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# Isolation of Highly Enriched Preparations of Two Types of Mucosal Cells of the Turtles Urinary Bladder (40247)

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The urinary bladders of the toad *Bufo marinus,* and the turtle *Pseudemys scripta,* share many transport properties with the mammalian distal nephron. For this reason, the biochemical and physiologic properties of these tissues have been extensively studied. Although the mucosal epithelium of these tissues comprises a barrier only one cell layer thick, there are at least three clearly defined morphologic cell types (3–4). The contribution of each cell type to the transport activities of the tissues cannot be measured *in situ* and must be approached indirectly. Although coincident changes in transport and morphology in the toad bladder have been interpreted as evidence that the "mitochondria-rich" (MR) and "granular" mucosal cells are the primary loci of fluxes of sodium (5) and water (6), respectively, most studies ignore the possibility that the various transport functions might not be uniformly shared by all cell types.

In another approach to this problem, we have sought to separate the morphologically distinct cell types by density gradient centrifugation and to correlate the biochemical properties of these cells with the transport characteristics of the intact bladder (7). The turtle urinary bladder actively transports sodium (8) and chloride (9) and acidifies the urine (10, 11). Acidification and chloride transport are both inhibited by acetazolamide (12) and are presumably related to the modest amounts of carbonic anhydrase found in the mucosal epithelium (13). Because recent histochemical evidence suggests that this enzyme is found primarily in the MR cell (14), the cellular content of this enzyme was taken as one index of cellular transport activity as well as a biochemical marker for the MR cell.

*Materials and methods. Preparation of epithelial cells. Pseudemys scripta,* maintained in tanks of tap water at ambient temperature, were exsanguinated and their urinary bladders removed. The bladders, in the form of closed sacs, were each tied to the outlet of a Luer-lock syringe and filled with and immersed in a Ringers' solution composed of (in m*M*/liter); NaCl, 83.5; $NaHCO_3$, 17.7; KCl, 4.0; $KH_2PO_4$, 0.8; NaEDTA, 1.5; $MgSO_4$, 0.8; glucose, 11; and equilibrated with 99% $O_2$/1% $CO_2$ (pH 7.25–7.3). The bladders were incubated for 45 min at room temperature and the disaggregated mucosal cells recovered from the intraluminal solution by centrifugation (3500*g* for 10 min).

*Density gradient centrifugation of mucosal cells.* The disaggregated mucosal cells were suspended in a small volume (2–3 ml) of EDTA-Ringers and layered over a discontinuous density gradient of Ficoll (Pharmacia Co., Piscataway, NJ) in EDTA-Ringers. The gradient was composed of four solutions having densities of 1.017, 1.035, 1.070, and 1.105 g/cm$^2$ as determined in an Abbe' refractometer. The mucosal cell preparation was centrifuged at 27,000 rpm for 45 min in a Beckman Model L5-50 preparative ultracentrifuge (SW-27 rotor). Four distinct bands of material were visible. Each band of material was carefully recovered with a pipette, suspended in 40 ml EDTA-Ringers, and collected by centrifugation at 22,000*g* for 20 min.

*Preparation of tissues for electron microscopy.* The banded material collected from the density gradient was fixed in 3% glutaraldehyde buffered with 0.1 *M* cacodylic acid (pH 7.3). The cells were rinsed, suspended in a 2% aqueous osmium tetroxide solution, dehydrated, and embedded in Epon 812 (15). Sectioned material was stained with uranyl acetate and lead citrate and examined in a Philips 300 electron microscope. For study of the intact bladder, tissue was cut into cubes of approximately 2–3 mm and treated similarly.

*Morphology of separated mucosal cells.* To quantitate the relative distribution of mito-



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chondria-rich and granular cells in Bands 2 and 3, mucosal cells from two representative experiments were rated for their content of mitochondria and granules. In a single-blind study, 210 cells from Band 2 and 234 cells from Band 3 were each rated 0–3+ for the relative number of granules and of mitochondria identified in the sectioned cells. Each cell appearing in the randomly selected fields was included in the study *regardless* of the plane of section through the cell.

*Measurement of cytochrome oxidase.* The cells separated by density gradient centrifugation were washed, suspended in 1 ml distilled $H_2O$ and sonicated for two 15-sec intervals. Cytochrome oxidase in the sonicate was determined in a Gilford model 220 spectrophotometer by the method of Smith, using a value of $2.10 \times 10^4\ M^{-1}$ for the $\epsilon_{550}$ (reduced-oxidized) (16).

*Assays of carbonic anhydrase activity.* Suspensions of cells were immersed in an ice bath, sonicated with a Branson sonifier for two 15-sec intervals and centrifuged at 48,000*g* for 20 min. Carbonic anhydrase activity was measured in the supernatant fraction by the method of Maren, Ash and Bailey (17).

*Results. Electron micrographs of intact turtle bladder.* Micrographs of the intact turtle bladder mucosa show a heterogeneous cell population in which the MR and granular cells are the most prominent of the three morphologically distinct cell types (Fig. 1).

The predominant cell type (the granular cell) is characterized by a large number of electron-dense granules which are often found in close proximity to the plasma membrane. This cell *also* contains a large number of mitochondria. The next most abundant cell, the MR cell, is characterized by having a flask-shaped contour, many mitochondria, and a paucity of granules. The MR cells have a relatively small luminal surface covered with microvilli. The cytoplasm of the MR cell appears more electron-dense than that of the granular cell. A third type of cell, the "basal" cell, fewer in number, has a large nuclear/cytoplasmic ratio, a dense cytoplasm, and does not penetrate to the luminal surface of the bladder.

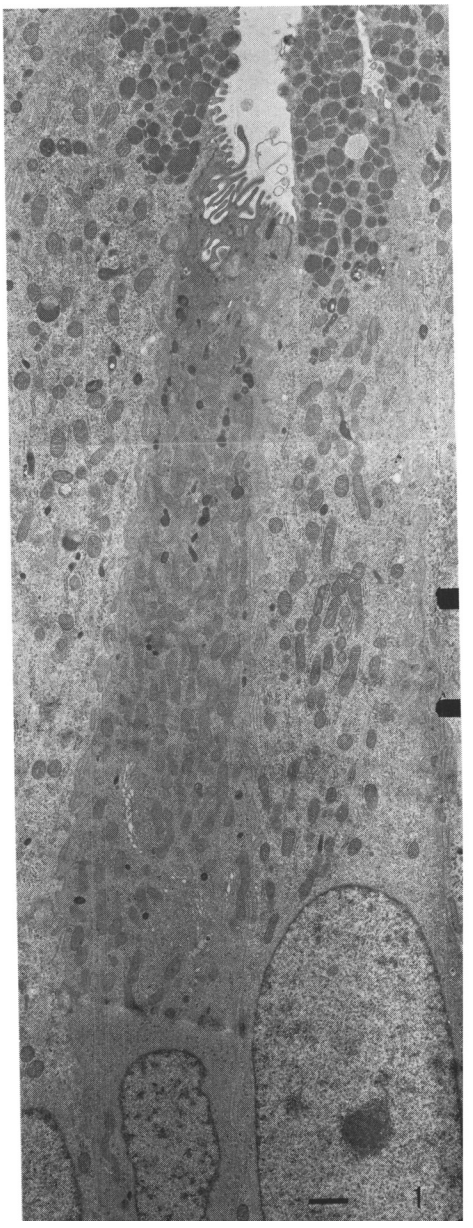
*Isolation of mucosal cells on density gradients of Ficoll.* Electron micrographs of the material in the four bands of material obtained by density gradient centrifugation (Fig. 2) showed that the most buoyant layer (Band 1) was composed almost exclusively of heterogeneous acellular material. This material, which is PAS-positive, may represent the coating of mucus normally found on the luminal surface of the intact bladder.

Band 2 was composed of intact, rounded epithelial cells (Fig. 3), which contained large numbers of mitochondria and few electron-dense granules. The cortex in the region of the mitochondria has prominent microvilli, while the remainder of the cortex is devoid of microvilli and has instead a ruffled appearance. These cells apparently represent the MR cells seen in micrographs of the intact bladder.

Band 3 contained intact epithelial cells characterized by a large number of electron-dense granules (Fig. 4). The mitochondria, though present in significant numbers, had a more compact structure than those in the cells of Band 2. These cells are assumed to represent the granular cells of the intact epithelium. Other cells occasionally seen in Band 3 contained few granules, a considerable number of mitochondria, a dense cytoplasm, and a high nucleus/cytoplasm ratio. These cells may be the basal cells identified in the intact tissue.

Band 4, containing a smaller amount of material, was composed primarily of cellular debris. This material presumably represents the damaged cells and cell fragments occasionally seen in the transmission micrographs of the uncentrifuged material.

*Organelle content of separated cells.* Our efforts to quantitate morphologically the relative enrichment of the two preparations of mucosal cells included a study of the relative incidence with which mitochondria and granules each appeared in sectioned cells from Bands 2 and 3. Our results, illustrated in Fig. 5, showed that granules were identified in only 15% of the mucosal cells in the Band 2 preparation, confirming our impression that Band 2 was a highly enriched preparation of MR cells. Although many more of the cells in Band 3 were found to contain granules, the plane of section in a significant number of cells (32%) did not include granules. This result presumably reflects both the assym-



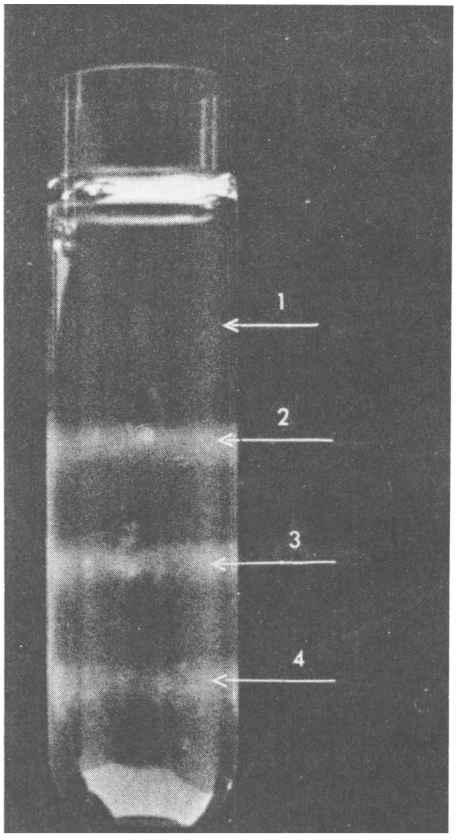



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FIG. 1. The turtle urinary bladder mucosal epitheium consists primarily of "granular" and "mitochondria-rich" cells. In the center of this section, a typical slender mitochondria-rich cell is covered on its luminal surface with microvilli. There are a small number of dark granules scattered through the cytoplasm. The granular cells adjoining on either side contain large numbers of electron-dense granules, located primarily near the luminal membrane, as well as significant numbers of mitochondria. ×5900.

FIG. 2. Separation of disaggregated mucosal cells on density gradients of Ficoll. Bands 2 and 3 contained intact mucosal cells, as illustrated in Figs. 3 and 4. Band 1 was composed of amorphous material and Band 4 of cellular debris. Erythrocytes sedimented to the bottom of the tube.

metrical distribution of granules within the granular cells and possibly the presence in the Band 3 material of some contamination by MR cells.

*Cytochrome oxidase of banded material.* Cytochrome oxidase, found only in the mitochondrial membrane (18), has been used to assay the relative abundance of mitochondria

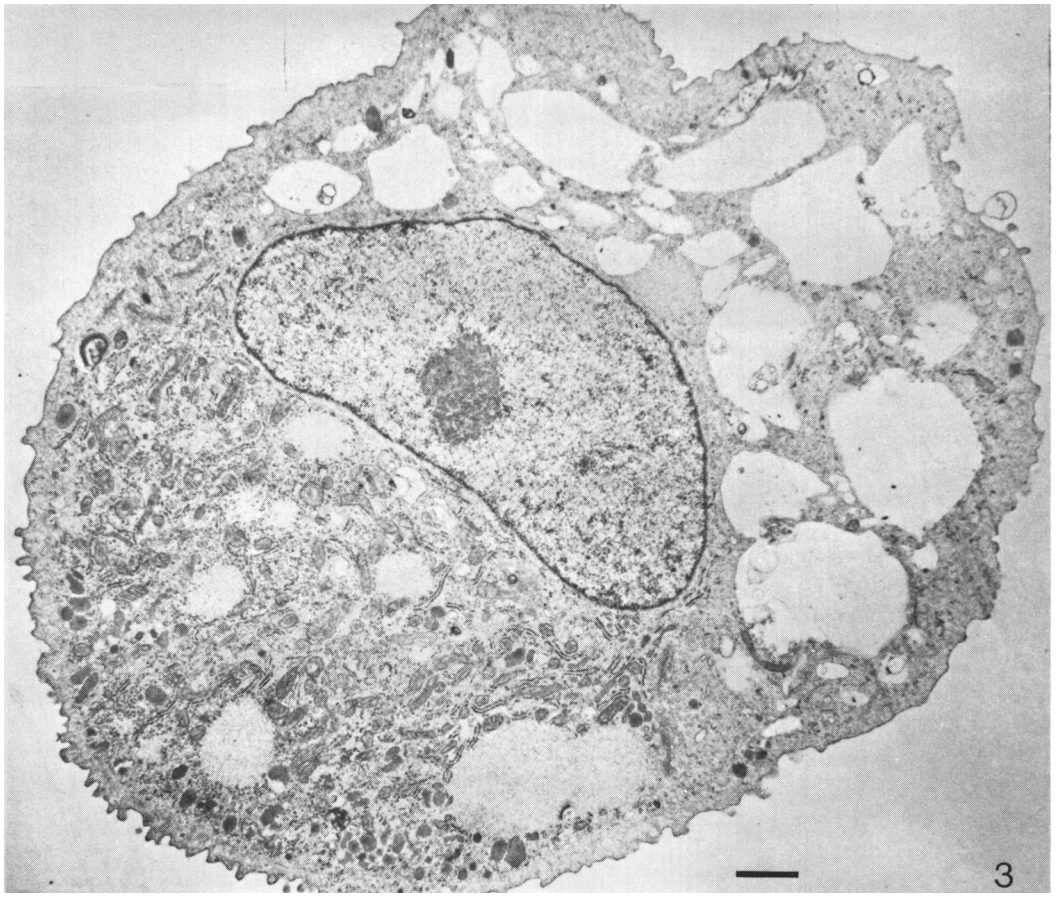


FIG. 3. A typical mucosal cell recovered in Band 2. The cell is characterized by large numbers of mitochondria and only an occasional small electron-dense granule. ×8450.

in vertebrate tissues. As shown in Table I, the cytochrome oxidase activity of the sonicated material of Band 2 amounted to 152.9 $\mu M$/min/mgm protein activity while that of Band 3 contained only 87.7 $\mu$M/min/mgm protein.

*Carbonic anhydrase activity in banded material.* The carbonic anhydrase activity in the supernatant fraction of the sonicated cells recovered from Bands 2 and 3 was measured by the $CO_2$-hydration method of Maren *et al.* (17). As shown in Table II, carbonic anhydrase activity in the supernatant fraction of cells from Band 2 was 0.79 EU/mg protein, significantly higher than the enzyme activity in the supernatant fraction of the cells of Band 3, 0.32 EU/mg protein.

*Discussion.* Although the urinary bladders of the toad and the turtle have been extraordinarily useful in studying active transport processes, few attempts have been made to assign the transport of each of the various substrates to one or another cell type. Saladino, Bentley and Trump (5) speculated that the marked changes in active sodium transport caused by the polyene antibiotic amphotericin B were related to morphologic alterations in the MR cells of the toad bladder; DiBona, Civan and Leaf (6) observed that only the granular cell population exhibited significant morphologic changes, i.e., an increase in profile and a fall in apparent cytoplasmic density, under conditions of vasopressin-induced water flux; histochemical localization of carbonic anhydrase in the MR cell of the turtle bladder was interpreted as evidence that acidification of the urine was accomplished by this cell type (19, 20); and

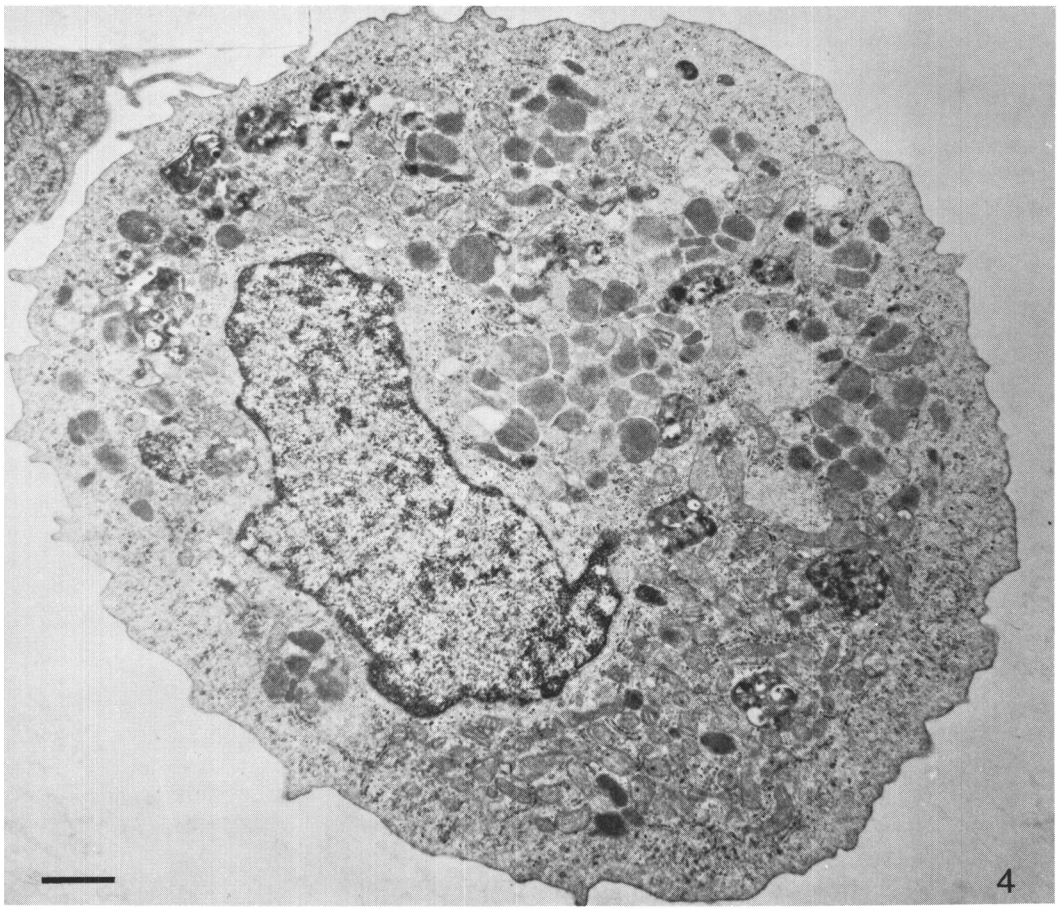


FIG. 4. A representative mucosal cell recovered from Band 3. The cytoplasm contains many large granules in addition to a significant number of mitochondria. ×9700.

evidence for the localization in the toad bladder MR cell of receptors for vasopressin (7) and for aldosterone (21) has been obtained in this laboratory.

It has been shown that intact, respiring mucosal cells can be removed from the bladder epithelium by incubating the tissue in EDTA-Ringers solution (22). This offered the opportunity to physically separate the constituent cell types. Using density gradient centrifugation, a technique often applied to the separation of different cell types from heterogeneous mixtures (23), we sought more direct evidence of differences in those biochemical properties of the MR and granular cells that could be correlated with the transport properties of the intact urinary bladder of the turtle. Turtle bladder mucosal cells prepared by this technique yield two bands of intact cells on density gradient centrifugation. Our conclusion that the two sets of cells consist of highly enriched fractions of the two major morphologic cell types is supported by the granule content of the separated cells, by the distribution of cytochrome oxidase activity between the cell preparations and by the predominance of carbonic anhydrase in the mucosal cells of Band 2. The latter observation, that the activity of carbonic anhydrase is approximately 2 ½-fold greater in the preparation of mucosal cells enriched in MR cells, is consistent with histochemical evidence that the enzyme is found in much higher concentration in this cell in the intact tissue (20).

Acetazolamide-inhibited ion transport, which has been reported in a number of epithelial tissues (9, 12, 24), is presumably related to the activity of carbonic anhydrase.

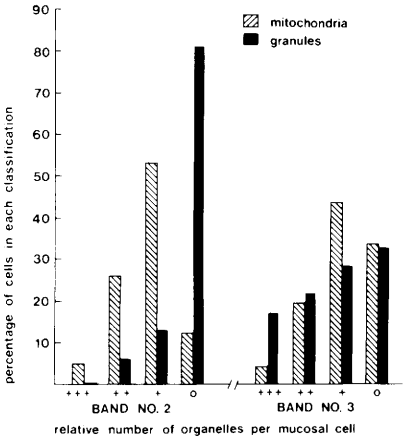


FIG. 5. The relative numbers of mitochondria and of granules were each evaluated in 210 cells from Band 2 and 234 cells from Band 3. The ratings of each organelle in the two sets of mucosal cells are given in the abscissa and the percentage of cells occurring in each rating is given on the ordinate.

TABLE I. CYTOCHROME OXIDASE ACTIVITY IN CELLS FROM BANDS 2 AND 3.[a]

| Cytochrome Oxidase Activity (μmoles/mgm/protein/min) | | |
|---|---|---|
| Band 2 | Band 3 | Ratio of Bands 2/3 |
| 107 | 85.6 | 1.25 |
| 162.6 | 103.1 | 1.58 |
| 180 | 78.6 | 2.29 |
| 185 | 103 | 1.80 |
| 130 | 68 | 1.91 |
| 152.9 ± 33.5 | 87.7 ± 15.4 | |

[a] Cells from bladders of 8–12 turtles were prepared on Ficoll gradients. Cells from Bands 2 and 3 were sonicated and aliquots assayed in triplicate by the method of Smith (16). Cytochrome *c* was reduced with hydrogen gas (using a palladium catalyst) immediately before use. The oxidation of cytochrome *c* was recorded at 550 nm in a Gilford Model 220 spectrophotometer at 25°.

The distribution of the enzyme between the MR and the granular cells in this tissue suggests that the biochemical processes related to chloride transport and to urinary acidification (either $H^+$ secretion or bicarbonate absorption) are predominantly localized in the MR cell, as suggested by Schwartz *et al.* (20). However, it is not possible to dismiss the significance of the enzyme assayed in the granular cells because this cell type accounts

TABLE II. CARBONIC ANHYDRASE ACTIVITY IN BANDS 2 AND 3.[a]

| Carbonic anhydrase activity (Enzyme units/mg protein) | | |
|---|---|---|
| Band 2 | Band 3 | Ratio of Bands 2/3 |
| 0.61 | 0.34 | 1.79 |
| 0.86 | 0.53 | 1.62 |
| 0.84 | 0.14 | 6.0 |
| 0.72 | 0.31 | 2.32 |
| 0.68 | 0.32 | 2.13 |
| 1.03 | 0.25 | 4.12 |
| 0.79 ± 0.15 | 0.32 ± 0.13 | |

[a] Mucosal cells from 8 to 12 bladders were separated on Ficoll gradients and the banded material was collected, washed, and sonicated. The sonicate was centrifuged at 48,000*g* (20 min) and the carbonic anhydrase activity determined by the method of Maren *et al.* (17). Enzyme units (EU) are calculated by: EU = $(Time_{uncatalyzed} - Time_{catalyzed})/Time_{catalyzed}$, when Time is the interval for the pH to change from approximately 8.0 to 6.0. Results are given as EU/mg protein.

for such a large proportion of the epithelial mass. One must also consider the possibility that the carbonic anhydrase localized in each cell type consists of a different isozyme having distinct physiologic, histochemical, and pharmacologic properties (24). Further application of this cell separation technique may provide answers to these unresolved questions regarding the precise mechanisms of transport of ions by heterogeneous epithelial tissues.

*Summary.* Enriched preparations of the two major cell types of the mucosal epithelium of the turtle's urinary bladder can be obtained by density gradient centrifugation. Carbonic anhydrase activity is much greater in the mitochondria-rich than in the granular cell, suggesting the former cell type is the site of acetazolamide-sensitive transport. This technique should be useful in further defining the biochemical and transport properties of epithelial tissues composed of heterogeneous mucosal cells.

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