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Immunocytochemical localization of γ-glutamyltransferase in rat kidney with protein A–horseradish peroxidase

(cytochemistry/plasma membrane/brush border/proximal convolution/glutathione metabolism)

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ABSTRACT **Immunocytochemistry with a specific antiserum and protein A–horseradish peroxidase permits visualization of the sites of γ-glutamyltransferase [(5-glutamyl)-peptide:amino-acid 5-glutamyltransferase, EC 2.3.2.2] at both the light- and the electron-microscope levels. As seen by light microscopy, the enzyme is localized only in the proximal convolutions of the renal tubules. Electron microscopy reveals dense deposits of 3,3′-diaminobenzidine reaction product embedded in the glycocalyx along the entire luminal surface of the microvilli and in the basolateral membranes.**

γ-Glutamyltransferase [γGluTase; (5-glutamyl)-peptide:amino-acid 5-glutamyltransferase, EC 2.3.2.2] is an enzyme of major importance in mammalian tissues (1). Its concentration is highest in kidney, where it is involved in glutathione metabolism. Therefore, the kidney is a favorable organ for histochemical and cytochemical studies of this enzyme. Previous studies have been restricted largely to the light-microscope level, and sites of enzyme activity were indicated by utilizing an azo dye procedure (2, 3). Because the reaction generated during such a procedure is soluble in the organic reagents required for electron microscopy, it is of little value for this technique.

The procedure used in the present study is based on immunocytochemistry rather than enzyme activity and utilizes 3,3′-diaminobenzidine (DAB) rather than a diazonium salt as the substrate. Sections of well-preserved tissue are exposed to antiserum specific for γGluTase and then to protein A–horseradish peroxidase (PrA–HRP). Subsequently, the sections are incubated for the DAB reaction generated by the bound HRP. The reaction product, oxidized DAB, is insoluble and produces a brown color at the light-microscope level. Its uniform electron opacity makes it eminently suitable for electron microscopy (4–6).

The results are of interest at different levels of inquiry. Light microscopy is sufficient for describing the topographical distribution of the enzyme—i.e., its presence in the proximal convolutions of the renal tubules. Because the diffusion of both the immunoreagents and the DAB reaction product are extremely limited, electron microscopy provides valid images of the intracellular sites of γGluTase. This report shows the enzyme to be present in the proximal convolution at the basolateral and the microvillar surfaces.

MATERIALS AND METHODS

Materials. PrA–HRP was obtained from E. Y. Laboratories, San Mateo, CA. Glutathione, γ-glutamyl-*p*-nitroanilide, and DAB were purchased from Sigma. Glutaraldehyde was obtained from Ladd Research Industries, Burlington, VT. Other reagents used were of analytical grade.

Enzyme and Antisera. γGluTase was purified from rat kidney by using the papain digestion method as described (7). γGluTase activity was measured by the method of Orlowski and Meister (7). After sodium dodecyl sulfate/polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue R-250, two polypeptide bands, which represent the two subunits of the enzyme, were observed without visible contaminant bands (8). Antiserum against purified γGluTase was produced in New Zealand White rabbits as described (9). A single precipitin line was observed upon double immunodiffusion of the antiserum with the Triton X-100-solubilized fraction of rat kidney homogenate. The antibody inhibited the transferase activity of the purified enzyme by 55%. Control serum was obtained from unimmunized rabbits.

Preparation of Tissue. Male Sprague–Dawley rats (Marland Farms, Hewitt, NJ) weighing 90–150 g were anesthetized with ether, and the kidneys were fixed by perfusion through the abdominal aorta. Perfusion was a two-step process: (*i*) 0.9% saline at 20°C for 2.5 min and (*ii*) 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4/5% sucrose, designated buffer PS, at 20°C for 15 min. Both solutions were applied at the rate of 7.5 ml/min. The kidneys were removed and transferred to buffer PS at 4°C and cut transversely into 2-mm slices. Selected areas from the midcortical region were excised, and sections (≈40–80 μm) from these areas were cut on an Oxford Vibratome (Ted Pella, Tustin, CA) into cold buffer PS. The sections were rinsed for 15 min twice in buffer PS and then stored in fresh buffer overnight at 4°C to remove residual glutaraldehyde.

Immunocytochemical Procedure. After the overnight rinse, all of the sections were immersed in buffer PS containing 10 mg of ovalbumin and 10 mg of L-lysine per ml and gently agitated every 10 min for 1.5 hr at 20°C; this was followed by two 10-min rinses in buffer PS at 20°C.

The sections were then divided into four groups and treated as follows. Group 1 was treated with the specific antiserum to γGluTase, diluted 1:100 in buffer PS. Group 3 was treated with unimmunized rabbit serum diluted 1:100 in buffer PS. Groups 2 and 4 were treated only with buffer PS without serum. The sections, as grouped, were kept at 4°C overnight. They were then washed twice in buffer PS at 20°C for 10 min each. All sections, except for those in group 4, were transferred to buffer PS containing PrA–HRP at 5 μg/ml at 20°C and gently agitated intermittently for 1 hr. The sections again were washed for 10 min twice in buffer PS and transferred to 0.1 M Tris·HCl, pH 7.6/5% sucrose, designated buffer TS, at 20°C for 10 min. Finally, all of the groups were incubated in the DAB medium.

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Abbreviations: DAB, 3,3′-diaminobenzidine; γGluTase, γ-glutamyltransferase; HRP, horseradish peroxidase; PrA, protein A.

Group 4 sections were not treated with PrA–HRP; instead, they remained in buffer PS only for 1 hr prior to incubation in the DAB medium.

DAB Incubation. The DAB medium contained 15 mg of DAB, 30 ml of buffer TS, and 0.3 ml of 1% hydrogen peroxide. Sections were incubated for 8 min at *ca.* 20°C. After incubation, sections were rinsed three times in cold 7.5% sucrose, treated with 1% OsO_4 in phosphate buffer for 1 hr, and processed further for electron microscopy, which included uranyl acetate *en bloc* treatment (10). A number of sections from each group were left untreated with uranyl acetate. Thin sections were not stained prior to examination in the electron microscope.

RESULTS

Light Microscopy. Fig. 1*a* shows the distribution of γGluTase as seen in sections treated with specific antiserum against γGluTase (group 1). Brown reaction product was seen in the brush borders of the proximal convolutions. No heterogeneity was apparent along the lengths of the tubules. When examined at higher magnification (oil immersion), the basolateral membranes showed reaction product (Fig. 1*b*).

LM Controls. The control sections of groups 3 (exposed to serum) and 4 (not exposed to serum or PrA–HRP) were totally negative. However, the control sections without exposure to serum (group 2) showed reaction product at the bases of the brush borders (Fig. 2*a*).

Electron Microscopy. Reaction product was embedded in the glycocalyx of the microvilli, endocytic vacuoles, and the basolateral membranes (Fig. 3). The tight junctions (arrowheads, Fig. 3 *a* and *b*) and all intracellular organelles were devoid of reaction product (Fig. 3 *a* and *b*).

There was no indication of diffusion of reaction product away from microvilli or basolateral membranes to adjacent sites as has been described in earlier studies (11). Indeed, there was no perceptible diffusion except at the bases of the cells near the basement membranes. In these regions (arrows), the reaction product (oxidized DAB) took the form of granules in the basement membranes and of smooth linear deposits on the surfaces of erythrocytes (Fig. 3*b*, arrow) and endothelial cells (EN, Fig. 3*a*).

EM Controls. Sections of groups 3 and 4 showed no reaction product anywhere except in erythrocytes with peroxidative activity due to hemoglobin, resulting in oxidized DAB (not illustrated).

Fig. 2*b* shows the distribution of reaction product in sections not treated with any serum before the PrA–HRP treatment. The reaction product was restricted to the interior of the endocytic vacuoles and to a variable degree at the proximal end of some microvilli. Such binding of reaction product may be due to PrA–HRP receptor interaction at the base of the microvilli; this interaction was completely blocked in the presence of serum. The basolateral membranes did not show any reaction product.

DISCUSSION

The most significant observation of this study is the presence of γGluTase in the basolateral membranes. This finding establishes γGluTase as the first brush border enzyme known to exist

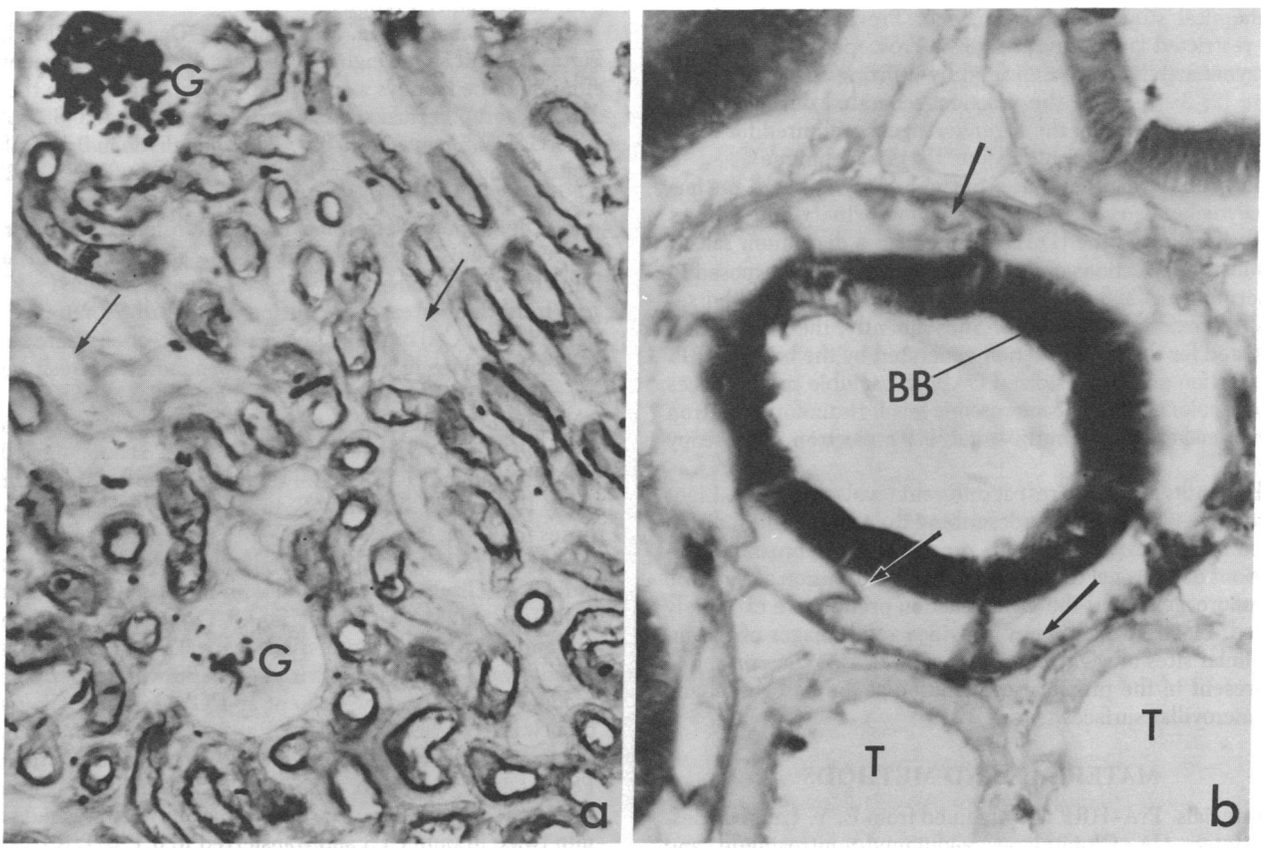

FIG. 1. (*a*) Section treated with PrA–HRP after incubation with antiserum specific for γGluTase. Reaction product is present in the brush borders of the proximal convolutions. Thick limbs of Henle and other portions of the kidney tubule are without reaction product; they are barely evident in the micrograph (arrows). The two glomeruli, labeled G, contain darkly stained erythrocytes. (×220.) (*b*) Higher magnification of a "thick" (3 μm) Epon section treated as in *a*. Reaction product is seen in the brush border (BB) and in the basolateral membrane (arrows). Two of the thick limbs of Henle are seen at T; they are devoid of reaction product. (×1700.)

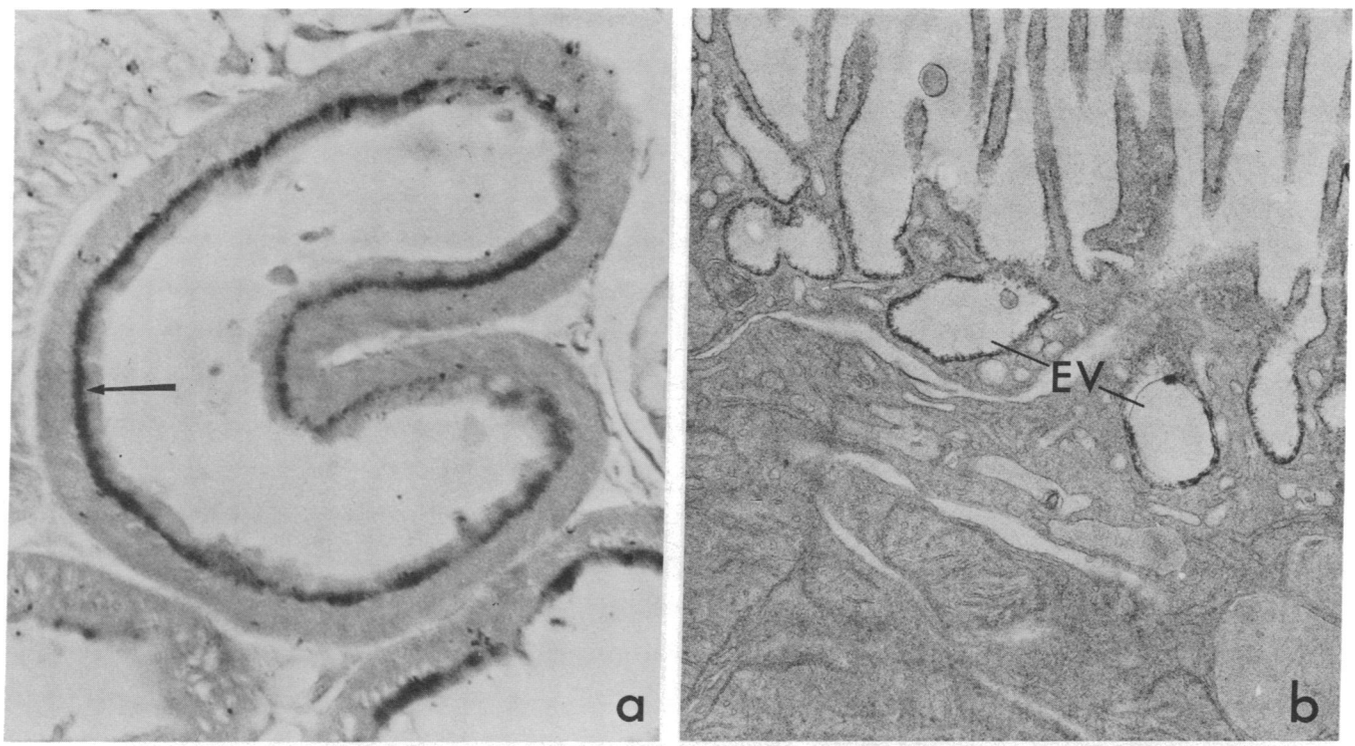

FIG. 2. (*a*) Control section, no serum treatment; PrA–HRP incubation. Reaction product is seen at the base of the brush border, (arrow). (×900.) (*b*) Portion of proximal convolution from a section treated as in *a*. A thin layer of reaction product is seen within the endocytic vacuoles and surfaces at the proximal ends of some of the microvilli (EV). The remainder of the cell, including the distal portions of the microvilli, is without reaction product. (×19,000.)

in that site in the proximal convolution and at the luminal surface.

Our observations also show the validity of using PrA–HRP after a specific antiserum to an antigen in an immunocytochemical procedure at both the light- and electron-microscope levels. In this study, glutaraldehyde fixation, adequate for good preservation of cell structure, did not destroy the enzyme's capacity for binding to specific antibody for γGluTase. Specific immunoreagent permits a specific localization not often possible with enzyme-generated reaction product in other cytochemical reaction methods.

Our findings are firm regarding the localization of γGluTase in the microvilli and basolateral membranes because there is only a minimal indication of artifact occurring only at the basement membrane (Fig. 3 *a* and *b*).

Azo dye histochemistry (2, 3) also has shown γGluTase to be localized in the proximal convolutions. Use of this azo dye method in our laboratory shows a much higher level of activity in the P_3 segment, or *pars recta*, than in the remainder of the proximal convolution. The significance of this segmental heterogeneity along the convolution is not known. The immunolocalization of the enzyme (Fig. 1) is in sharp contrast to the diffuse azo dye reaction and gives no sign of heterogeneity. The chief advantage of the immunocytochemical method is its validity at the electron-microscope level (Fig. 3 *a* and *b*).

Silbernagl *et al.* (12) have used ferritin conjugated to anti-γGluTase antibody, to localize the transferase in *isolated* proximal convolutions. The ferritin was observed at the tips and bases of the microvilli; however, none was present at the basolateral surfaces of the cells. To us it would seem likely that the IgG–ferritin conjugate failed to penetrate to the basolateral cell surfaces in the isolated tubules.

Enzyme in basolateral location may play an important role in the clearance of plasma glutathione, ≈50–60% of which occurs by nonglomerular filtration mechanisms (13, 14). Confirming data should be obtained by biochemical studies on isolated basolateral membranes.

Another observation of interest is the abrupt termination of the γGluTase localization at the endocytic vacuoles (Fig. 3) without accompanying evidence of breakdown of membrane (Fig. 3 *a* and *b*). Moreover, reaction product is not seen more deeply in the cell, in lysosomes, or other organelles.

One should be alerted to the false positive reaction in sections not treated with any serum prior to the PrA–HRP treatment and subsequent visualization (Fig. 2 *a* and *b*). It may have general significance in cytochemistry. Biochemical studies on isolated brush borders (unpublished observations) indicate that ovalbumin might be responsible for the spurious binding of PrA–HRP to the membrane surface of cells not exposed to serum prior to PrA–HRP treatment.

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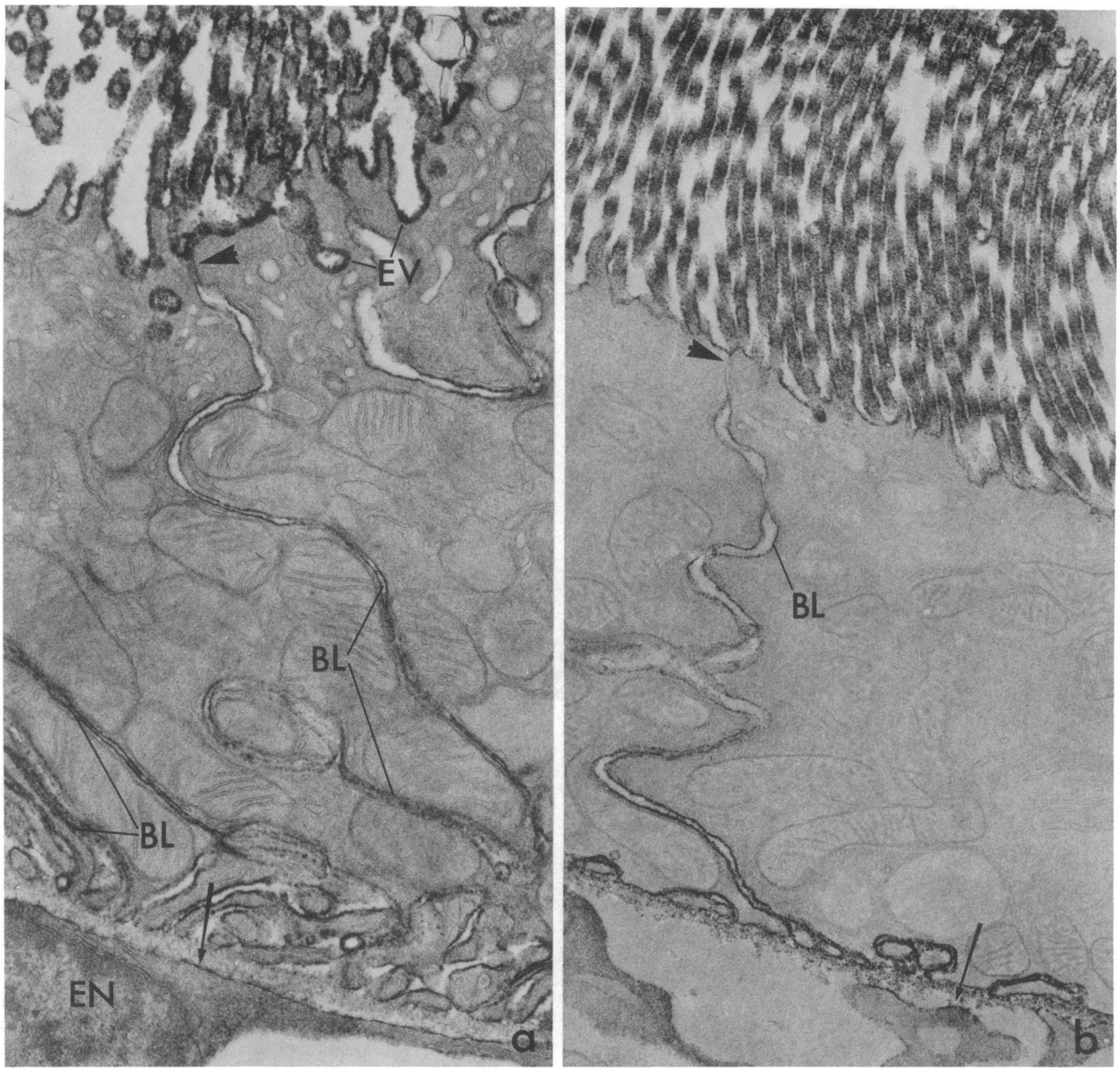

FIG. 3. (*a*) Portion of a cell from the proximal convolution of a section treated as in Fig. 1 *a* and *b*. Electron-opaque reaction product is embedded in the glycocalyx of the microvilli (top of micrograph), the endocytic vacuoles (EV), and the basolateral membranes (BL). No reaction product is present in the tight junction (arrowhead). At the base of the cell, reaction product takes the form of granules within the basement membrane (arrow) and an incomplete layer between the basement membrane and the endothelial cell (EN). (×16,000.) (*b*) Portion of a proximal convolution from a section treated as in Fig. 3*a* except that tissue was not treated with uranyl acetate *en bloc*. Tight junction (arrowhead) shows no reaction product. Reaction product is seen in the microvilli (at the top of the micrograph) and the basolateral membranes (BL). Reaction product also forms an incomplete layer on the surface of the erythrocytes (arrow) and in the basement membrane (granules). (×14,000.)

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