoplasm, zymogen granules and parts of the Golgi complex. A general increase in contrast is evident when compared with Fig. (The micron marker in this figure is in error. It should measure 46 mm.)

FIG. 6 is from a section stained with a saturated solution of monobasic lead acetate for 10 minutes. A portion of the nucleus with a nuclear pore appears at the lower left and a large mitochondrion near the lower right surrounded by ergastoplasm. Toward the upper part of the figure are zymogen granules and parts of the Golgi complex. In this case the ribonucleoprotein granules are selectively stained; *e.g.*, mitochondrial and Golgi membranes exhibit no greater contrast than those in Fig. 3 while the ribonucleoprotein granules show greater contrast than those in any of the other figures.



(Dalton and Zeigel: Lead hydroxide staining of biological material)