

## Morphologic identification of microperfused-fixed single nephrons during renal micropuncture

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Sometimes in micropuncture experiments, simultaneous evaluation of function and structure is required in single nephrons under identical conditions. Hence, there is a need for a simple method for fixing *in vivo* and then identifying the nephron under study. We describe a very simple method which fulfills such a need, thus allowing the examination of the cellular structure with light and electron microscopy at the end of a micropuncture experiment.

**Methods.** Nonfasted Wistar rats, weighing 200 to 300 g, were anesthetized with sodium pentobarbital (Nembutal, 60 mg/kg of body wt) intraperitoneally and prepared for micropuncture as previously described [1]. The animals were given a sustaining infusion of saline solution at 0.02 ml/min and physiological measurements were completed in another program of micropuncture studies. The latter involved puncture of superficial loops of proximal tubules, measurement of free-flow intratubular pressure and/or timed collection of tubular fluid samples for ultramicroanalysis. After each collection for functional measurements, the individual tubules were reentered with a micropipet filled with 2% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH, 7.3) plus Alcian blue at a final concentration of 1% (osmolality, 520 mOsm/kg of H<sub>2</sub>O).<sup>1</sup>

The tubules were perfused for five to ten minutes at a pressure slightly exceeding the average free-flow intratubular pressure previously measured in the same animal. At the end of the experiment, the kidney was

<sup>1</sup> When preparing this solution special care should be taken to prevent impurities. Thus, the fixative should be filtered through a Millipore filter (0.25  $\mu$ ) immediately before use. Then the Alcian blue is added and the solution is spun down (do not filter) and the supernatant utilized for the microperfusion.

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removed and several fragments of tissue were cut with a razor blade and removed, each of them containing a perfusion-fixed tubule; the latter were recognized easily because of the blue color due to the Alcian blue added to the fixative.

Each fragment of renal tissue containing a stained tubule was fixed for an additional three hours in fresh fixative at room temperature without Alcian blue; the tissue was then rinsed several times in 0.1 M sodium cacodylate (pH, 7.3) and stored overnight at 4°C. The small blocks of tissue were postfixed in 1% osmium tetroxide buffered with sodium cacodylate (pH, 7.3) [3], dehydrated in acetone and embedded in Araldite (ACM Fluka).

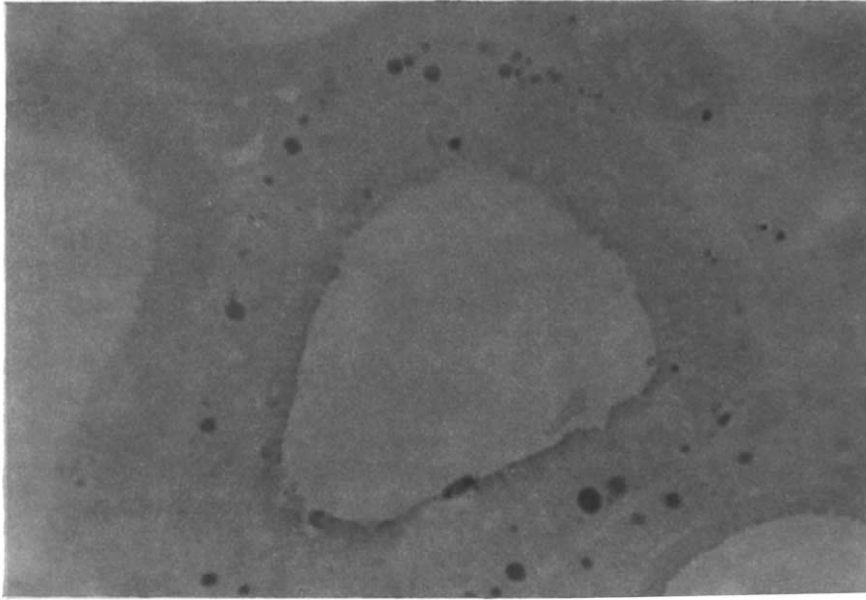
Sections of the plastic-embedded material were examined unstained by light microscopy for the purpose of identifying the stained tubules. Thin sections of the selected areas were then cut with a glass knife, stained with uranyl acetate and lead citrate and examined with an electron microscope (Siemens Elmiskop I). Unstained sections were also examined.

**Results.** The proximal tubules microperfusion-fixed *in vivo* were easily identified by light microscopy by the blue color of the brush border on 1  $\mu$ -thick sections (Fig. 1).

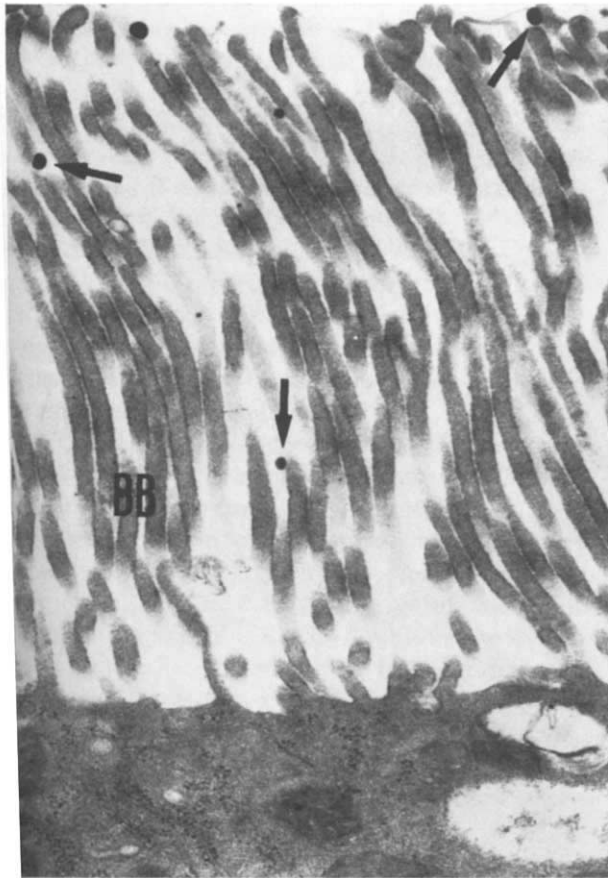
By electron microscopy electron-opaque granules were clearly seen between the microvilli of the brush border (Fig. 2).

A linear staining of the cell surface made the identification of distal tubules possible in the thick sections. A few granules on the plasma membrane were also visible by electron microscopy (Fig. 3).

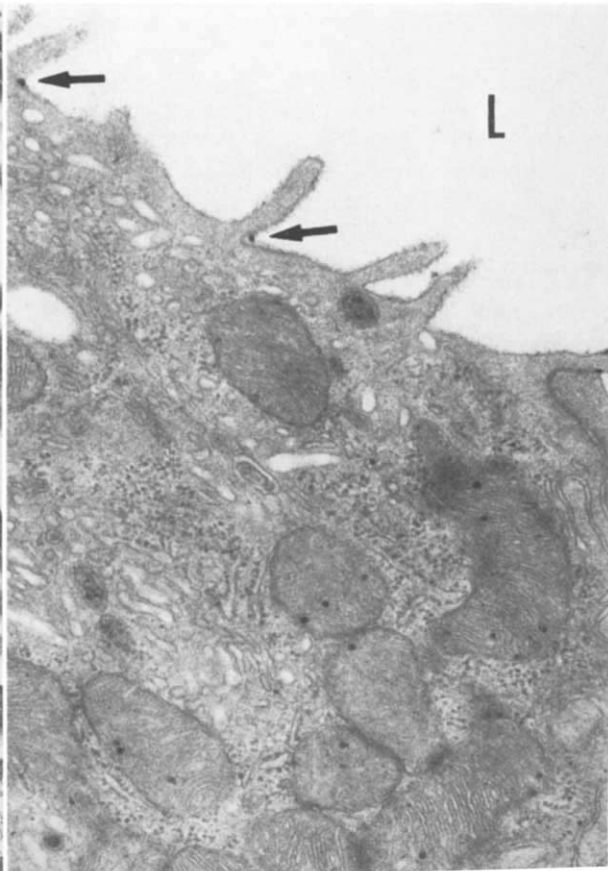
**Discussion.** Tisher and Clapp [2] have recently described a method for fixing *in vivo* and then identifying a given tubule. The method involves perfusion of the tubule with a fixative stained with lissamine green followed by intraluminal microinjection of latex. This



**Fig. 1.** Photomicrograph of a proximal tubule microperfusion-fixed in vivo with glutaraldehyde-Alcian blue. The blue color of the brush border allows identification of the tubule (unstained 1  $\mu$  thick section,  $\times 1220$ ).



**Fig. 2.** Electron micrograph of the brush border (BB) of a proximal tubule microperfusion-fixed in vivo. Electron opaque granules of Alcian blue mucosubstance complex (arrows) are clearly seen between the microvilli (uranyl acetate and lead citrate,  $\times 60,000$ ).



**Fig. 3.** Electron micrograph of a distal tubule microperfusion-fixed in vivo. A few electron-opaque granules of Alcian blue mucosubstance complex (arrows) are visible on the cell surface. L, lumen (uranyl acetate and lead citrate,  $\times 42,000$ ).

implies that a double puncture is necessary. Since their technique was designed for the evaluation of tubular structure after functional measurements had been completed, its use requires at least three micropunctures of the same tubular loop: for *a*) functional measurement, *b*) fixation and *c*) intraluminal injection of latex.

We describe a simple method for fixing and simultaneously staining the tubule during a single micropuncture. By perfusing the tubule with 2% glutaraldehyde buffered with 0.1 M sodium cacodylate plus Alcian blue, the tubule is fixed and stained at the same time.

Therefore, two main advantages may be ascribed to our technique, as compared to that of Tisher and Clapp: *a*) it requires one less puncture and *b*) it allows easy identification of the micropunctured tubule while minimizing the risk of damage to the cell surface and the microvilli of the brush border (which might be compressed by latex injection).

Furthermore, since only a few granules are scattered for identification purposes in the electron micrographs, the latter will not be disturbed by foreign material within the tubular lumen, as with latex.

Alcian blue is a dye used in histochemistry for staining acid mucopolysaccharides at a pH of approximately 1. At a neutral pH, Alcian blue does not allow histochemical reaction; but with glutaraldehyde it renders mucosubstances insoluble [3]. Thus, an Alcian blue mucosubstance complex is formed which is osmiophilic and electron dense, therefore easily distinguishable on the surface of the cells of the per-

fused tubules. The double staining with uranyl acetate, and lead citrate makes this complex more electron dense.

Several factors may account for the scattered distribution of these electron dense granules rather than a continuous layer on the cell surface, e.g., low concentration of Alcian blue in the perfusing solution, high pH, and diffusion of dye into the buffer while rinsing with sodium cacodylate. For identification of the tubules, however, these scattered granules are sufficient.

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The content of this note was presented as a demonstration at the Tenth Congress of the European Dialysis and Transplant Association in Vienna; it has appeared in abstract form in the *Proceedings of the EDTA*.

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#### References

1. ANDREUCCI VE, HERRERA-ACOSTA J, RECTOR FC JR, SELDIN DW: Measurement of single-nephron glomerular filtration rate by micropuncture: Analysis of error. *Am J Physiol* 221: 1551-1559, 1971
2. TISHER CC, CLAPP JR: Intraluminal latex injection: An aid to the histological identification of renal tubules. *Kidney Int* 2:54-56, 1972
3. BEHNKE O, ZELANDER T: Preservation of intercellular substances by the cationic dye Alcian blue in preparative procedure for electron microscopy. *J Ultrastruct Res* 31:424-438, 1970