Histol Histopath (1991) 6: 309-315

Histology and Histopathology

Alkaline phosphatase, 5'-nucleotidase and magnesium-dependent adenosine triphosphatase activities in the transitional epithelium of the rat urinary bladder

S.X. Zhang, T. Kobayashi, T. Okada, E. García del Saz and H. Seguchi
Department of Anatomy and Cell Biology, Kochi Medical School, Kohasu, Okoh-cho, Nankoku, Kochi, Japan

Summary. The cerium-based method was used to demonstrate cytochemically the ultrastructural localization of alkaline phosphatase (ALPase), 5'-nucleotidase (5'-Nase) and magnesium-dependent adenosine triphosphatase (Mg-ATPase) on the transitional epithelium of the rat urinary bladder. The reaction product for ALPase was found on the plasma membrane of all epithelial cells, except the luminal surface of superficial cells. The activity of 5'-Nase appeared on the plasma membrane of all bladder transitional epithelial cells, including the free surface of superficial cells. The Mg-ATPase reaction product was seen on the plasma membrane of superficial, intermediate and basal cells, but never on the luminal surface of superficial cells and it was only occasionally seen on the basal surface. The possible functions of these phosphatases have been discussed, and it was emphasized that the 5'-Nase activity present on the luminal surface of superficial cells may play a special role in the membrane movement of these cells in the transitional epithelium.

Key words: ALPase, 5'-Nase, Mg-ATPase, Cerium, Transitional epithelium

Introduction

The chemical and physiological configuration of urine remains unchanged during the period it is retained in the bladder. However, the presence of intramembranous particles has been reported on the plaque region of the luminal plasma membrane in the superficial cells of the transitional epithelium, and intramembranous particles are often associated with enzyme proteins (Seguchi et al., 1980b; Seguchi, 1989).

Offprint requests to: Dr. H. Seguchi, Department of Anatomy and Cell Biology, Kochi Medical School, Kohasu, Okoh-cho, Nankoku, Kochi 783, Japan

In order to know the nature of these intramembranous particles we previously investigated the activities of several phosphatases on the plasma membrane of urinary bladder epithelial cells using the lead method (Seguchi et al., 1979, 1980a, 1982; Okada et al., 1982). However, in view of the inherent drawback of the lead-based cytochemical reaction for the phosphatases, we have recently adopted the cerium-based method, now commonly used for the ultrastructural demonstration of phosphatases because of its precise localization of the enzyme activity. In the present study, we used cerium as capture agent to demonstrate the distribution of ALPase, 5'-Nase and Mg-ATPase, in order to investigate the relationship between function and ultrastructure in the transitional epithelial cells of the rat urinary bladder.

Materials and methods

Urinary bladders of Sprague-Dawley strain rats weighing 200-300 g were used in this study. After the animals had been perfused via the heart with physiological saline and the adequate fixatives, tissue blocks were fixed by immersion for 30 min at 0° C. The fixatives used were 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) with 5% sucrose for ALPase (Robinson and Karnovsky, 1983a), 1% glutaraldehyde in 0.1 M Tris-maleate buffer (pH 6.0) containing 5% sucrose for 5'-Nase (Robinson and Karnovsky, 1983a), and 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) with 6.8% sucrose for Mg-ATPase (Hardonk et al., 1985). Subsequently, the blocks were washed overnight at 0-4° C in the same buffers as those used in the fixatives, and cut into 40-60 μm sections with a Microslicer (Dosaka EM, Co., Kyoto, Japan). The cytochemical reactions were carried out at 37° C in various media for 10-60 min with gentle shaking. The sections were then washed several times in the appropriate buffers and post-fixed for 60 min in 1% OsO_4 in 0.1 M cacodylate buffer (pH 7.2). The tissues

were then dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin (Spurr, 1969). Ultrathin sections were counterstained with uranyl acetate and lead citrate and observed under a JEM-1200EX (Jeol, Co., Tokyo, Japan) electron microscope operated at 80 Kv.

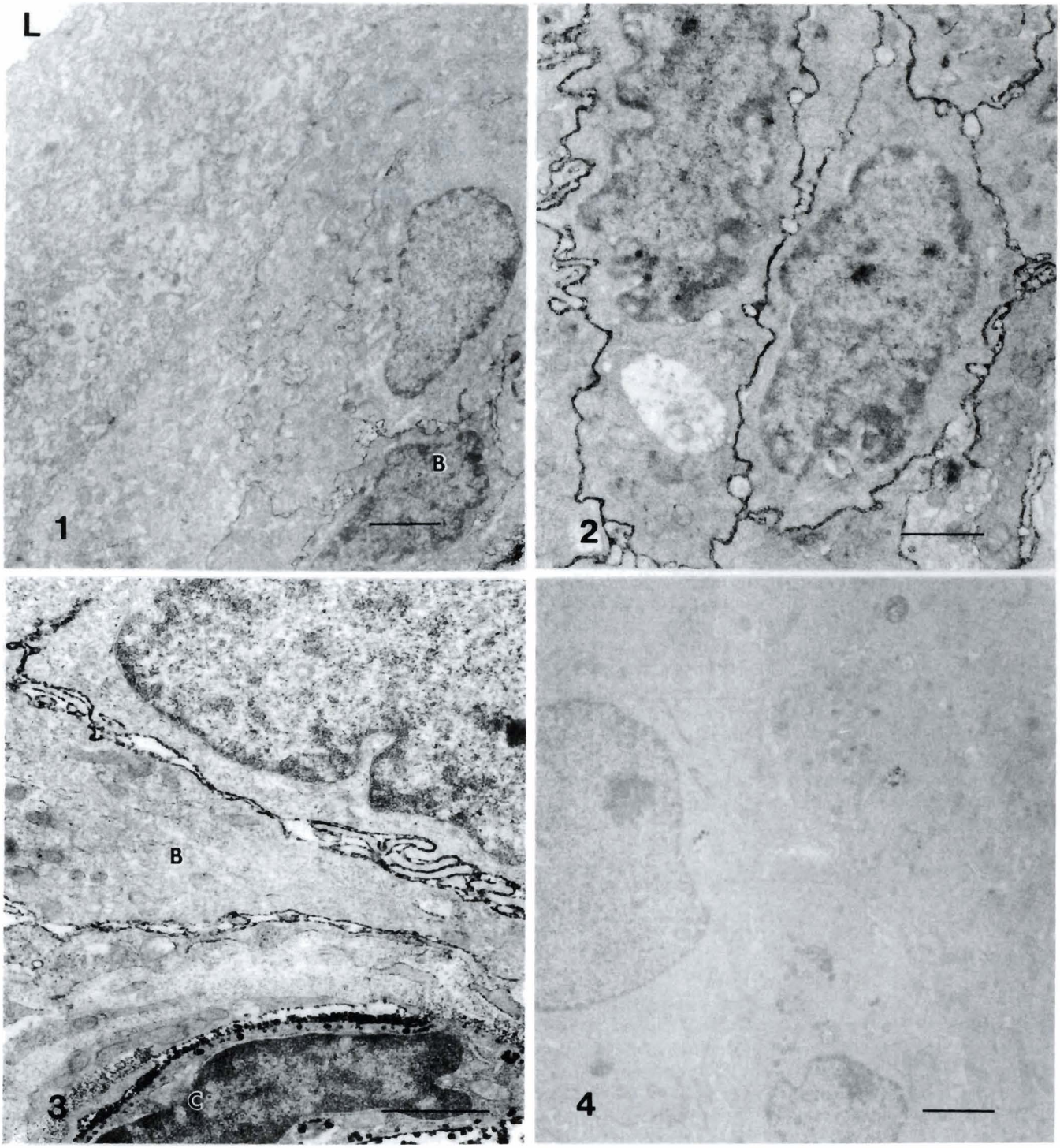


Fig. 1. ALPase activity in the transitional epithelium. The intensity of labelling decreases from base to top of the epithelium. B, basal cell; L, lumen × 7,000, bar = 1 μm.

Fig. 2. Localization of ALPase activity on intermediate cells. × 13,000, bar = 1 μm.

Fig. 3. ALPase positive reaction is detected on basal cells (B). C, capillary. × 17,500, bar = 1 μm.

Fig. 4. ALPase activity is inhibited when the tissue is incubated in a medium without substrate. × 12,000, bar = 1 μm.

The various incubation media used in this study were prepared as described below, just before incubation. The ALPase incubation medium contained 0.1 M Tris-maleate buffer (pH 8.0), 1mM ß-glycerophosphate (disodium salt), 2mM $CeCl_3$ and 5% sucrose; that for 5'-Nase contained 0.1 M Tris-maleate buffer (pH 7.4), 1mM AMP, 2mM $MgCl_2$, 2mM $CeCl_3$, 2.5 mM levamisole and 5% sucrose; and that for Mg-ATPase

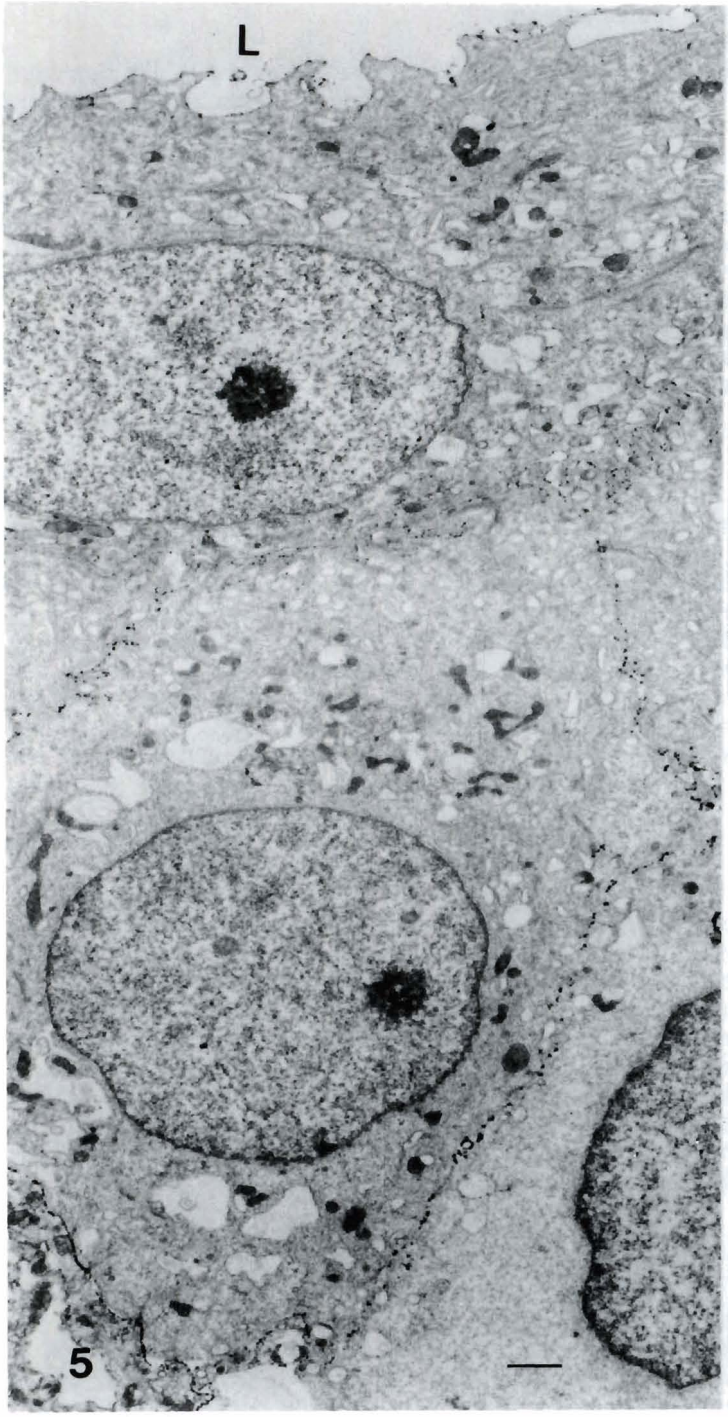


Fig. 5. Distribution of the 5'-Nase reaction product on the three layers of epithelial cells. L, lumen. × 7,000, bar = 1 μm.

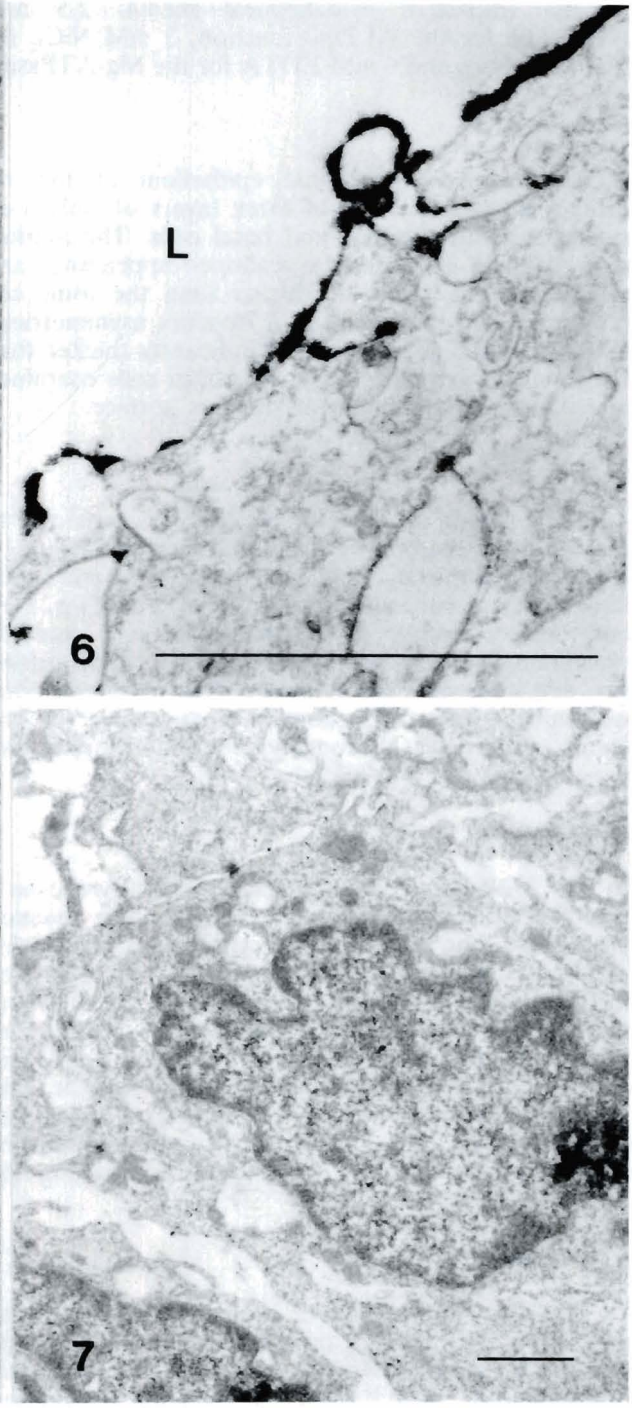


Fig. 6. Distribution of 5'-Nase reaction deposits on the luminal plasma membrane of superficial cells. L, lumen. × 58,000, bar = 1 μm.

Fig. 7. Control specimen showing no reaction product when $NiCl_2$ was added to the incubation medium. × 13,000, bar = 1 μm.

contained 70 mM Tris-maleate buffer (pH 7.2), 5 mM Mg $(NO_3)_2$, 2.3 mM ATP, 1 mM $CeCl_3$ and 2.5 mM levamisole.

Various control experiments were performed without substrates, and by adding different inhibitors to the respective cytochemical media: 2.5 mM levamisole for the ALPase reaction, 3 mM $NiCl_2$ for that of 5'-Nase and 6 mM EDTA for the Mg-ATPase.

Results

The normal transitional epithelium of the rat urinary bladder consists of three layers of cells, i.e., superficial, intermediate and basal cells. The luminal surface has a characteristic scalloped appearance and the plasma membrane is thicker than the usual cell membrane. In cross-section it appears asymmetrical, with the outer dense leaflet significantly thicker than the inner dense leaflet. The superficial cells contained many fusiform vesicles near the free surface.

ALPase

The reaction product for ALPase was restricted to the external face of the epithelial cell surface. It could be found around the basal and intermediate cells and on the lateral surface between two superficial cells (Figs. 1-3), but the free surface of the superficial cells, and the cytoplasm of all cells was always negative. No deposition was detected when the substrate, ß-glycerophosphate, was omitted from the incubation medium (Fig. 4). The enzyme activity was thoroughly inhibited by levamisole, a potent inhibitor for ALPase.

5'-Nase

The 5'-Nase activity on the plasmalemma was clearly detected by the cytochemical method. The reaction product was confined to the external side of the plasma membrane in the three layers of epithelial cells, including the luminal surface of superficial cells (Figs. 5, 6), where the reaction was less intense than at other parts of the plasma membrane. However, no reaction product was observed on other membrane structures inside the cells. The reaction product was not seen in controls without the substrate AMP. The 5'-Nase activity was inhibited by $NiCl_2$ (Fig. 7), but not by levamisole, proving the authenticity of the reaction.

Mg-ATPase

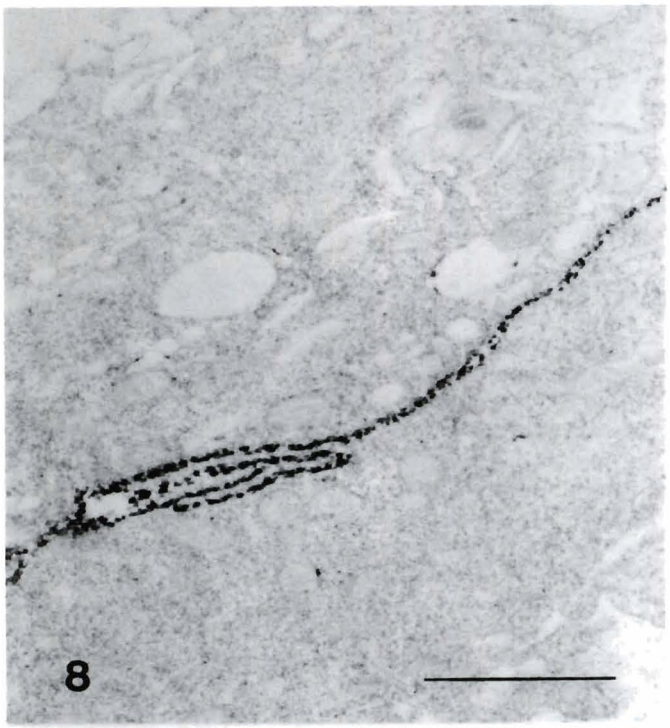
The Mg-ATPase reaction product was found along the plasma membrane of the epithelial cells, but not on the luminal surface. No reaction product was present inside the cells, including the nucleus and membranous structures in the cytoplasm. The basal plasma membrane of the basal cells revealed hardly any reaction deposits. Mg-ATPase activity was stronger between superficial and intermediate cells than between adjacent superficial cells or between intermediate and basal cells (Figs. 8, 9). The enzyme reaction was negative when the tissue was incubated in a medium without substrate, that is, ATP (Fig. 10) or Mg ions. The enzyme activity was completely inhibited by EDTA (Fig. 11).

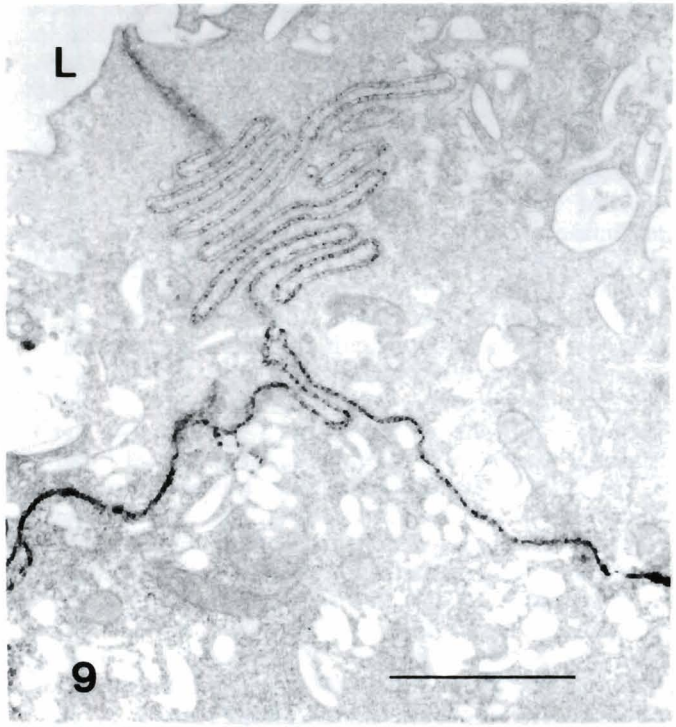
Discussion

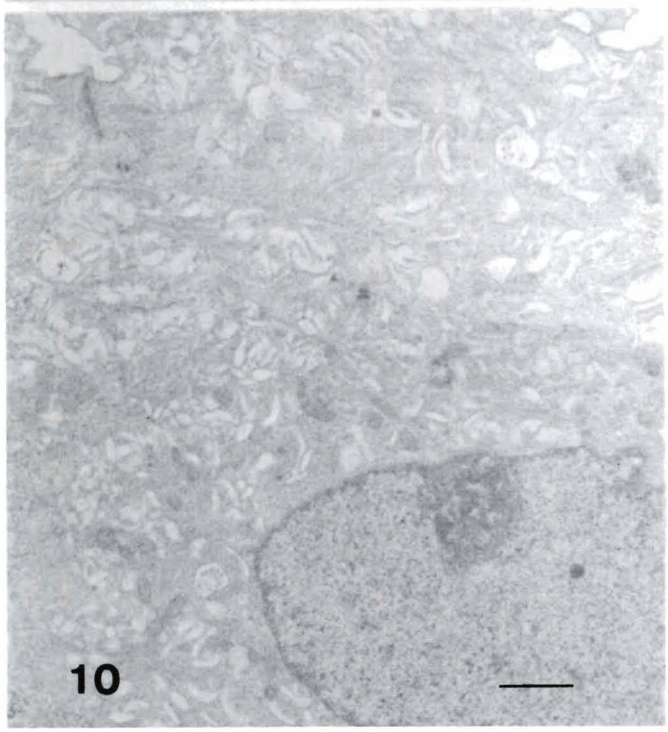
The cerium-based method was successfully employed for the detection of ALPase, 5'-Nase and Mg-ATPase in the transitional epithelium of rat urinary bladder. The present results were similar to those obtained in the rabbit (Seguchi et al., 1979). Using lead as capture agent for the detection of phosphatases, nonspecific deposits of lead phosphate are often encountered. However, with the present method, nonspecific reaction products were absent in the sections.

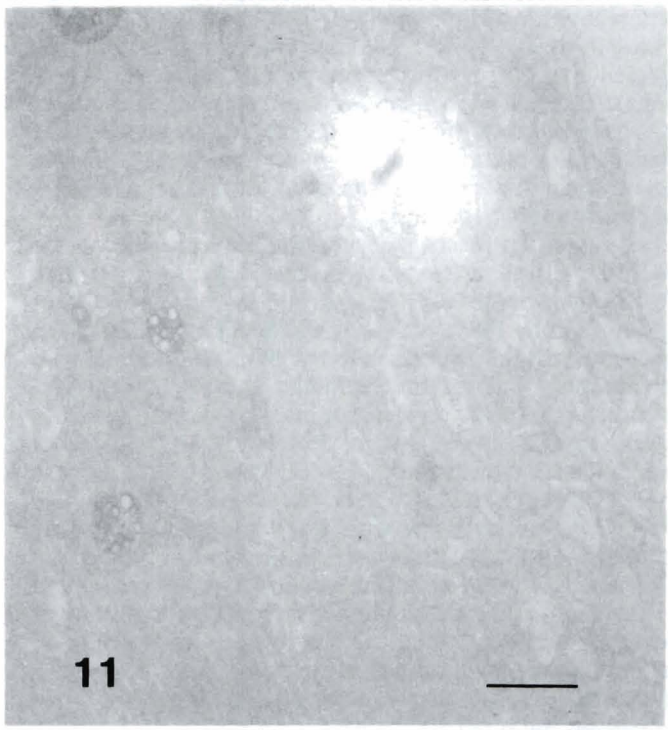
The ALPase activity in the mammalian urinary bladder epithelium has already been demonstrated by several authors (Mende and Chambers, 1957; Martin, 1958) at the light microscopical level. Using the Gomori type incubation medium (Gomori, 1950), ALPase activity was found in the peripheral part of the cytoplasm of lining epithelial cells, but it was thought to be absent from the superficial layer of the transitional epithelium (Martin, 1958). Later, it was shown that ALPase activity was associated with the plasma membrane of basal and intermediate cells of the bladder epithelium of the rat (Wilson and Hodges, 1979) and the rabbit (Seguchi et al., 1979), and its presence was also demonstrated in culture cell lines derived from human bladder carcinomas (Benham et al., 1977), using the lead-based method. In some cases, the ALPase reaction product was found in the endoplasmic reticulum and the mitochondria of transitional epithelial cells (Benham et al., 1977). In the present study, the reaction product was observed on the plasma membrane of basal, intermediate and superficial cells. However, the luminal surface of the superficial cells showed no reaction product. We consistently found a gradual decrease in the distribution of the reaction product from basal to superficial cells. No reaction product was seen in any of the cytoplasmic organelles. ALPase constitutes a heterogeneous group of enzymes which may occur as one of several isozymes in normal tissue (Boyer, 1963). High levels of ALPase activity have been reported in some culture cells derived from human bladder carcinoma (Benham et al., 1977) as well as a loss of the enzyme activity in the bladder epithelium of rats and mice treated with chemical carcinogens (Highman et al., 1975; Kunze et al., 1975). It seems that ALPase levels are related to location, tissue, pH, and to physiological conditions and stage of differentiation. According to its distribution in the transitional epithelium of rat urinary bladder, it is reasonable to think that the function of ALPase in this tissue might be associated with the division and differentiation of basal cells, and with the exchange of materials between basal cells and capillaries (Seguchi, 1985; Ishikawa and Seguchi, 1985), rather than with the reabsorption of water and creatinine from the urine by the superficial cells (Kerr et al., 1963).

5'-Nase has been regarded as a marker enzyme for plasma membrane in cell fractionation studies. Its reaction product has been observed ultracytochemically on the plasma membrane of lymphocytes (Uusitalo and Karnovsky, 1977), cartilage cells (Rodan et al., 1977), urinary bladder epithelium of rat (Wilson and Hodges, 1979) and rabbit (Seguchi et al., 1980a), liver cells (Berman et al., 1980), fibroblasts (Berman et al., 1980), ventricular choroid plexus epithelial cells (Masuzawa et al., 1982), and frog retina (Hussain and Baydoun, 1985).









Figs. 8 and 9. The reaction product for the Mg-ATPase activity is localized between basal and intermediate cells, and between adjacent superficial cells. L, lumen. × 25,000, (Fig. 8). × 24,000, (Fig. 9) bar = 1 μm.

Fig. 10. Control experiments without substrate reveal no enzyme activity. × 9,700, bar = 1 μm.

Fig. 11. Addition of EDTA to the incubation medium completely inhibits the enzyme activity. × 12,500, bar = 1 μm.

The activity of 5'-Nase has also been found in the lysosomes (Masuzawa et al., 1982; Wada et al., 1987) and Golgi complex (Little and Widnell, 1975; Brandan and Fleischer, 1982; Kaur et al., 1984). All these studies were done with the lead-based method. Blok et al. (1982), using cerium as the trapping agent, showed strong activity of the plasma membrane 5'-Nase in macrophages, with some diffusion of reaction product. Robinson and Karnovsky (1983a) indicated that the cerium-based method gives better results for ultrastructural localization of 5'-Nase than conventional lead-based methods. Wilson and Hodges (1979) reported positive 5'-Nase reaction on the plasma membrane of basal, intermediate and superficial cells, including the asymmetric membrane with the lead-based method. According to previous researchers, 5'-Nase might function in association with the production of adenosine, permeability of the plasma membrane and regulation of glycolysis (Masuzawa et al., 1982). Also, 5'-Nase may possibly accelerate the rate of actin polymerization. Membrane-bound 5'-Nase may act as an anchoring protein for intercellular actins, and modify their availability for cellular motile response (Rohr and Mannherz, 1979). There are many hexagonal mosaic protein structures implanted in the plasma membrane of the luminal surface, and a lot of actin in the cytoplasm has been observed attached to the membrane of such structures (Seguchi et al., 1978; Seguchi, 1989). The lumenal plasma membrane of superficial cells was internalized with 5'-Nase when the urinary bladder contracted or bacteria were phagocytized by the transitional epithelium (Edelson and Cohn, 1976; Fukushi et al., 1979). We, therefore, suggest that the presence of 5'-Nase in the luminal plasma membrane of superficial cells of the bladder might play a special role in the movement of the plasma membrane by accelerating the rate of actin polymerization under the plasma membrane. More direct evidence is, however, necessary to confirm this hypothesis.

It is well known that Mg-ATPase is a member of the ATPase group. It has been studied cytochemically and functionally in several organs and in animal embryos (Avner et al., 1983; Ishikawa and Seguchi, 1982, 1985). However, Mg-ATPase in the transitional epithelium of the urinary bladder has been the subject of very few ultracytochemical investigations. In our previous cytochemical study using the lead-based method, we have demonstrated Mg-ATPase activity in the transitional epithelium of the rabbit urinary bladder. The Mg-ATPase reaction product was present on the plasma membrane of basal cells only (Seguchi et al., 1979). In this study, cerium chloride was used as capture agent (Hardonk et al., 1985), and the reaction product for Mg-ATPase was found on the plasma membrane of intermediate cells adjacent to basal and superficial cells, and on the adjacent plasma membrane of two neighbouring superficial cells. In contrast to our previous results, the reaction product was hardly seen along the plasma membrane of basal cells facing the basement membrane. The reason for this difference, however, is still unknown. According to the cytochemical results obtained from this study, the physiological function of Mg-ATPase seems to be related to the activity of Na-K-ATPase (Coleman and Finean, 1968). But further investigations will be needed to explain in detail the role of this enzyme in the transitional epithelium of rat urinary bladder.

Acknowledgements. Part of this work was supported by a Grant-in-Aid for scientific research, No. 61480092, from the Ministry of Education Science and Culture, Japan.

References

Avner D.L., Larsen R. and Berenson M.M. (1983). Inhibition of liver surface membrane Na^+, K^+-adenosine triphosphatase, Mg^{+2}-adenosine triphosphatase and 5'-nucleotidase activities by protoporphyrin. Observations in vitro and in the perfused rat liver. Gastroenterology 85, 700-706.

Benham F., Cottell D.C., Franks L.M. and Wilson P.D. (1977). Alkaline phosphatase activity in human bladder tumor cell lines. J. Histochem. Cytochem. 25, 266-274.

Berman J.J., Tong C. and Williams G.M. (1980). 5'-Nucleotidase activities in cultured rat liver epithelial and fibroblast cells. J. Histochem. Cytochem. 28, 174-177.

Blok J., Onderwater J.J.M., de Water R. and Ginsel L.A. (1982). A cytochemical method for the demonstration of 5'-nucleotidase in mouse peritoneal macrophages, with cerium ions used as traping agent. Histochemistry 75, 437-443.

Boyer S.H. (1963). Human organ alkaline phosphatases: discrimination by several means including starch gel electrophoresis of antienzyme-supernatant fluids. Ann. the N. Y. Acad. Sci. 103, 938-951.

Brandan E. and Fleischer B. (1982). Orientation and role of nucleosidediphosphatase and 5'-nucleotidase in Golgi vesicles from rat liver. Biochemistry 21, 4640-4645.

Coleman R. and Finean J.B. (1968). Cell-surface membrane. In: Comprehensive biochemistry Vol. 23 Cytochemistry. Florkin M. Stotz E.H. (eds). Elsevier. Amsterdam. pp 99-126.

Eldeson P.J. and Cohn Z.A. (1976). 5'-nucleotidase activity of mouse peritoneal macrophages. II. Cellular distribution and effects of endocytosis. J. Exp. Med. 144, 1596-1608.

Fukushi Y., Orikasa S. and Kagayama M. (1979). An electron microscopic study of the interaction between vesical epithelium and *E. coli.* Invest. Urol. 17, 61-68.

Gomori G. (1950). An improved histochemical technic for acid phosphatase. Stain Technol. 52, 81-85.

Hardonk M.J., Kalicharan D. and Hulstaert C.E. (1985). Cytochemical demonstration of ATPase activity in the rat kidney basement membrane using the cerium-based method. Acta Histochem. Suppl. Band XXXI, S. 253-261.

Highman B., Frith C.H. and Littlefield N.A. (1975). Alkaline phosphatase activity in hyperplastic and neoplastic urinary bladder epithelium of mice fed 2-acetylaminofluorene. J. Natl. Cancer Inst. 54, 257-261.

Hussain S.T. and Baydoun E.A.H. (1985). Cytochemical localization of 5'-nucleotidase in the frog (*rana pipiens)* retina; A histochemical and cytochemical study. J. Histochem. Cytochem. 33, 1067-1072.

Ishikawa T. and Seguchi H. (1982). Localization of phosphatase activities on mouse teratocarcinoma embryoid bodies. Exp. Cell Res. 140, 427-429.

Ishikawa T. and Seguchi H. (1985). Localization of Mg^{++}-dependent adenosine triphosphatase and alkaline phosphatase activities in the postimplantation mouse embryos in day 5 and 6. Anat. Embryol. 173, 7-11.

Kaur C., Ling E.A. and Wong W.C. (1984). Cytochemical localisation of 5'-nucleotidase in amoeboid microglial cells in postnatal rats. J. Anat. 139, 1-7.

Kerr W.K., Barkin M., D'Aloisio J. and Menczyk Z. (1963). Observations on the movement of ions and water across the wall of the human bladder and ureter. J. Urol. 89, 812-819.

Kunze E., Schauer A. and Krüsmann G. (1975). Focal loss of alkaline phosphatase and increase of proliferation in preneoplastic areas of the rat urothelium after administration of N-butyl-N-(4-hydroxybutyl) nitrosamine and N-4-(5-nitro-2-furyl)-2-thiazolyl) formamide. Z. Krebsforoch. 84, 143-160.

Little J. and Widnell C.C. (1975). Evidence for the translocation of 5'-nucleotidase across hepatic membranes *in vivo*. Proc. Natl. Acad. Sci. USA. 72, 4013-4017.

Martin B.F. (1958). Histological and histochemical studies on the bladder and ureter, with particular reference to alkaline phosphatase and Golgi material. J. Anat. 92, 286-297.

Masuzawa T., Shimabukuro H. and Sato F. (1982). Enzyme histochemistry of ventricular choroid plexus (Part V): Localization of nucleosidophosphatase and 5'-nucleotidase activities. Neurol. Med. Chir. (Tokyo) 22, 875-878.

Mende T.J. and Chambers E.L. (1957). Distribution of mucopolysaccharide and alkaline phosphatase in transitional epithelia. J. Histochem. Cytochem. 5, 99-104.

Okada T.,Seguchi H. and Ogawa K. (1982). Acid phosphatase activities in the transitional epithelium of canine urinary bladder. Acta Histochem. Cytochem. 15, 639-647.

Robinson J.M. and Karnovsky M.J. (1983a). Ultrastructural localization of 5'-nucleotidase in guinea pig neutrophils based upon the use of cerium as capturing agent. J. Histochem. Cytochem. 31, 1190-1196.

Robinson J.M. and Karnovsky M.J. (1983b). Ultrastructural localization of several phosphatases with cerium. J. Histochem. Cytochem. 31, 1197-1208.

Rodan G.A., Bourret L.A. and Cutler L.S. (1977). Membrane changes during cartilage maturation. Increase in 5'-nucleotidase and decrease in adenosine inhibition of adenylate cyclase. J. Cell. Biol. 72, 493-501.

Rohr G. and Mannherz H.G. (1979). The activation of actin: DNase I complex with rat liver plasma membranes. The possible role of 5'-nucleotidase. FEBS. Lett. 99, 351-356.

Seguchi H., Okada T. and Ogawa K. (1978). Morphology and function of membrane subunits of the transitional epithelial cells. Research Reports for Membrane Subunits and Localization of Physiologically Active Substances. (in Japanese pp 1-3.

Seguchi H., Okada T., Fujimoto T., Mayahara H. and Ogawa K. (1979). Phosphatases in transitional epithelia of the rabbit urinary bladder. Acta Anat. Nippon. 54, 186-187.

Seguchi H., Okada T. and Ogawa K. (1980a). Localization of 5'-nucleotidase activity in the transitional epithelium. Research Reports for Membrane Subunits and Localization of Physiologically Active Substances. (in Japanese). ppl 3.

Seguchi H., Okada T. and Ogawa K. (1980b). Ultracytochemical demonstration of functional groups in the lumenal plasma membrane of rabbit transitional epithelium. Acta Histochem. Cytochem. 13, 660-678.

Seguchi H., Okada T. and Ogawa K. (1982). Localization of Ca^{++}-activated adenosine triphosphatase activities in the transitional epithelium of the rabbit urinary bladder. Acta. Histochem. Cytochem. 15, 76-89.

Seguchi H. (1985). Phosphatase I: Alkaline phosphatase and acid phosphatase. Histo- and Cytochemistry. pp. 7-21.

Seguchi H. (1989). Urinary bladder. In: Textbook of Human Histology, 5. Respiratory and Urinary System. Chap. 12 Asakura Shoten. (in Japanese). pp. 370-397.

Uusitalo R.J. and Karnovsky M.J. (1977). 5-nucleotidase in different populations of mouse lymphocytes. J. Histochem. Cytochem. 25, 97-103.

Spurr A.R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26, 31-43.

Wada I., Eto S., Himeno M. and Kato K. (1987). 5'-nucleotidase in rat liver lysosomes. J. Biochem. 101, 1077-1085.

Wilson P.D. and Hodges G.M. (1979). Focal distribution of surface marker enzymes after long-term culture of adult rat bladder epithelium and methylnitrosourea (MNU)-induced bladder tumors. J. Histochem. Cytochem. 27, 1236-1246.

Accepted December 20, 1990