The Demonstration of Alkaline Phosphatase in the Electron Microscope.* By Elisabeth R. G. Mölbert, Franz Duspiva, and Otto H. von Deimling. (*From the Ludwig Aschoff-Haus, Pathologisches Institut der Universität Freiburg i. Br., Germany.*)‡

At the present time, attempts are being made in many laboratories to adapt histochemical methods for electron microscopy (1–4, 6, 8, 13, 15, 16, 20–22) and thus localize in greater detail sites of enzymatic activity within the cell, and Barrnett and Palade (5) have recently discussed the problems involved.

We asked ourselves whether the well known reaction of Gomori and Takamatsu might be suitable for the demonstration of alkaline phosphatase in the electron microscope. For our experiments we derived valuable suggestions from the investigation of Essner, Novikoff, and Masek (7) on adenosintriphosphatase and 5-nucleotidase localization in liver cells which were recently published in this *Journal*. As demonstrated by these authors, enzyme inactivation by osmium tetroxide fixation shows a gradient from the periphery to the center of tissue blocks. Applying their technique of prefixation, but for a shorter time (about 3 minutes) we carried out the Gomori reaction (9) at pH 9.6 in the epithelial cells of the kidney tubules of mice. At the end of the incubation period we changed the method of Essner *et al.* and used a postfixation of 2 hours in osmium tetroxide solution as employed by Caulfield (11).

The product of the enzymatic reaction, *i.e.*, calcium phosphate, is absent in the outermost layers of the tissue because enzymatic activity is totally lost at this level. But the activity increases towards the center of the tissue-block. Fixation being conversely best for our purposes at the periphery and worst in the center, it follows that only a small layer, which is wide enough for 20 to 30 sections, shows both the reaction product and a well preserved structure.

Calcium phosphate deposits show only little contrast in the electron microscopic image because of their small electron-scattering power. To obtain a better contrast we replaced calcium chloride by lead nitrate in the incubation mixture. But here we ran into several difficulties for most phosphatase substrates give precipitates with the heavy metal around pH 7. We obtained a stabilization of the reaction mixture by using phenyl phosphate as a substrate and adding a chelating agent at pH 7.6–7.7.

Procedures

Tissue blocks of 1 mm.^{3} were prefixed with 1 per cent osmium tetroxide at 0°C. and incubated for 20 minutes at room temperature in the following reaction mixture: 2 gm. of potassium sodium tartrate dissolved in 45 ml. of tris buffer (tris (hydroxymethyl) aminomethane-HCl, 0.05 M, pH 7.6) and mixed with 5 ml. of 0.04 M disodiumphenylphosphate and 1 ml. each of 2 per cent manganese chloride and magnesium chloride. Finally, while shaking the mixture, 2.5 ml. of lead nitrate (0.1 M) were added drop by drop. The pH of the complete mixture was about 7.6–7.7. When carefully prepared, the solution was stable for about 30 minutes. As a control, the same mixture was used without the substrate.

After a brief fixation, the tissue blocks were washed several times with the incubation mixture. To avoid unspecific precipitation by residual components of the incubation mixture the fixative was changed several times during postfixation. For the rest of the preparation usual electron microscopic techniques were followed. Embeddings were made in methacrylate and the thin sections were studied in a Siemens Elmiskope I at 60 kv.

OBSERVATIONS

Under the conditions described, the enzymatic activity is referable mainly to alkaline phosphatase (17, 19) but the participation of other phosphatases is not excluded. With the technique outlined here we obtained excellent preservation of cellular fine structure as well as deposits of high contrast at the precipitation sites. In our micrographs, a fine precipitate can be seen on all outer cellular membranes of the tubule; *i.e.*, on the basal membrane infoldings, the microvilli of the brush border, the cell membranes of the endothelium of both peritubular and glomerular capillaries, and on the epithelial membranes of the glomerulus (Figs. 1 and 2).

In places where the membranes are cut normally it is clear that lead phosphate crystals lie always in one row on the side facing the cytoplasm. With the phosphatase reaction, therefore, the plasma membrane stands out as being very active. The sharp localization of the precipitate suggests a localization of the enzymes within or

* The results of this investigation have been presented at the Ninth Meeting of the German Society for Electron Microscopy, held October 19–21, 1959 in Freiburg i. Br. Fuller presentation will appear in the *Zeitschrift für Histochemie.*

‡ *Received for publication, November 15, 1959.*

very closely below the cell membrane. In cases in which the cell membrane was normally cut we never observed crystals in the extracellular spaces.

In control preparations, which were treated in exactly the same way (that is in the same reaction mixture but without a substrate), the precipitate was completely absent (Fig. 3).

In principle spurious localization of the precipitate could be due to two different causes. During incubation the enzyme molecules might migrate away from their original site in the cell, or, the molecules of the reaction product might diffuse away from their site of formation. Enzyme diffusion may be reduced or prevented by suitable prefixation. If indeed the enzyme is bound in the cell to definite structures it should be possible to preserve its original high local concentration by suitable prefixation. Under these conditions, the product of the enzymatic reaction (the orthophosphate ion) is formed at a high rate and is present in a high enough concentration to precipitate as lead phosphate in the immediate vicinity of the original site of the enzyme. Under these conditions, the objections raised by Johansen and Linderström-Lang (10–12) about the sharp localization of the end product (14, 18) in the Gomori reaction are not valid.

Our experiments show that a good localization of enzymatic activity within the cell and a quantitative measurement of the same activity, cannot be obtained at the same time. Other methods are available for quantitative studies. For the purposes of this study and the procedures used by us it is essential that the enzymatic activity in the cell be neither weak nor diffusely distributed, but present in reasonably high concentrations and bound to definite structures. The histochemical reactions employed here are therefore essentially qualitative methods. They demonstrate the localization of the enzymatic activity on the fine structures of the cell.

Addendum.—While this paper was in press, we read the article published by Kaplan and Novikoff (in *J. Histochem. and Cytochem.*, 1959, **7,** 295). These authors found, in agreement with us, enzymatic activity localized in the endothelium. In disagreement with our findings they detected phosphatase activity only in the apical part of the nephron epithelium.

BIBLIOGRAPHY

1. Barrnett, R. J., Histochemical demonstration of dehydrogenase systems requiring pyridine nucleotide coenzymes with the electron microscope, *Anat. Rec.*, 1957, **127,** 395.
2. Barrnett, R. J., Histochemical demonstration of the sites of acetylcholinesterase activity with the electron microscope, *J. Histochem. and Cytochem.*, 1957,
3. Barrnett, R. J., Histochemistry and electron microscopy, *4th Internat. Conf. Electron Micr.*, Berlin, 1958.
4. Barrnett, R. J., and Palade, G. E., Histochemical demonstration of the sites of activity of dehygenase systems with the electron microscope, *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 577.
5. Barrnett, R. J., and Palade, G. E., Application of histochemistry to electron microscopy, *J. Histochem. and Cytochem.*, 1958, **6,** 1.
6. Barrnett, R. J., and Palade, G. E., Enzymatic activity in the M band, *J. Biophysic. and Biochem. Cytol.*, 1959, **6,** 163.

6 *a*. Caulfield, J. B., Effects of varying the vehicle for OsO_4 in tissue fixation, *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 827.

7. Essner, E., Novikoff, A. B., and Masek, B., Adenosinetriphosphatase and 5-nucleotidase activities in the plasma membrane of liver cells as revealed by electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 711.
8. Gersh, J., Selective and cytochemical staining of frozen-dried preparations for study with the electron microscope, *4th Internat. Conf. Electron Micr.*, Berlin, 1958.
9. Gomori, G., and Benditt, E. P., Precipitation of calcium phosphate in the histochemical method for phosphatase, *J. Histochem. and Cytochem.*, 1953, **1,** 114.
10. Johansen, G., and Linderström-Lang, K., Precipitation of phosphate in the Gomori test, *Acta chem. Scand.*, 1951, **5,** 965.
11. Johansen, G., and Linderström-Lang, K., Liberation, diffusion and precipitation of phosphate in the Gomori test, *Acta med. Scand.*, 1952, **142,** 601.
12. Johansen, G., and Linderström-Lang, K., Liberation, diffusion and precipitation of phosphate in the Gomori test, *J. Histochem. and Cytochem.*, 1953, **1,** 442.
13. Kaplan, S. E., and Novikoff, A. B., The localisation of adenosine triphosphatase activity in rat kidney: Electron microscopic examination of reaction product in formol-calcium-fixed frozen section, *J. Histochem. and Cytochem.*, 1959, **7,** 295.
14. Lison, L., La recherche histochimique des phosphates. Etude critique, *Bull. Histol. appl. et Techn. micr.*, 1948, **25,** 23.
15. Mölbert, E., Duspiva, F., and von Deimling, O., Die Darstellung von Phosphatsens im elektronenmikroskopischen Bild, 9th Meeting of

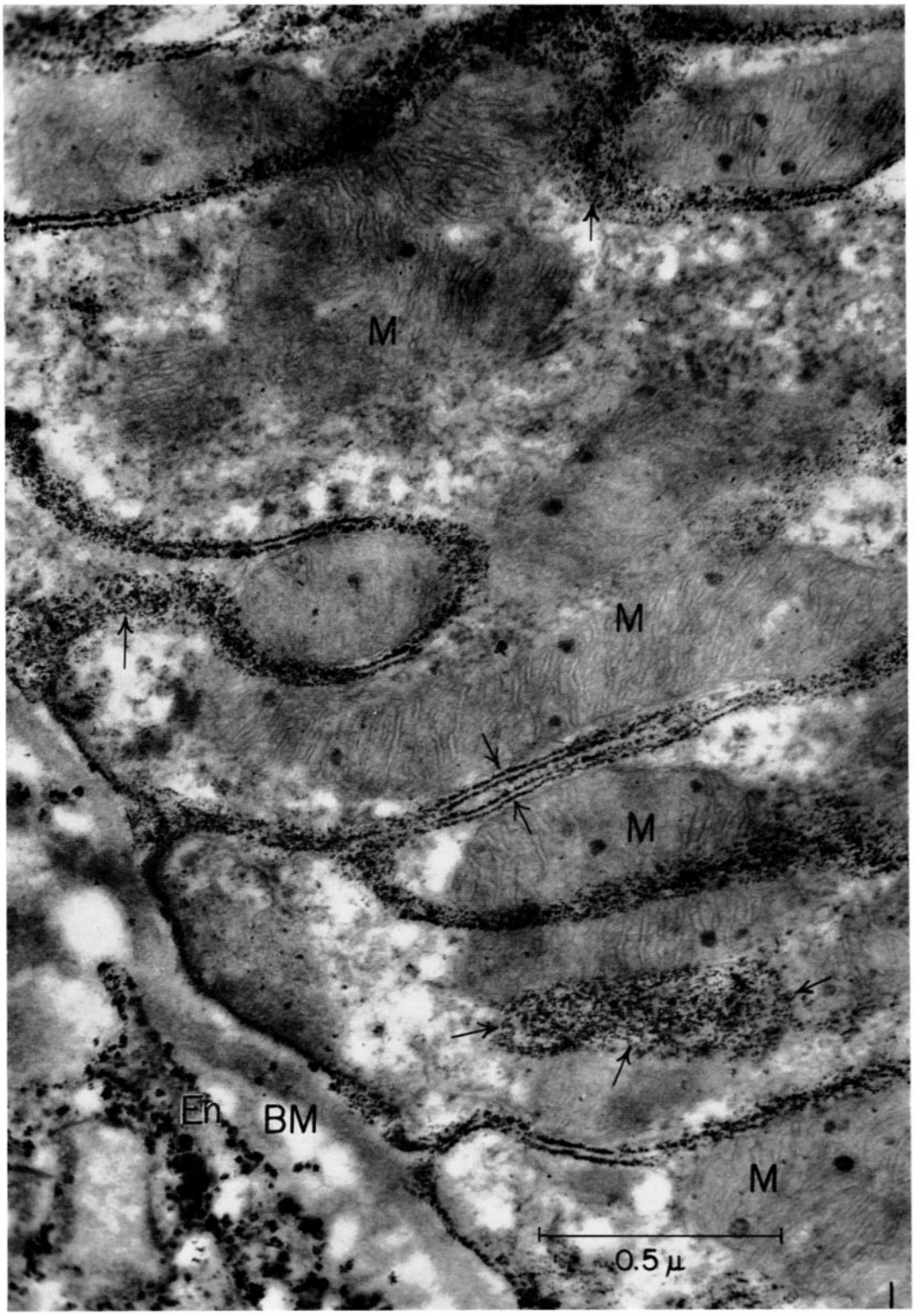
the German Society for Electron Microscopy, Oct. 19–21, 1959 in Freiburg i. Br.

16. Mölbert, E., Duspiva, F., and von Deimling, O., Die histochemische Lokalisation der Phosphatase in den Tubuluszellen der Mausniere im elektronenmikroskopischen Bild, *Z. Histochem.*, 1960, in press.
17. Novikoff, A. B., The validity of histochemical phosphatase methods on the intracellular level, *Science*, 1951, **113**, 320.
18. Pearce, A. G. E., Histochemistry, London, Churchill, 1954.
19. Spater, H. W., Novikoff, A. B., and Masek, B., Adenosintriphosphatase activity in the cell membranes of kidney tubule cells, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 765.
20. Unakar, N. J., and Sirsat, S. M., Distribution of succinic dehydrogenase in the human spermatozoa as revealed in the electron microscope, *4th Internat. Conf. Electron Micr.*, Berlin, 1958.
21. Watson, M. L., Staining of tissue sections for electron microscopy with heavy metals, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 475.
22. Watson, M. L., Staining of tissue sections for electron microscopy with heavy metals. II. Application of solutions containing lead and barium, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 727.

Explanation of Plates

Plate 211

Fig. 1. Basal zone of epithelial cell of the tubule as shown by the phosphatase reaction with lead nitrate. The infoldings of the cell membrane are marked as phosphatase-active by a fine crystalline precipitate of lead phosphate. Where the membranes are sectioned normally (single arrow), the lead phosphate crystals are observed as lying in a *single* row on the inner cytoplasmic surface of the membrane. Tangential sections of the membrane infoldings (double arrow) stand out clearly by the impregnation with lead. Usually such tangential sections of the membrane are not to be seen in the electron microscope because of their low contrast. The mitochondria (*M*) do not show an enzymatic reaction with this test; they are phosphatase-negative. Because of sufficient prefixation, the inner membranes of the mitochondria are clearly to be seen. The basement membrane (*BM*) shows no reaction. The cell membrane of the endothelial cells (*En*) is enzymatically active. × 76,000.

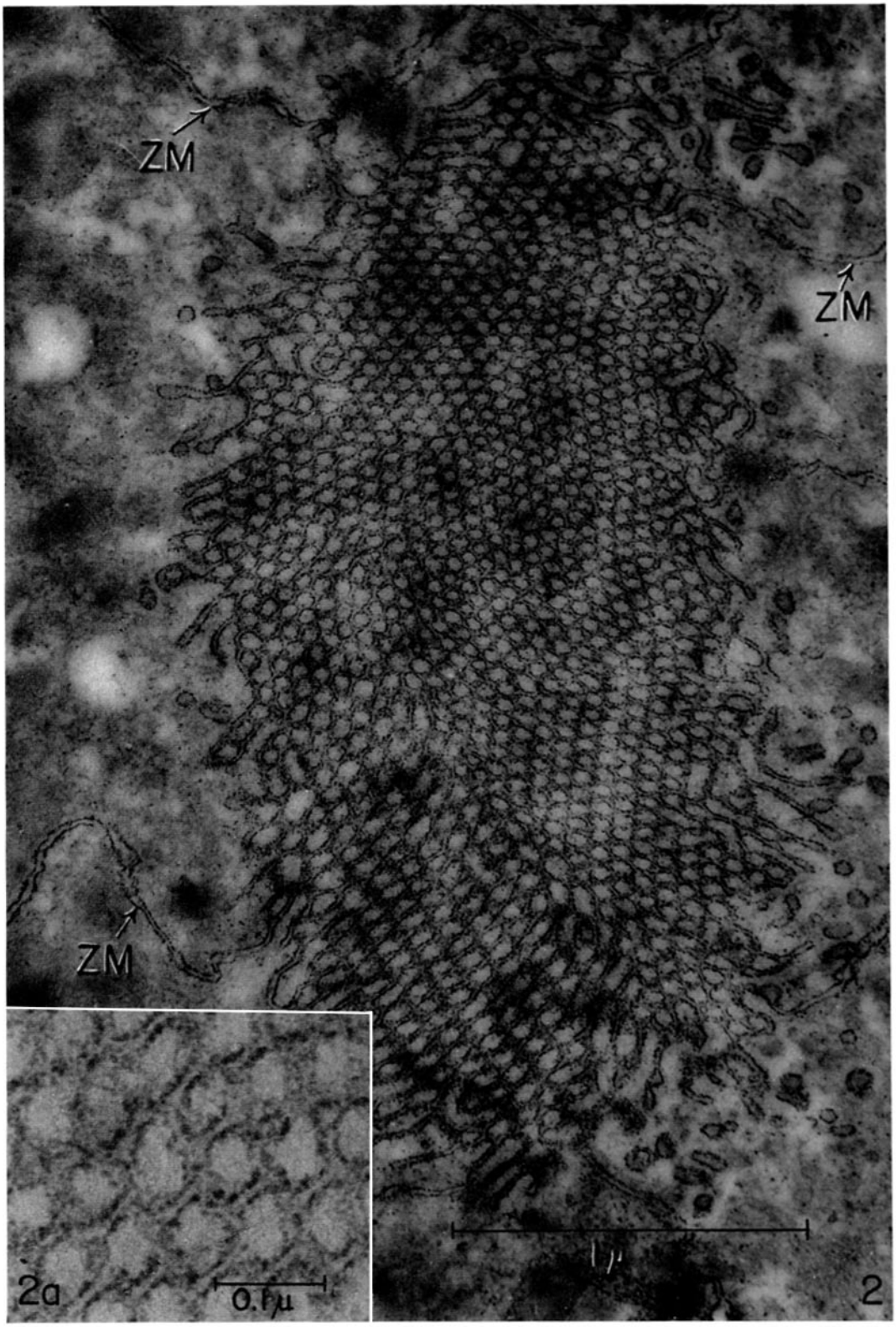


(Mölbert *et al.*: Alkaline phosphatase in the electron microscope)

PLATE 212

FIG. 2. Transversely cut brush border of epithelial cell of a mouse kidney tubule. Reaction with lead nitrate. The phosphatase activity is confined to the cell membrane of the microvilli as is shown by the small crystals attached to the cell membrane. In the extracellular space between the microvilli no crystals are visible. The limiting membranes of the cell (*ZM*) are also enzymatically active. × 56,000.

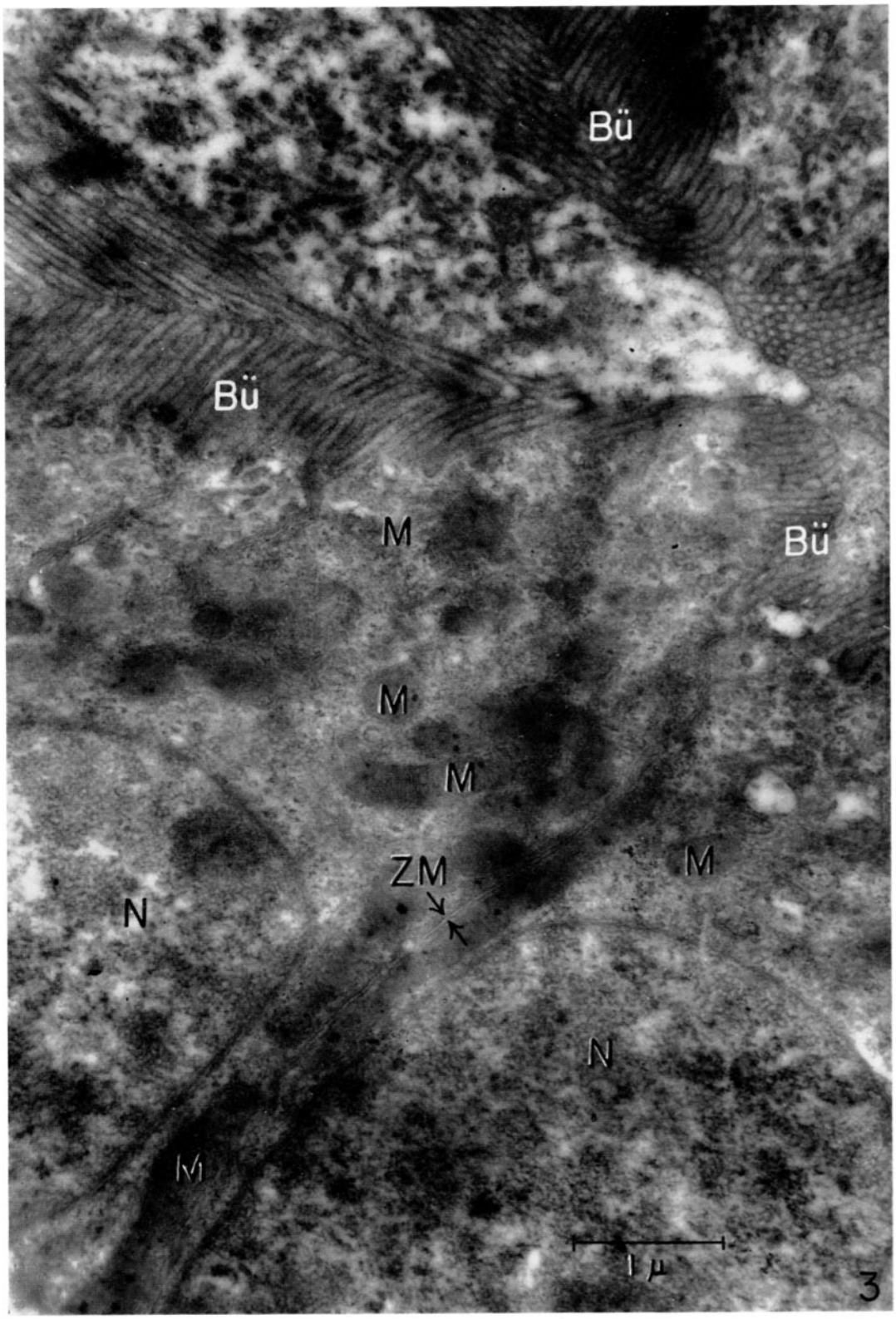
FIG. 2 *a*. Greater enlargement of transverse section of microvilli. × 170,000.



(Mölbert *et al.*: Alkaline phosphatase in the electron microscope)

PLATE 213

FIG. 3. Epithelial cells of the mouse kidney tubule. Control preparation for the phosphatase reaction. The control preparation has been incubated with all the reagents of the phosphatase reaction including lead nitrate and omitting only the substrate. No unspecific lead precipitations are evident in the cytoplasm. Only in the cell nuclei (*N*) few unspecific deposits of lead bound to the nucleic acids. The same phenomenon has been observed in tissues incubated with substrate. Cell membrane (*ZM*), brush border (*Bü*), Mitochondria (*M*). × 24,000.



(Mölbert *et al.*: Alkaline phosphatase in the electron microscope)