

# ULTRATHIN FROZEN SECTIONS

## II. Demonstration of Enzymic Activity

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### ABSTRACT

Endogenous enzyme activity can be readily and routinely demonstrated in ultrathin, frozen sections for electron microscopy. The procedure employed to obtain the best structural preservation as well as enzyme activity in thin sections involved fixation in glutaraldehyde, embedding in thiolated gelatin or pure gelatin, partial dehydration in glycerol, and sectioning in a cryostat at  $-35^{\circ}\text{C}$  with a slightly modified Porter-Blum microtome on which the tissue is maintained at  $-70^{\circ}\text{C}$  and the knife at  $-23^{\circ}\text{C}$ . Kidney cortex was used as test tissue, but a few other organs were occasionally used. Thin sections were floated on the surface of several incubation media routinely employed for enzyme cytochemistry. Positive, specific reactions were obtained for alkaline phosphatase in kidney brush border, for adenosine triphosphatase in brush border and in basal membranes of distal tubules, for acid phosphatase and esterase in lysosomes, and for NADH diaphorase in mitochondria. Mitochondrial ATPase was sporadically evident only in the distal tubule of the kidney. Localizations of enzyme activity reported by other technical approaches were confirmed and in some cases somewhat improved.

### INTRODUCTION

The classical techniques of enzyme histochemistry for optical microscopy are usually carried out on frozen sections, 4–40  $\mu$  or more in thickness, although some enzymes survive sufficiently even in sections of paraffin-embedded tissues. The routine procedure of fixation and embedding for good structural preservation of tissues for electron microscopy, involving osmium-tetroxide fixation and plastic embedding, do not permit enzyme cytochemistry on ultrathin sections. Instead, very successful localization of the products of enzyme activity at the ultrastructural level has been accomplished by preincubating 50- $\mu$  thick, frozen

sections with the enzyme substrates and by subsequently processing them by the routine osmium-tetroxide-epoxy-resin methods (2, 3, 10, 12, 13, 20, 32, 33). More recently, good results have also been obtained with 10–50- $\mu$  sections cut with a tissue sectioner that avoids freezing (11). It still seemed desirable, however, to develop a relatively simple method that would permit us to carry out these reactions directly on ultrathin sections. This would permit us to carry out several reactions on a single block of tissue or even on a single cell. Attempts to achieve this goal with aldehyde-fixed tissues embedded in water-miscible glycolmeth-

acrylate (GMA)<sup>1</sup> and hydroxypropylmethacrylate (HPMA) (16, 17) were without success, although thin sections of such tissues were amenable to other types of cytochemical reactions. Since in this investigation the tissues were not embedded in plastics, we eliminated the possibility of enzyme inactivation during polymerization. Furthermore, the tissues underwent less dehydration, and they were sectioned at a lower temperature than in previous experiments: two other factors which may have contributed to better preservation of enzyme activity.

## MATERIALS AND METHODS

### *Tissues*

Experiments were carried out with the cortex of the rat kidney, since all of the enzymes investigated occur there in abundance. The kidneys of 14 rats were used. In a few special cases other tissues were also employed, namely, colon epithelium, liver parenchyma, and diaphragm muscle.

### *Fixatives*

Fixation of the tissues was carried out at 3°C in solutions buffered to pH 7.2 with 0.1 M sodium cacodylate. Routine fixation involved the use of 1.25 and 2.5% glutaraldehyde for 15 min and 1 hr each. Eventually, our standard fixation became 2.5% glutaraldehyde for 1 hr. Other fixation schedules were occasionally employed, including 1.25 and 4.0% glutaraldehyde for 10 min and 4 hr, respectively, and 4% formaldehyde plus 0.25 M sucrose for 10 min and 1 and 30 hr.

After fixation the 1.5 mm<sup>3</sup> blocks of tissue were washed overnight in two changes of the cacodylate buffer on an agitator in a cold room at 3°C.

### *Embedding Procedures*

Embedding was carried out primarily in a mixture of 10% Thiogel A plus 10% Thiogel B (Schwarz Bio Research, Inc., Orangeburg, N. Y.) cross-linked by the action of 5% dimethylsulfoxide (DMSO) (6). Trials were also made with 20% Thiogel A, 20% Thiogel B, and 20% concentration of a purified, non-thiolated gelatin (Rousselot, Paris 16e., France) (5-7).

### *Sectioning Procedure*

The preparation of the tissue blocks and method of cutting sections were those described by Bernhard and Leduc (6). The sections were picked up from

<sup>1</sup> Leduc, E. H., and W. Bernhard. 1967. *J. Ultrastruct. Res.* 19: 196.

the trough of the knife with plastic rings designed by Marinozzi (16), were removed from cryostat, and were floated for a few minutes on distilled water for removal of the DMSO on which they had floated in the trough and for thawing and flattening out.

### *Enzyme Reactions*

Enzyme activities studied included those of adenosine triphosphatase (ATPase), acid and alkaline phosphatases, esterase, and NADH diaphorase. Control sections were either inactivated by heating to 80°C for 15 min or were incubated in media lacking substrates. The sections were floated on the surface of the incubating medium, either while they were still in the plastic rings or after they had been picked up on Formvar- and carbon-coated grids, and were air-dried. The latter was preferable for those reactions in which lead salts were formed in neutral or alkaline solutions, in that cleaner preparations were obtained. After incubation, the sections were rinsed in two changes of distilled water. Acid rinses or poststaining in phosphotungstic acid (PTA) had to be avoided with reactions in which lead phosphate is deposited at the site of enzyme activity, because the reaction product is partly dissolved (12) and in some cases is redeposited at other sites.

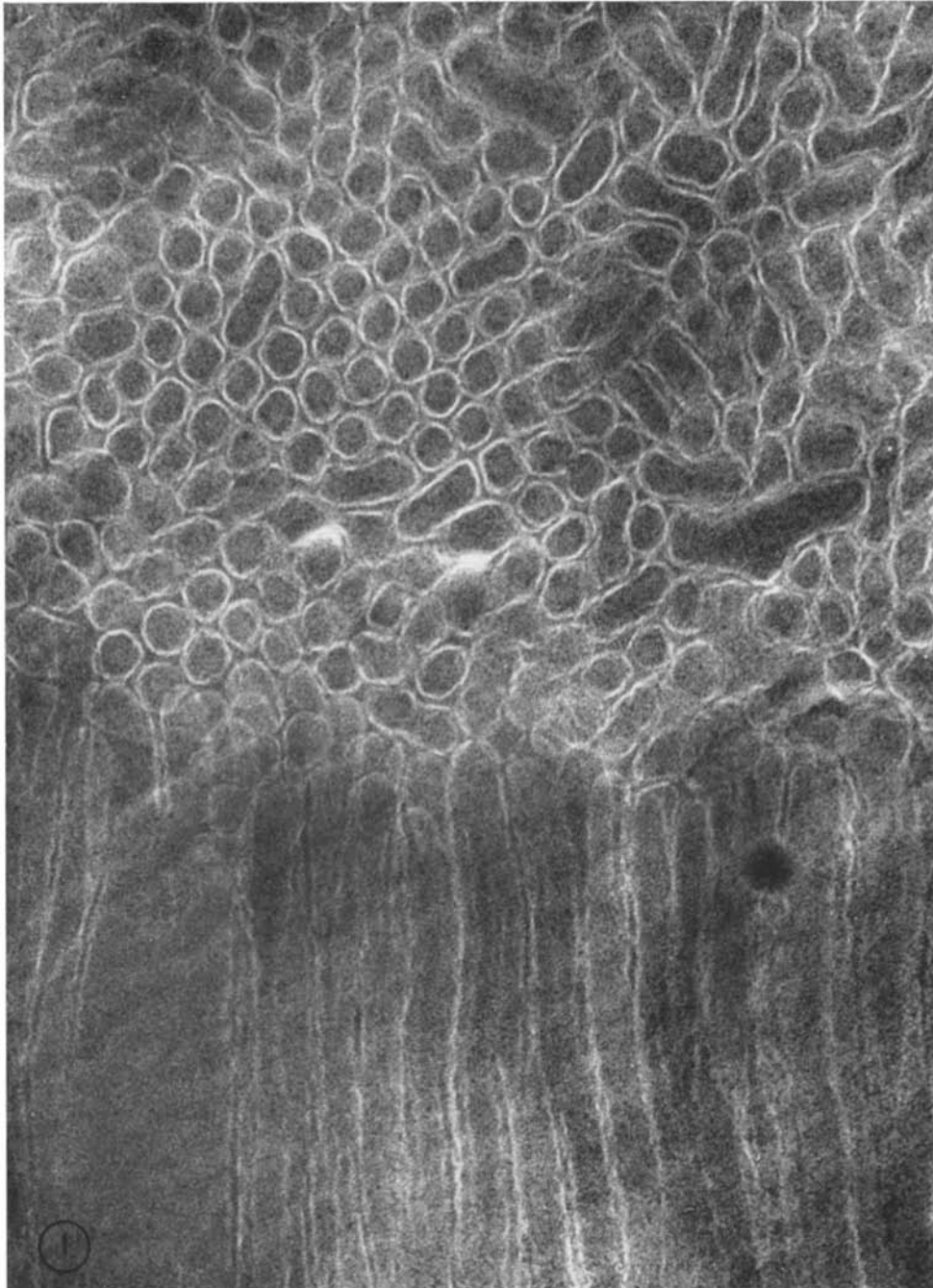
The incubating medium for revealing ATPase activity was that of Wachstein and Meisel (37), modified only by the addition of 0.25 M sucrose. Incubation was carried out at room temperature (22°-24°C) for 30 min and 1 and 2 hr.

The substrate for acid phosphatase was cytidine-5'-monophosphate (12) with manganese as activator. The constituents of the medium were as follows: 0.05 M acetate buffer pH 5.0, 7.9 ml; 2% lead nitrate, 0.6 ml; 0.1 M manganese chloride, 0.5 ml; 2 M sucrose, 1.0 ml; cytidine monophosphate, 10 mg. Incubation was carried out at room temperature for 30 min and 1 and 2 hr.

Three media for alkaline phosphatase (pH 8.5-9.0) were utilized: (a) that of Hugon and Borgers (15) with glycerophosphate, tris maleate buffer, and lead nitrate, (b) that of Tranzer (36) with glycerophosphate, Veronal buffer, and lead citrate; and (c) a modification of the Tranzer medium<sup>2</sup> in which 1% disodium 1-naphthylphosphate is employed as substrate. Incubation was carried out at room temperature for 5, 10, and 15 min and occasionally 30 min.

Esterase activity was revealed by the indoxyl acetate-hexazotized pararosaniline method investigated by Holt and Hicks (14) in which the azo dye derived from indoxyl becomes complexed with divalent osmium tetroxide during postfixation to form an electron-opaque reaction product. Incubation was carried out in an ice bath for 3 hr. The sections

<sup>2</sup> Tranzer, J. P. Unpublished observations.



All figures are of frozen ultrathin sections of the cortex of the rat kidney. The tissues in Figs. 8 and 10 were fixed in a 4% glutaraldehyde for 4 hr; the rest were fixed in 2.5% glutaraldehyde for 1 hr. All were embedded in Thiogel, impregnated with glycerol before sectioning, mounted on grids covered with Formvar and carbon, and stained or incubated with enzyme substrates.

**FIGURE 1** Brush border of the proximal convoluted tubule epithelium stained with PTA to demonstrate morphological preservation. To be compared with Figs. 2-5. Microvilli are cut in cross-section at top of figure, in longitudinal section at bottom of figure. The unstained cell membrane stands out in negative image against the heavily stained cytoplasm within the microvilli and the extracellular coat.  $\times 90,000$ .

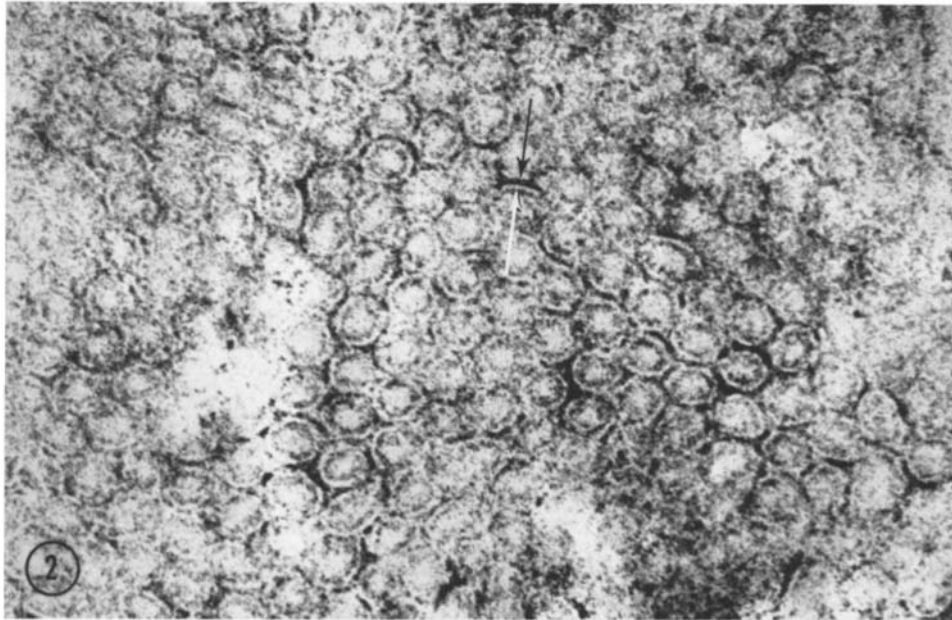


FIGURE 2 Alkaline-phosphatase activity, 10 min incubation at 22°C in Hugon and Berger's medium, in cross-sections of microvilli. The enzyme product outlines the cell membrane with a heavier precipitate on the external surface of the cell membrane (black arrow) and a somewhat lighter precipitate on the internal surface (white arrow).  $\times 80,000$ .

were then floated for 1 hr at room temperature on 2% osmium tetroxide in phosphate buffer at pH 7.3 and containing 1% formaldehyde or ethyl alcohol. This ensured reducing conditions for the formation of the osmium-tetroxide chelate of the azo dye better than those provided by the very small amount of tissue in the ultrathin sections.<sup>3</sup>

NADH diaphorase (25) was demonstrated by incubating the sections in the following medium: 0.2 M tris buffer pH 7.2, 0.4 ml; 0.1% tetra Nitro BT, 0.5 ml; NADH, 6 mg. Incubation was carried out at 37°C for 1½ hr with three changes of the substrate solution.

## OBSERVATIONS

### *Fixation*

Concentrations of 1.25 and 2.5% glutaraldehyde, for 15 min and 1 hr each, conserved equivalent degrees of activity of adenosine triphosphatase and acid and alkaline phosphatase. Because structural preservation was best with 2.5% glutaraldehyde for 1 hr, as judged from parallel sections stained with PTA, this was selected as our standard

<sup>3</sup> Holt, S. J. Unpublished observations.

fixative. Formaldehyde-fixed tissues were also phosphatase-positive but structural preservation was very poor. The demonstration of esterase activity was carried out primarily after 4% glutaraldehyde for 4 hr, for comparison with other procedures of embedding and sectioning (14), and the enzyme was also preserved after 2.5% glutaraldehyde-fixation for 1 hr. NADH diaphorase was more active after fixation in 4% formaldehyde for 10 min (25) than in 1.25% glutaraldehyde for 10 min, but structural preservation in the latter was preferable. It must be emphasized that after fixation all tissues were washed in the cacodylate buffer for at least 16 hr. With a few minor exceptions, our results corroborate in general the observations of Goldfischer et al. (12) on the relative capacities of formaldehyde and glutaraldehyde to preserve the enzymic activity of tissues.

### *Embedding*

All of the enzymes were demonstrated in tissues embedded in the mixture of Thiogel A plus Thiogel B. In addition, alkaline phosphatase, the enzyme most sensitive to inhibitory effects of various

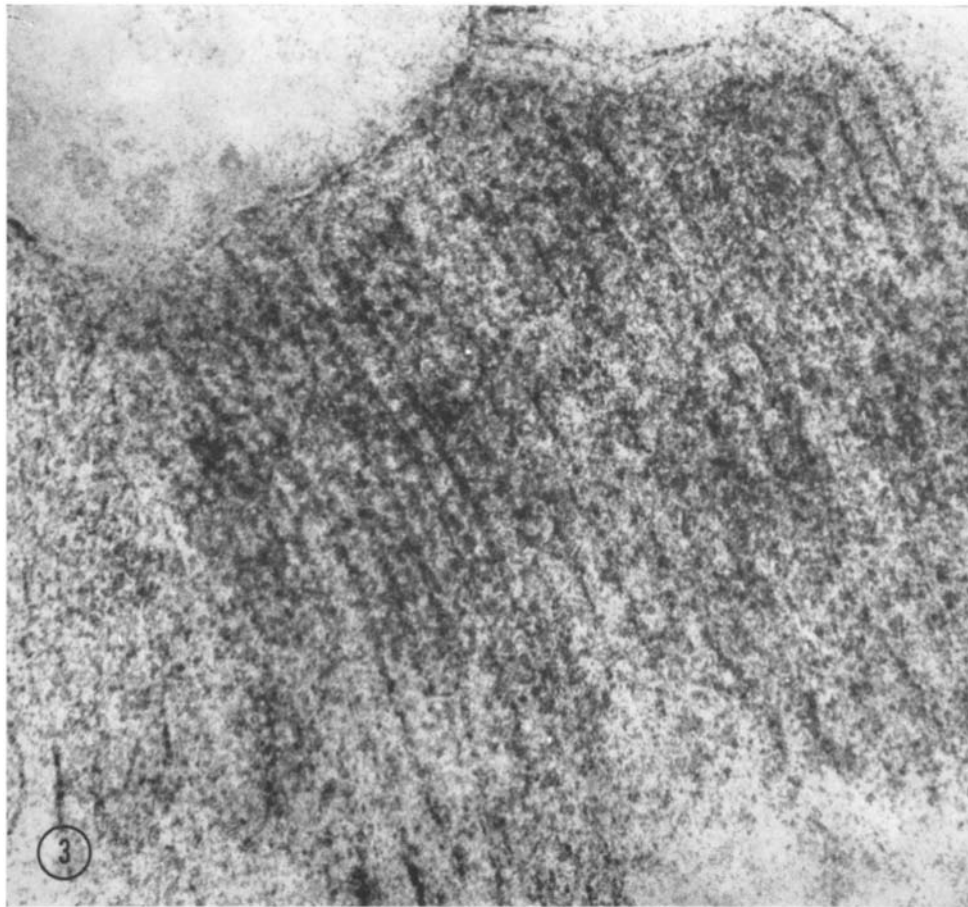


FIGURE 3 Alkaline-phosphatase activity, 5 min incubation, in longitudinal sections of microvilli.  $\times 60,000$ .

technical procedures, was found to be equally active in blocks of the same kidney which were also embedded in (a) the Thiogel A + B mixture but without preliminary impregnation, (b) Thiogel A alone, (c) Thiogel B alone, and (d) nonthiolated gelatin.

#### *Alkaline Phosphatase*

All three incubating media gave the same results, but in our hands the technique of Hugon and Borgers (15) was most consistently satisfactory, in that nonspecific precipitates were minimal. After a 5 min incubation period a fine precipitate of the reaction product was obtained; even after only 15 min a coarser, crystalline precipitate was formed and after 30 min there was a superposition of still larger particles. The latter may well have

been of enzymic origin but their size and abundance obscured underlying structure. Hence, 5–10-min incubation periods were preferable for enzyme localization.

In the kidney cortex, alkaline-phosphatase activity was found in the brush border of the proximal convoluted tubule. The greatest activity occurs on the external surface of the microvilli (Figs. 2 and 3) and is abundant along the entire length of a microvillus (Fig. 3). In addition, some activity occurs on the lining of some, but not all, of the tubular invaginations of the cell membrane in the apical cytoplasm. The latter is especially evident in overincubated (30 min) preparations. Finally, in cross-sections of the microvilli a small accumulation of reaction product is found in the form of a ring within the cytoplasm (Fig. 2).

This ring, or cylinder, exhibits a constant relative distance from the active external surface of the cell membrane and probably corresponds to the internal surface of the membrane (Fig. 1).

### *Adenosine Triphosphatase*

1–2-hr incubation periods were necessary to reveal the presence of this enzyme. As a result, there was, in addition to the precisely localized enzyme product, a superimposed flocculent material (Fig. 7) which was always most abundant in areas of highest enzyme activity but which could be readily distinguished from the reaction product.

In kidney cortex there are two major sites of adenosine-triphosphatase activity that are invariably demonstrable: the brush border of the proximal convoluted tubule cells and the basal membrane of the distal convoluted tubule cells (12, 32). Activity is greater in the latter, and after the shorter incubations of 30 min only cells of the distal tubule are reactive. With longer incubation the enzyme product appears on the brush border, at first only near the tips of the microvilli and later throughout the length of the microvillus.

As in the alkaline-phosphatase preparations, the major activity in the brush border is found on the external surface of the cell membrane, with a lesser accumulation of lead phosphate on the internal surface of the membrane (Figs. 3–5). Unlike alkaline-phosphatase activity, ATPase activity could not be demonstrated in the tubular infoldings of the cell membrane in the apical cytoplasm at the base of the microvilli.

The degree of activity associated with the deep infoldings of the basal membrane of the distal tubule cells varies to some extent from one animal to another after 30-min–1-hr incubation periods. As in the microvilli, most activity, that is, the heaviest resulting reaction product, occurred on the external surface of the cell membrane, whereas a smaller degree of activity is discernible on the internal surface of this membrane (Fig. 7). Thus, the cell membrane is outlined by the lead-phosphate deposits on its two surfaces. The extracellular space between the folds of the membrane becomes filled with the reaction product after 2 hr of incubation (Fig. 7). Localization of activity, in this case in particular, is facilitated by comparing the enzyme preparations with corresponding sections stained with PTA (Fig. 6). Our interpretation corresponds with that of Pease (28) (see Fig. 4).

We were led to make extensive attempts to demonstrate mitochondrial ATPase by positive reactions in some of the mitochondria of the distal tubule cells. In some kidneys, many mitochondria exhibited an accumulation of reaction product. In other kidneys, only a trace was found in an occasional mitochondrion. The lead-phosphate precipitate was very fine compared to the heavier deposit on nearby basal membranes. It outlined the cristae mitochondriales with a continuous accumulation on the matricial surface of the inner mitochondrial membrane (Fig. 8). Mitochondria of the proximal tubule cells were not reactive. Attempts to activate mitochondrial ATPase with dinitrophenol or cysteine or by quenching in liquid nitrogen before fixation (1) and attempts to find it in other cell types including diaphragm muscle and colon epithelium (26) were unsuccessful.

### *Acid Phosphatase*

This reaction gave very variable results. In some kidneys a strongly positive reaction could be obtained after 30 min of incubation, whereas in others it occurred only after 1–2 hr. Activity was limited to the lysosomes or some of the “protein droplets” in the proximal convoluted tubule. Not all of the lysosomes in a section, or a tubule or even one cell were reactive. The lead phosphate reaction product is distributed throughout the lysosome (Fig. 9). A sharp line of demarcation exists at the edge of the lysosome, but in some preparation there was a diffuse deposit in the neighboring cytoplasm (Fig. 9).

### *Esterase*

All of the lysosomes in the proximal convoluted tubule and in hepatic parenchymal cells appear to be esterase-positive. The intensity of reaction may vary slightly from one particle to another, even within a single cell, but in general is relatively intense in all of them. The density of the lysosomes is about the same both in unincubated sections and in those floated on solutions containing the hexazotized pararosaniline but without indoxyl acetate, and the lysosomes can just be discerned in these preparations. Incubation with indoxyl acetate together with hexazotized pararosaniline produces a marked increase in their electron opacity. This is further enhanced by subsequent coupling of the reaction product with reduced osmium tetroxide (Fig. 10). The osmium tetroxide

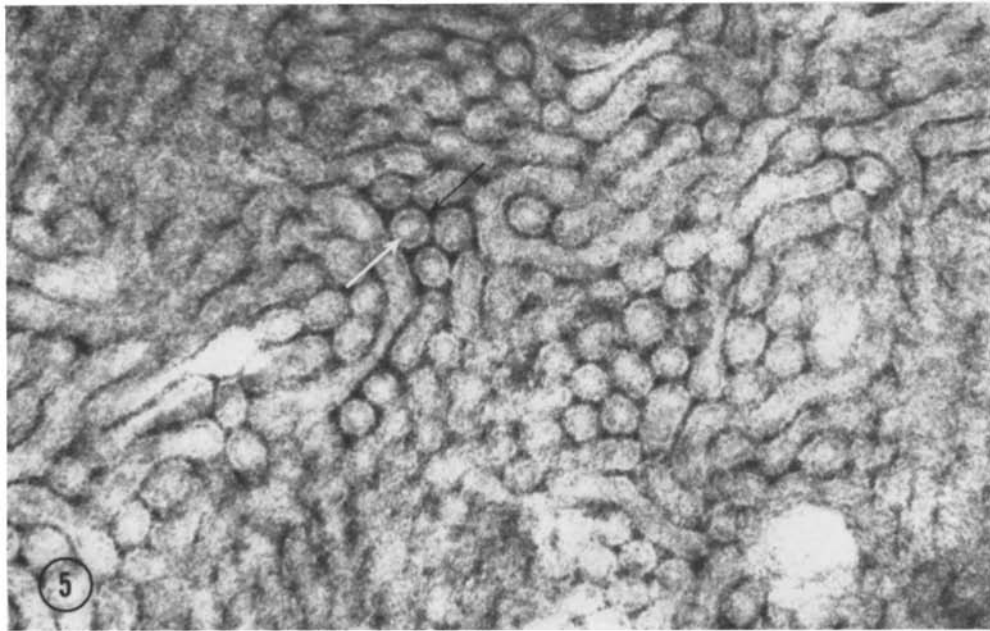
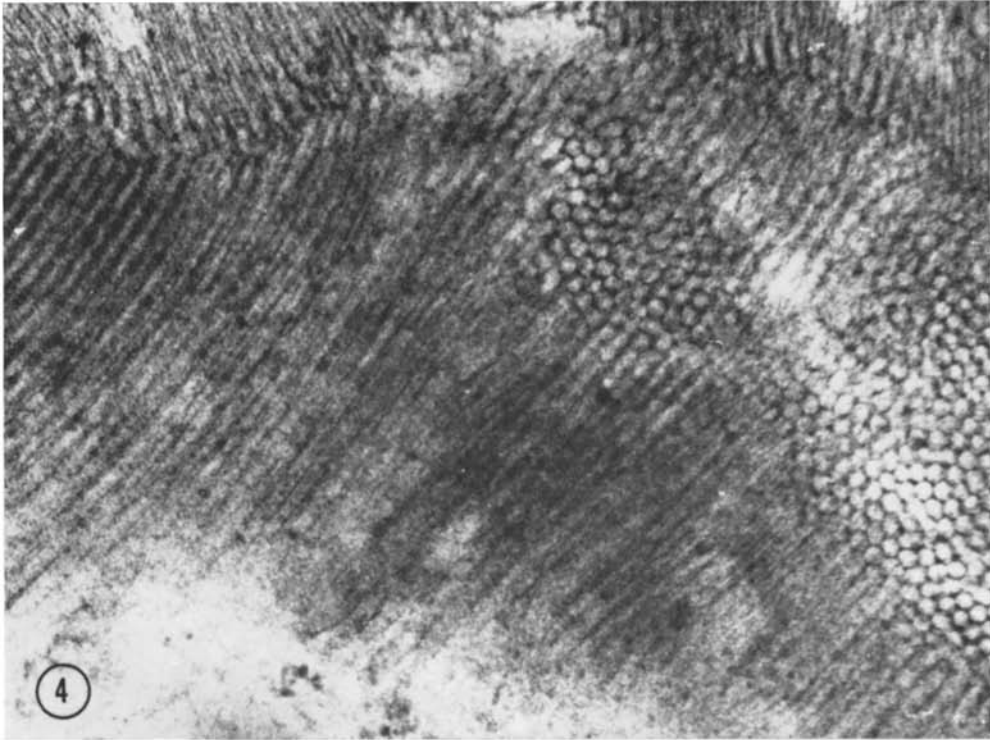


FIGURE 4 Adenosine-triphosphatase (ATPase) activity in brush border after 2 hr incubation at 22°C in Wachstein and Meisel's medium.  $\times 30,000$ .

FIGURE 5 ATPase reaction product, like that of alkaline phosphatase in Fig. 2, is heaviest on the external surface of the cell membrane and fills the extracellular space between the microvilli (black arrow). A faint but consistently reproducible accumulation of lead also is located on the internal surface of the cell membrane (white arrow); incubation for 2 hr in Wachstein and Meisel's medium.  $\times 60,000$ .

concomitantly produces a faint positive stain of mitochondrial membranes.

### *NADH Diaphorase*

This respiratory enzyme was tried because it was the one most likely to survive fixation (25). The reaction was first carried out on 1  $\mu$  sections for control by optical microscopy. In kidneys fixed 10 min with 4% formaldehyde a color reaction was discernible with the naked eye after 15 min of incubation and was intense after 30 min. After being fixed 10 min with 1.25% glutaraldehyde the formazan reaction product showed slower and less intense development, but development was visible after 45 min. In the optical microscope, the mitochondria in the formaldehyde-fixed kidney were deep blue and those in glutaraldehyde-fixed kidney were pale mauve. The reaction was limited to the mitochondria.

For electron microscopy, ultrathin sections were incubated for 1½ hr after which a color reaction was visible with the naked eye. With the electron microscope, in formaldehyde-fixed tissues examined at low magnifications the mitochondria were denser than the rest of the cell; this was not the case in control or unincubated sections. At higher magnifications, however, the mitochondria were too diffusely stained to determine the precise localization of the site of reaction within these organelles. In glutaraldehyde-fixed kidney, on the other hand, it was just possible to discern that the mitochondrial membranes were denser than the matrix. The electron opacity was too slight, however, to permit reasonable micrographs. Thus, precise localization of the reaction in ultrathin, frozen sections must await a means of rendering the formazan reaction product more electron opaque, without resorting to osmium tetroxide which stains the membranes per se.

### DISCUSSION

This investigation has shown that it is possible to retain enough endogenous enzyme activity in an ultrathin section to obtain cytochemical localization of activity directly on the section. Several factors have been changed since our earlier, unsuccessful attempts with ultrathin sections of tissues embedded in glycolmethacrylate (GMA) or hydroxypropylmethacrylate (HPMA). In the first place, the tissues underwent less dehydration. In previous work they were impregnated with a final mixture of 97% hydroxypropylmethacrylate and only 3% water; in this study they were impregnated and stored in 20% Thiogel or gelatin, then treated with 50% glycerol before sectioning. The second change was the absence of plastic embedding. This eliminated the possibility of chemical, physical, or thermal inactivation of enzymes which might occur during the polymerization of the embedding medium. Finally, sectioning was carried out at a lower temperature. It has been suggested (2) that sufficient heat might be generated at the moment that ultrathin (but not thick) sections were cut to inactivate enzymes. The exact temperature at the surface of the gelatin-embedded or Thiogel-embedded blocks at the moment of sectioning in the cryostat is not known, but the temperature of the block as a whole was  $-70^{\circ}\text{C}$ , that of the knife was  $-23^{\circ}\text{C}$ , and that of the cryostat was  $-35^{\circ}\text{C}$ . The results of two other successful demonstrations of enzyme activity directly on ultrathin sections throw some light on this question of enzyme preservation. Chase (8) was able to demonstrate alkaline-phosphatase activity in frozen-dried tissue that was subsequently embedded, first at  $4^{\circ}\text{C}$  then at room temperature, by slow impregnation with prepolymerized methacrylate dissolved in ethylene dichloride and by

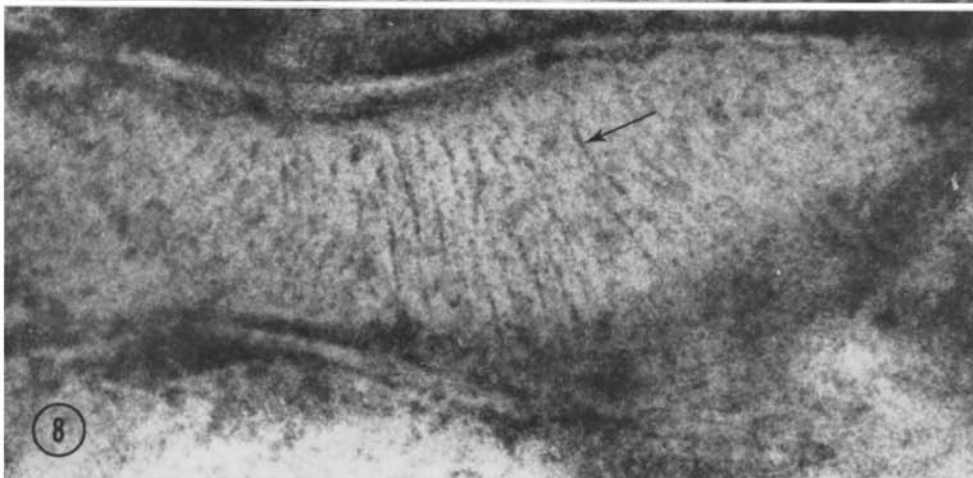
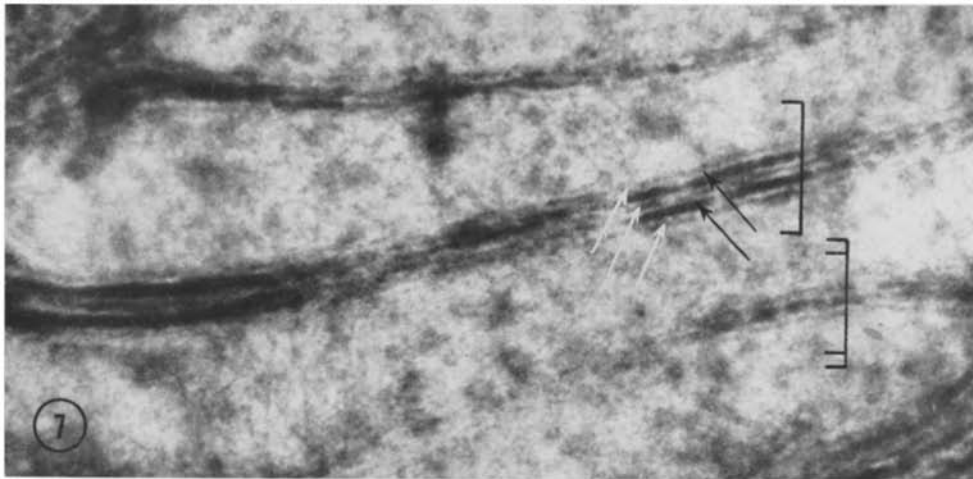
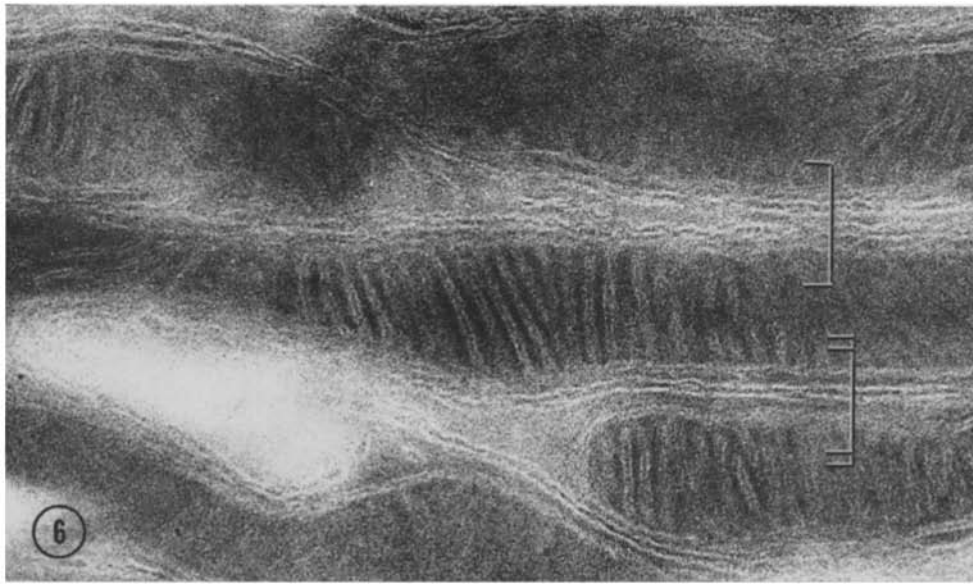
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FIGURE 6 PTA-stained section of the basal part of a cell lining the distal convoluted tubule. The infolded cell membranes between the mitochondria are to be compared with those that are similarly bracketed in Fig. 7.  $\times 90,000$ .

FIGURE 7 ATPase activity in the distal convoluted tubule after 3 hr incubation. Compare with similarly bracketed areas in Fig. 6. The reaction product is heaviest on the external surface of the cell membrane, fills the extracellular space (black arrows) and also lines the internal surfaces of the membrane (white arrows).  $\times 90,000$ .

FIGURE 8 ATPase reaction in a mitochondrion of the distal convoluted tubule. The reaction product forms a continuous fine precipitate on the matricial surfaces of the cristae (arrow) as described with other quite independent techniques.  $\times 90,000$ .





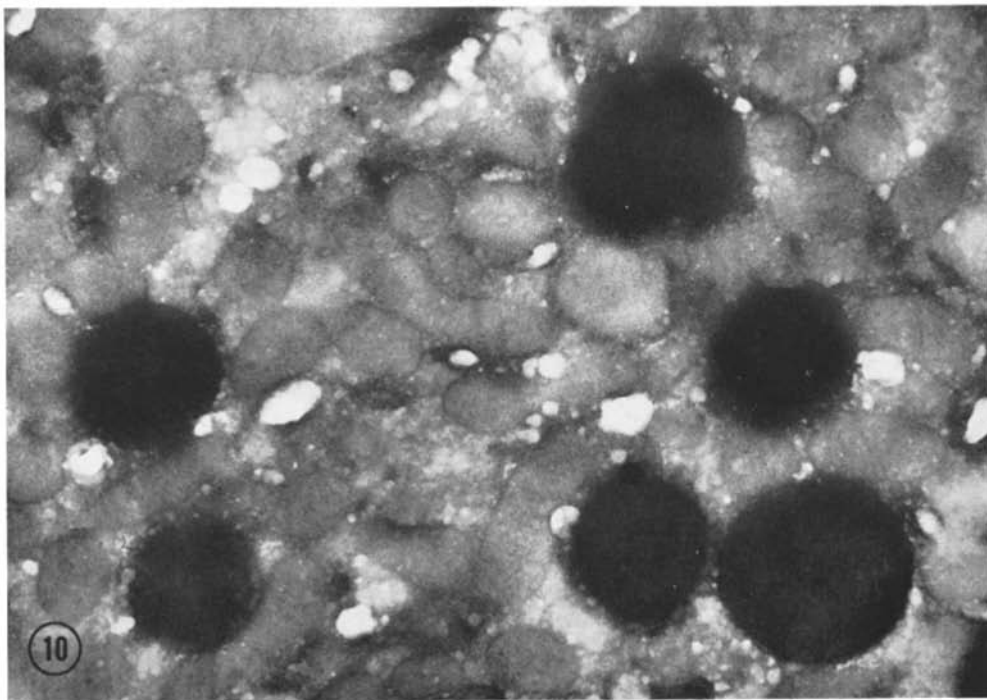
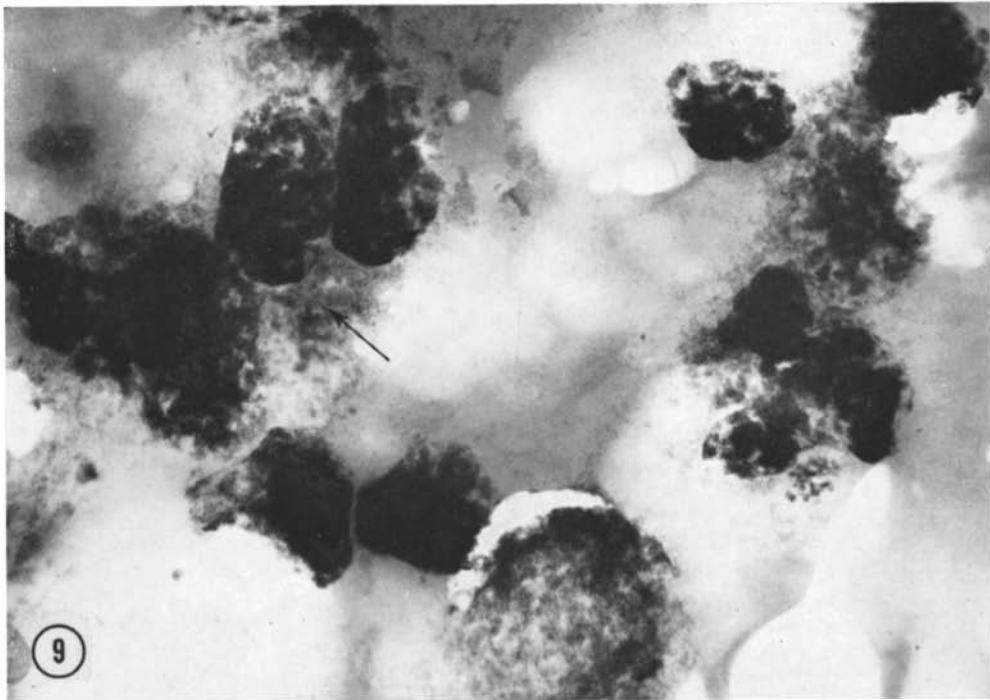


FIGURE 9 Acid-phosphatase activity, after 30 min incubation, localized in the lysosomes of the proximal convoluted cells. The reaction product nearly fills these organelles. In addition, some of the reaction product is present in the surrounding cytoplasm (arrow).  $\times 25,000$ .

FIGURE 10 Indoxyl acetate-esterase reaction, after 3 hr incubation, in the lysosomes of the proximal convoluted tubule.  $\times 21,000$ .

subsequent evaporation of the solvent. Tranzer (35) found high acid- and alkaline-phosphatase activity in glutaraldehyde-fixed tissue sectioned in a Pearse cryostat at 20  $\mu$  and dried in air. When adjacent 20- $\mu$  cryostat sections were dried onto the surface of a blank Epon block, ultrathin sections could be cut from them that still exhibited some enzymic activity, but the reactivity of the two enzymes was greatly reduced so that longer incubation times in more concentrated substrates were necessary. Furthermore, it is important to note here two other (unpublished) observations made in this laboratory. On the one hand, enzyme activity can be demonstrated with 97% HPMA-3% water-embedded tissues in sections that are 1  $\mu$  or more in thickness, but not less. On the other hand, ultrathin sections, cut in the same cryostat that was used in this study, of tissues embedded in gels consisting of 80% water, 5-10% water-miscible methacrylate, plus 5-10% carbowax or ethyleneglycol (4) were unreactive. Thus, it would seem that both the embedding procedure and the temperature of sectioning are important in the conservation of enzyme activity. Structural preservation varied with the conditions of fixation, and in briefly fixed material retraction artifacts occur even after only short exposures to stains (6). Hence, long exposure of fragile sections to incubation media presents the risk of loss of structural preservation and diffusion of enzymes.

The degree of reaction or staining varied directly with the thickness of the section. It is not surprising, therefore, that the most active of the enzymes investigated, alkaline phosphatase, could be demonstrated in the thinnest sections. Adenosine triphosphatase was also active in thin sections but after longer incubation. Esterase was strongly positive in the lysosomes in thin sections but it was rarely detectable in the cisternae of the ergastoplasm (14). Acid phosphatase, in contrast to the other enzymes, could be shown only in relatively thick sections. In sections which tapered in thickness from one side to the other, positive acid-phosphatase activity often occurred only in the thick part. This, plus the fact that frequently only a few lysosomes in a single cell were positive even in a thick section, suggests that the enzyme may have remained soluble and diffused out of some of the lysosomes either when the sections were thawed and flattened by floating on distilled water at room temperature or during incubation with enzyme substrates. This is supported by evidence of

diffusion of the reaction product into the cytoplasm around the lysosomes in occasional cells.

The reactions were highly specific according to the standards of cytochemistry at the light microscope level. Nuclear staining, that is a fine precipitate of lead particles on the chromatin (1, 9), occurred only once when the ATPase reaction was carried out at 37°C instead of 22°C. The question that this observation raises, whether nuclear staining is a diffusion artifact or a positive reaction that can be obtained only under certain conditions of fixation or incubation, has been discussed by Ashworth et al. (1). It might be related to non-enzymatic hydrolysis of ATP by the lead ions in the medium, a reaction which is pronounced at 37°C but not at 20°C.<sup>4</sup>

The same organelles were positive in this study as in others which had different technical approaches, but with some important differences. Perhaps the most striking difference was a more precise localization of the reaction product in adenosine-triphosphatase and alkaline-phosphatase preparations. We confirmed the already reported (1, 2, 8, 12, 20, 21, 32, 35) high level of activity on the external surface of reactive membranes and also revealed simultaneously a narrow continuous band of activity on the inner surface of these membranes. We have interpreted the cylinder of activity within the microvilli of the brush border as being related to the inner surface of the cell membrane because it corresponds in position to the inner surface of the phospholipid portion of the membrane seen in negative image in PTA-stained material. Furthermore, it appears too regularly disposed in relation to the highly active external surface of the membrane to be related to the filamentous extensions of the terminal web (1), and a similar zone of activity occurs in the basal membranes of the distal tubule cells. Overton (27), using the chopping procedure of Farquhar and Palade (11), has described ATPase activity associated with the inner layer of the unit membrane of duodenal microvilli, where the reaction product was distributed in small, discontinuous clumps. Another difference obtained when the reaction was carried out directly on ultrathin frozen sections, compared to thick sections, either frozen or not, followed by epoxy-embedding, is that in the former the reaction product is finer and is uniformly continuous along the reactive membranes instead of being in discontinuous

<sup>4</sup> Porter, K. R. Personal communication.

clumps as reported both in renal cells and in various other cell types (2, 11, 32). It remains to be determined whether the enzymes exist in a mosaic of patches visible at the magnifications employed here or whether they are uniformly distributed on the membrane. The acid-phosphatase reaction demonstrated here with cytidine monophosphate as substrate may have represented two acid hydrolases according to Novikoff (23), a "non-specific acid phosphatase" which also hydrolyses glycerophosphate and is not activated by manganese and an "acid nucleotidase" stimulated by manganese ions. In thick, frozen sections incubated with either glycerophosphate (13, 19) or cytidine monophosphate (12) before plastic embedding the reaction product is usually concentrated primarily in the zone adjacent to the limiting membrane of the lysosome. In our frozen thin sections the activity was more uniformly distributed throughout the lysosome. This might have been a function of a somewhat longer incubation time (24). On the other hand, like the more uniform distribution of the other phosphatases, this may have been the result of a better exposure of the cell organelles in the thin section to the components of the incubation media.

We did not review all sites of enzyme activity in detail, but concentrated on a few to determine the relative merits of this approach. In the kidney a positive ATPase reaction was also obtained in the cytoplasm of intertubular capillary endothelium and sometimes, but rarely, the reaction product was seen on adjacent basement membranes. Goldfischer et al. (12) report a positive reaction at this site after formaldehyde fixation but not after glutaraldehyde fixation, which was used here. Mitochondrial ATPase was found only in the distal convoluted tubule, never in other segments of the tubule, and there it was more regularly reactive in three kidneys fixed in 4% glutaraldehyde for 4 hr than in blocks of the same kidney and of other kidneys fixed in less concentrated glutaraldehyde for shorter periods of time. This suggests that mitochondrial ATPase is readily lost unless it is suitably fixed. It also suggests that the reaction product on the cristae probably was not the result of diffusion from adjacent highly active sites, because the basal infoldings were equally reactive in kidneys where little or no reaction product was found in nearby mitochondria. Although we did not get the striking activity demonstrated by Ashworth et al. (1) and Otero-Vilardebó

et al. (26), we did obtain the same localization in relation to the cristae. In diaphragm muscle fixed 1 hr in 2.5% glutaraldehyde we obtained positive ATPase activity in capillary endothelium, especially in its pinocytotic vesicles (18), and in the triads of the sarcoplasmic reticulum (12), but not in the myofibrils (34) or mitochondria. It is evident that considerable attention must be paid to fixation procedures in relation to different enzymes at specific sites with ultrathin, frozen sections as well as with thick, frozen sections stained before embedding (12, 31, 34).

In this preliminary work no attempt was made to recognize different ATPases by use of specific activators and inhibitors. It would be of interest to do so in light of Hoffman's hypothesis (see Farquhar and Palade, reference 11) that  $Mg^{++}$ -activated ATPase attacks extracellular ATP and  $Mg^{++} + Na^{+} + K^{+}$ -activated ATPase acts primarily on intracellular substrate. In a recent report Moses et al. (22) suggest that the ATPase reaction demonstrated in fixed tissue by the Wachstein and Meisel technique may be the result of nonenzymatic hydrolysis of ATP by the lead ions in the medium and that the plasma-membrane phospholipid could act as a phosphate acceptor. We cannot rule out these possibilities completely. However, the reactions in this study were carried out at room temperature and not at 37°C as in their work (22, 30). Furthermore, we carried out an additional control in which sections were heated to 80°C for 15 min to inactivate the enzyme and subsequently were incubated adjacent to nonactivated sections to determine whether the reaction product produced in the active sections could migrate and be trapped on the membranes of the inactivated sections. No lead precipitate was found in the heated sections.

In conclusion, a new procedure for demonstrating enzymic activity directly on ultrathin sections for electron microscopy has been developed. Its chief advantage over that reported by Chase (8) is that it is more rapid, and it is simpler in that it does not require elaborate equipment for freeze-drying. The problem of ice-crystal artifacts reported by Chase has been solved by Rebhun's (29) procedure of partial dehydration of tissues before freezing, and the dry-freeze-substitution method may very well become extremely useful in the localization of enzymes. With any of these approaches in which enzyme activity is localized directly on thin sections rather than on thicker

pieces, it is possible to avoid one of the pitfalls of cytochemistry, namely the problem of rapid and equal penetration of the tissue by all constituents of the incubation medium (12). On the other hand, the use of very thin sections might accentuate the possibility of the loss of diffusible enzymes from the tissue into the medium. It seems likely, however, that it will be possible to control this by the selection of the proper fixation and incubation schedules. The procedures outlined in this study

make possible not only a very precise localization of activity in some cases but also permit the investigation of several enzymes on a single small sample of tissue or even on a single cell.

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