Separation of Renal Medullary Cells: Isolation of Cells from the Thick Ascending Limb of Henle's Loop

JILL EVELOFF, W. HAASE, and ROLF KINNE

Max-Planck-Institut für Biophysik, 6000 Frankfurt am Main 71, Germany. Dr. Eveloff's and Dr. Kinne's present address is the Department of Physiology, Albert Einstein College of Medicine, Bronx, New York 10461.

ABSTRACT A homogeneous population of single cells from the thick ascending limb of Henle's loop (TALH) has been isolated from the rabbit kidney medulla. A total medullary cell suspension was prepared by a series of collagenase, hyaluronidase, and trypsin digestions and separated on a Ficoll gradient (2.6–30.7% wt/wt). Morphologically, the cells isolated from the TALH were homogeneous and showed polarity within their plasma membrane structure, with a few blunt microvilli on their apical surface and deep infoldings of the basal-lateral membrane. Biochemically, the TALH cells were highly enriched in calcitonin-sensitive adenylate cyclase and Na, K-ATPase. Alkaline phosphatase and arginine vasopressin-sensitive adenylate cyclase, highly concentrated in proximal tubule and collecting duct, were present only in low concentrations in the TALH cells. Additionally, furosemide, a diuretic inhibiting sodium chloride transport in the TALH in vivo, inhibited oxygen consumption of the TALH cells in a dose-dependent manner. The TALH cells were viable, as judged by morphological appearance, trypan blue exclusion, the response of oxygen consumption to 2,4-dinitrophenol, succinate and ouabain, and the cellular Na, K and ATP levels.

The kidney is a heterogeneous organ and each nephron is composed of a multitude of cell types having widely differing structures and functions. To understand the function of each cell type at the cellular and subcellular level, the isolation and separation of homogeneous cell populations in preparative amounts are necessary. This has been achieved to date only in the cortex of the kidney (9, 15, 16, 20). Using free-flow electrophoresis and density gradient centrifugation, it has been possible to isolate proximal tubule cells, distal tubule cells, and renin-rich cells. However, the preparation of homogeneous cell populations from the medulla has, heretofore, not been described.

The medulla contains at least six to seven cell types: cells of the pars recta of the proximal tubule, cells of the thin descending limb of Henle's loop, cells of the thick ascending limb of Henle's loop (TALH), cells of the collecting ducts as well as the interstitial cells, and cells of the blood vessels. Out of the medullary nephron segments, the TALH represents an especially interesting and important tubular segment for the analysis of the molecular mechanisms of chloride transport and the mode of action of the diuretics. The TALH functions integrally in the operation of the countercurrent mechanism for urinary concentration and dilution, which is effected by the transport of sodium chloride out of the tubular lumen in the absence of water movement. Chloride is transported actively in the TALH and its transport is inhibited by many diuretics, such as furosemide (4, 6, 10, 21).

This paper describes the attempt to isolate a homogeneous population of cells derived from the medullary TALH of the rabbit kidney. As judged by morphological, biochemical, and functional criteria, a population of viable cells was obtained which contained primarily cells from the TALH.

MATERIALS AND METHODS

Isolation of TALH Cells

Rabbits were killed by a blow to the head, and the kidneys were rapidly removed and placed in ice-cold Joklik's buffer, pH 7.4, a commercial isotonic saline medium enriched with the essential amino acids and substrates (Grand Island Biological Co. [GIBCO]-Biocult, Karlsruhe, Germany) which was used

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unmodified except for the addition of 10% fetal calf serum. The kidneys were cooled for 20 min and then perfused through the renal artery with 50 ml of ice-cold Joklik's containing 0.2% (wt/vol) collagenase-0.25% hyaluronidase. The medulla was excised and cut into small, uniform pieces (2–3 mm diameter) with scissors.

The isolation of the TALH cells is represented schematically in Fig. 1. The medullary fragments were first digested into tubules by a method similar to that described by Burg and Orloff (5) by incubating the pieces with gentle shaking in 40 ml of 0.2% collagenase–0.25% hyaluronidase Joklik's buffer for 1 h at 37°C, gassed with 95% O_2-5% CO_2. Periodically during the 1-h digestion, the fragments were sucked 10 times through a wide-mouth syringe to mechanically disrupt the sticky clumps which formed. The collagenase-hyaluronidase digestions produced tubules from 90 to 100% of the tissue. The tubules were sedimented by centrifugation at 50 *g* for 3 min and washed once with Joklik's to remove the collagenase-hyaluronidase. Cells were prepared from the tubules in a method similar to that described by Kreisberg et al. (15, 16). The tubules were stirred with 50 ml of 0.25% trypsin in Joklik's buffer for 20 min at 22°C, gassed with 95% O_2-5% CO_2. The tubules were sedimented at 750 *g* for 25 s and the released cells were collected by centrifugation at 500 *g* for 5 min. The cells were resuspended in 10 ml of Joklik's buffer and kept at 4°C until placed on the gradients. Frequently, the first trypsin digestion contained a large amount of red blood cells and, in this instance, it was discarded. The trypsin digestion of the medullary tubules was repeated eight successive times. The cells from all digestions were combined and recentrifuged at 500 *g* for 5 min, suspended in 20 ml of Joklik's buffer, and filtered through a teflon mesh (36-μm grid size). The majority of cells after filtration were single; infrequently, groups of two or three cells were seen, which did not affect the separation. The cells were then placed on two linear Ficoll-400 gradients, which were formed with a two-chamber gradient maker similar to that described by Pretlow and Boone (19). The Ficoll was dissolved in a buffer containing: 137 mM NaCl, 5 mM KCl, 0.33 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.8 mM $MgSO_4$, 20 mM Tris-HCl, pH 7.4. A 10-ml cushion of 30.7% (wt/wt) Ficoll was placed on the bottom of the tube (tube length, 16 cm; inside diameter, 13 mm) and a 60-ml gradient was constructed, which decreased from 30.7% (wt/wt) at the cushion-gradient interface to 2.6% (wt/wt) at the gradient-sample interface. 10 ml of cells in Joklik's buffer were put on top of the gradient and centrifugation was carried out for 40 min at 2,800 rpm, which corresponds to 1,400 *g* at the gradient-sample interface (2,100 *g* max) in a Damon/IEC (Damon/IEC Div., Damon Corp., Needham Heights, Mass.) refrigerated centrifuge DM6000, centrifuge rotor 259 (at 4°C). Cells were collected in 4-ml fraction by the use of a wide mouth pipette, the first 10-ml fraction, which contained the buffer of the cell suspension put on the gradient, was discarded. The Ficoll was removed by diluting the cells 10 times with Joklik's buffer and centrifuging at 4,000 *g* max for 5 min.

Enzyme Determinations

Na, K-ATPase activity was determined by coupling ATP hydrolysis to NADH oxidation as originally described by Schoner et al. (23). The reaction mixture contained: 100 mM imidazole, pH 7.3, 150 mM NaCl, 5 mM $MgCl_2$, 0.4 μM NADH, 100 mM NH_4Cl, 2.9 μM disodium ATP, 0.6 μM phosphoenolpyruvate, 3.9 U pyruvate kinase, 7.4 U lactate dehydrogenase, plus or minus 2.4 mM ouabain. The ATP hydrolysis was monitored at 37°C and 340 nm, as the disappearance of NADH.

Alkaline phosphatase activity was measured as the rate of hydrolysis of *p*-nitrophenylphosphate with a Merckotest® kit (Merk AG., Darmstadt, W. Germany, no. 3344). For the measurement of adenylate cyclase, the cells were suspended in a hypotonic medium as described by Morel et al. (18) containing: 8 mM Tris-HCl, pH 7.4, 0.8% bovine serum albumin, 1 mM $MgCl_2$, and 0.25 mM EDTA. The cells were then frozen, followed by thawing. 25-μl samples were incubated in 210 μl of buffer containing: 30 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 0.1 mM EDTA, 10 mM theophylline, 3 mM disodium ATP, 10 mM creatine phosphate, and 0.3 mg/ml creatine kinase. The hormone sensitivity of the enzyme was studied with human synthetic calcitonin (10^{-6} M) or 8-arginine vasopressin (AVP) (10^{-6} M). The reaction was run at 37°C for 15 min and stopped by boiling the samples for 3 min. The cyclic AMP (cAMP) formed was measured with an Amersham Test Kit (Amersham TRK 432, Amersham Corp., Arlington Heights, Ill.).

For protein determination, the samples were first precipitated with 10% trichloracetic acid, then dissolved in 0.1 N NaOH, and processed according to Lowry et al. (17) using bovine serum albumin as a standard.

Oxygen Consumption and Viability Measurements

Cellular respiration was measured with a Clark oxygen electrode (Yellow Spring Instrument Co., Yellow Springs, Ohio) in Joklik's buffer at 37°C. Intracellular Na and K levels were measured with an Eppendorf flame photometer (Netheler & Hinz, Hamburg, Germany) in cells incubated in Joklik's buffer for 15 min at 37°C with 95% O_2-5% CO_2. The cells were separated from the incubation medium by spinning a sample through silicone oil (AR 20:AR 200 = 3:1). On centrifugation for 0.5 min in a Beckman centrifuge (Beckman Instruments, Inc., Fullerton, Calif.), the cells with a small amount of adherent medium (0.1–0.5%), as determined by incubating the cells with [^{3}H]inulin, passed through the oil and sedimented. The oil was removed and the pellet taken up in distilled water. The amounts of sodium and potassium in the cells were corrected for the amount in the adherent buffer and divided by cellular water to yield intracellular concentrations. The measured intracellular concentrations are average values over all cellular compartments.

Perfusion of the kidney with 50 ml of Joklik's buffer to remove red blood cells

↓

Medulla is carefully excised from the cortical tissue and cut in small pieces

↓

Incubation of medulla in 0.2% collagenase-0.25% hyaluronidase-Joklik's buffer for 1 h, 37°C.

Yielding: Tubules

↓

Incubation of the tubules in 0.25% trypsin-Joklik's buffer, 20 min, eight times

↓

Tubules sedimented by centrifugation at 750 *g*, 25 s

Medullary cells collected by centrifugation at 500 *g*, 5 min

↓

Cells separated on Ficoll gradient (2.5–30.7% wt/wt), centrifuged at 1,400 *g*, 45 min.

FIGURE 1 Schematic of the procedure to isolate medullary cells from the rabbit kidney.

Intracellular water was measured as the difference between wet weight and dry weight by drying the cells overnight in a 60°C oven. The intracellular water was also corrected for extracellular water by determining the [^{3}H]inulin space.

ATP levels in the cells were determined by incubating the cells for 5 min at 37°C in Joklik's buffer with 95% O_2-5% CO_2. The cells were then boiled for 3 min and ATP levels were determined by the luciferin-luciferase method (Abimed test kit No. 4613 for nonbacterial ATP, (Lumac Systems A.G., Basel 2, Switzerland) and measured in a Bioluminescence analyzer XP 2000 (Scan AG., Switzerland).

Stainability by trypan blue was tested by mixing equal volumes of cells and 0.4% trypan blue solution in Joklik's buffer at room temperature. At least 100 cells were counted under a light microscope.

Electron Microscopy

For electron microscopy, the cells were washed several times in Joklik's buffer to completely remove the Ficoll and resedimented by centrifugation at 500 *g* for 5 min. The cells were fixed in 5% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.3, for 2 h at 0°C. Whole kidneys were additionally fixed by perfusion with 3% glutaraldehyde in 0.1 M sodium cacodylate containing 0.05% $CaCl_2 \times 2H_2O$. Postfixation was performed in 1% osmium tetroxide buffered with 0.05 M Veronal-HCl, pH 7.2, for 1 h, followed by a water rinse and staining with uranyl acetate. After rapid dehydration in a series of ethanol-water mixtures, the cells and medullary slices were embedded in an epoxy resin, cut, stained with lead citrate, and examined in a Philips 300 electron microscope.

Chemicals

All chemicals used were of the highest purity possible and most were purchased from Merck AG., Darmstadt, Germany. The Joklik's buffer (Minimum Essential Medium), fetal calf serum and trypsin (0.25%, lyophilized) were purchased from GIBCO. The following items were obtained from Boehringer Mannheim GmbH, Mannheim, Germany): hyaluronidase (1,175 U/mg), disodium ATP, ouabain, pyruvate kinase, phosphoenolpyruvate, lactate dehydrogenase, creatine phosphate, and creatine kinase. Collagenase, grade II (146 U/mg) was purchased from Worthington Biochemical Corp., Freehold, N. J.; Ficoll-400 from Pharmacia, Inc., Uppsala, Sweden; human synthetic calcitonin from Ciba-Geigy, Basel, Switzerland; 8-arginine vasopressin from Parke-Davis, München, Ger-

many. Furosemide was a gift from Hoechst Pharmaceuticals, Frankfurt, Germany.

RESULTS

During the isolation of the TALH cells, the various tissue fractions were identified morphologically, enzymatically, and by their response to furosemide, which is known to interfere with the chloride transport system in the TALH in vivo. These three criteria will be discussed separately in the following sections.

Morphological Identification

The outer medulla of the rabbit kidney, as shown in Fig. 2, contains collecting ducts, the straight portion of the proximal tubule (pars recta), the ascending thick limb of Henle's loop, and vasculature and interstitial cells (for a detailed morphological description of the rabbit kidney, see Kaissling and Kriz [12]). The TALH tubes (Fig. 3), obtained after collagenase-hyaluronidase digestion, are characterized by short blunt microvilli, numerous dark mitochondria, and a large nucleus. As in the intact kidney, numerous vesicles and tubulovesicular structures can often be observed below the luminal membrane (1).

The single cells obtained after digestion with trypsin are a mixture of cells from the numerous nephron segments (Fig. 4). The cells are difficult to identify when released from their tubular structure and neighboring landmarks, but certain features were found to be useful in distinguishing the various cells. These features are compiled in Table I. The proximal tubule cells (Fig. 5) are characterized by the microvilli that surround the cells and by the thick irregular basal infoldings. The proximal tubule cells also contain small, ovoid, dark mitochondria and a complex system of vacuoles, vesicles, and tubules within the cell which are also seen in the intact tissue (25). The TALH cells (Fig. 6) have a large indented nucleus, possess many round dark mitochondria, short blunt microvilli, and many "vacuoles." These vacuoles, and those of the proximal tubule cells (Fig. 5), represent not only naturally occurring cytoplasmic vesicles but also sections through the complex interdigitating basal-lateral infoldings, whose intercellular spaces spread when a cell is released from its epithelium and subsequently rounds up. This is evident from Figs. 3 and 4, in which a TALH cell is cross sectioned in the basal portion, so that a nuclei is not seen (arrows) and the cell appears as consisting of only dark mitochondria and vacuoles. In fact, in isolated epithelial cells, the appearance of vacuolization seems to correlate with extensive basal-lateral membrane infoldings observed *in situ*. An isolated collecting duct cell, in comparison (Fig. 7), where the contraluminal membrane is relatively undifferentiated, has no evidence of vacuolization.

The collecting duct cells, identifiable as light or principal cells (Fig. 7), have small ovoid or round mitochondria, a centrally located nucleus surrounded by clear, less electron-dense cytoplasm which contains a few small vesicles. Cells from the thin descending and thin ascending limbs of Henle's loop, interstitial and endothelial cells, are difficult to distinguish outside of their tubular structure. Intercalated cells from the collecting duct, which are characterized by large number of mitochondria, a prominent Golgi apparatus, a large number of polysomes and abundant coated, and spherical and flat-stacked vesicles, were not seen. These cells which were not clearly identifiable composed ~15% of the total medullary cell population.

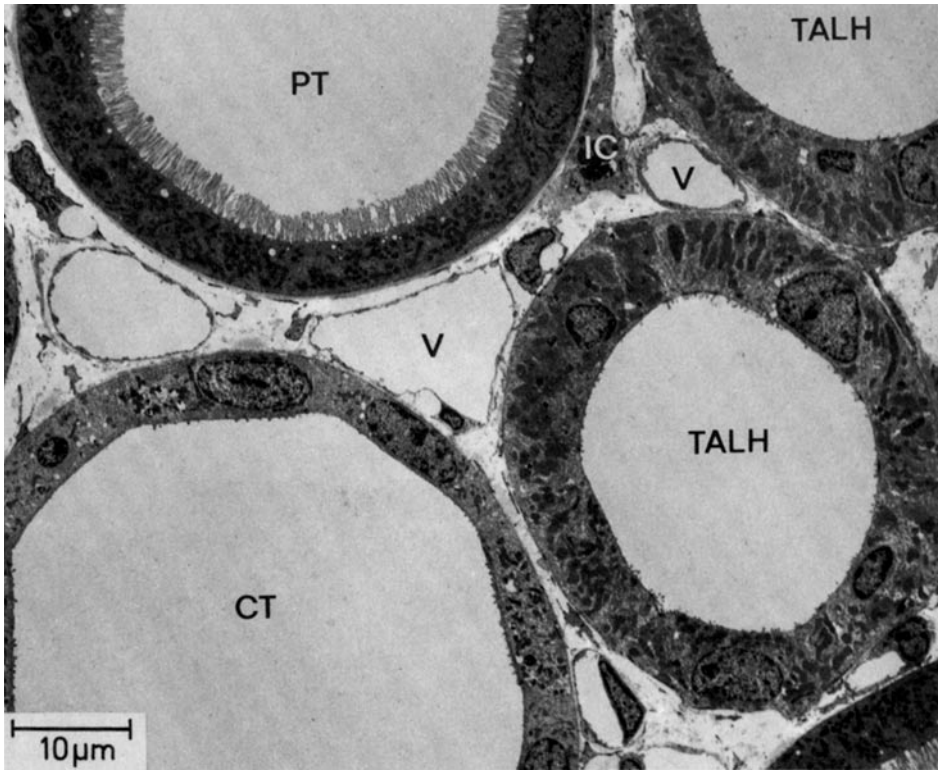

FIGURE 2 Electron micrograph of rabbit outer medullary tissue. The tissue is composed of the pars recta of the proximal tubule (*PT*), collecting tubules (*CT*), thick ascending limb of Henle's loop (*TALH*), interstitial cellular elements (*IC*), and the vasculature (*V*). × 1,500.

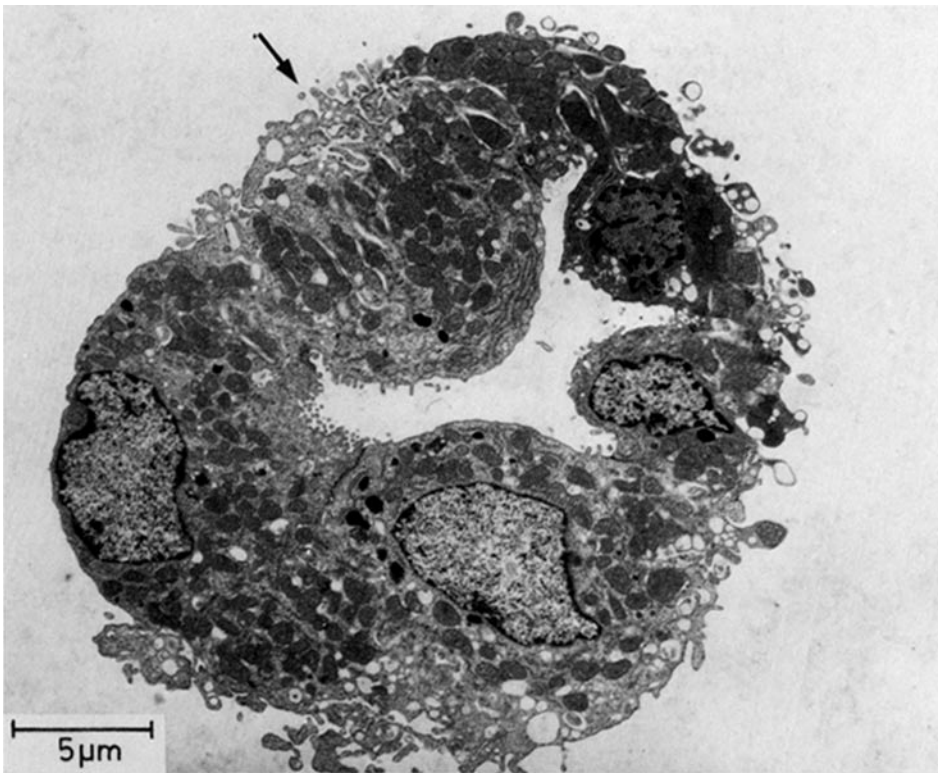

FIGURE 3 Electron micrograph of an isolated TALH tubule from the rabbit medulla after incubation of the renal medulla in 0.2% collagenase–0.25% hyaluronidase for 1 h at 37°C. The arrow indicates a TALH cell sectioned without a nucleus, thus, the cell appears to consist of only mitochondria and vacuoles. × 3,600.

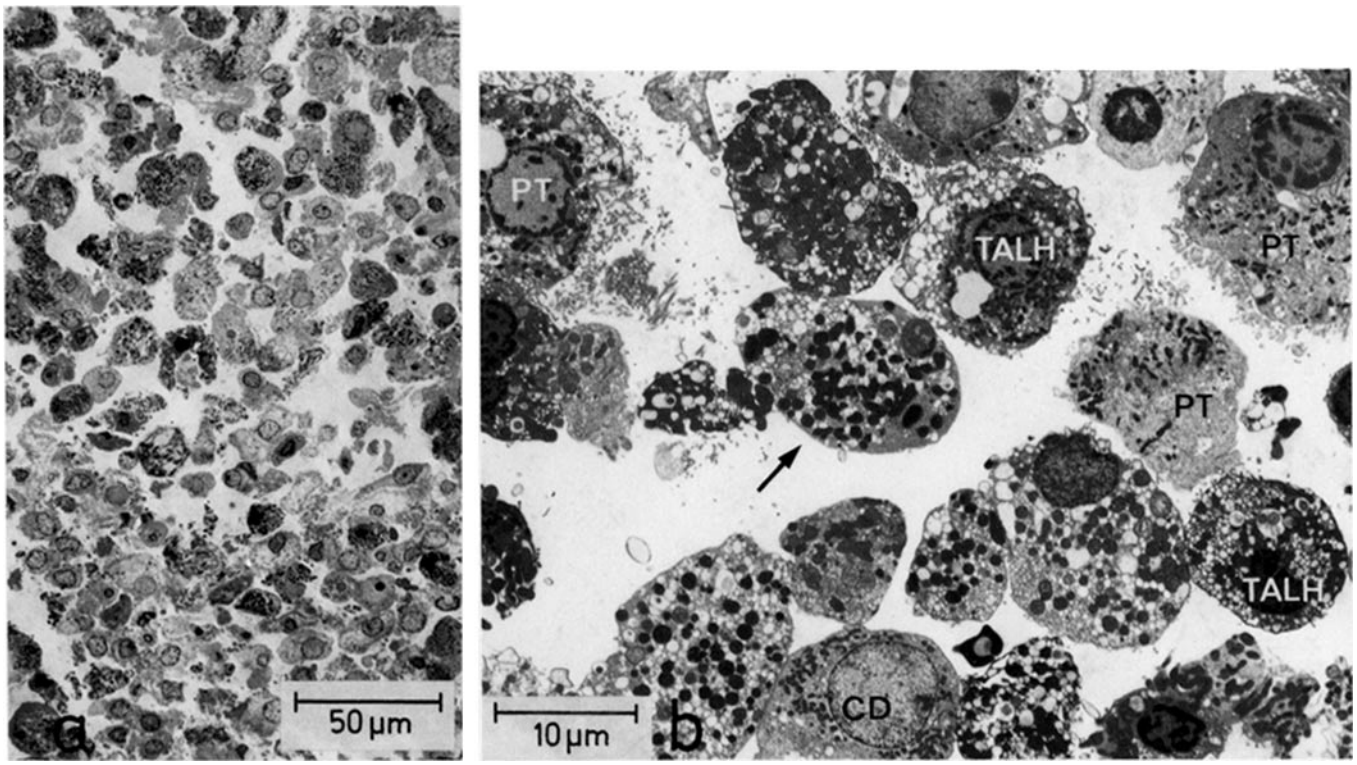

FIGURE 4 Electron micrograph of the total medullary cell population before being centrifuged on the Ficoll gradient. The recognizable cells are derived from the proximal tubule (*PT*), collecting duct (*CT*), and the thick ascending limb of Henle's loop (*TALH*). The arrow indicates a TALH that was sectioned through the basal portion of the cell and can be compared with the cell indicated by the arrow in Fig. 3. *a*, × 340; *b*, × 1,800.

TABLE I

Structural Markers for Identification of Single Isolated Cells from the Rabbit Renal Medulla

Cells*	Plasma membrane	Nucleus	Mitochondria	Cytoplasmic inclusions
Proximal tubule (pars recta)	Long microvilli, thick basal-lateral membrane infoldings	Round, elongated, often indented	Small, ovoid, dark	Numerous vesicles (mean diameter ~0.7 μm), electron-dense material‡
TALH	Few short microvillus projections, basal-lateral membrane is highly infolded and appear as vesicles	Elongated with indentations	Numerous, large, dark, densly packed, filling up most of cell, mostly round, larger than in proximal and collecting tubule cells, cristae visible	Vesicles mostly smaller than mitochondria‡
Collecting duct (light or principal cells)	A few short microvilli, no infoldings	Large round	Dark, small less numerous than in proximal tubule	Small, light vesicles (mean ~0.3 μm diameter)
Thin loop, interstitial, endothelial cells	Irregular surface with microvillous like extrusions	Irregular shape	Very few	Some vesicles, cytoplasm is only a small ring around nucleus

* All cells have a more or less round shape on separation.

‡ Vesicles may include naturally occurring vesicle structures and cross sections of basal-lateral membranes.

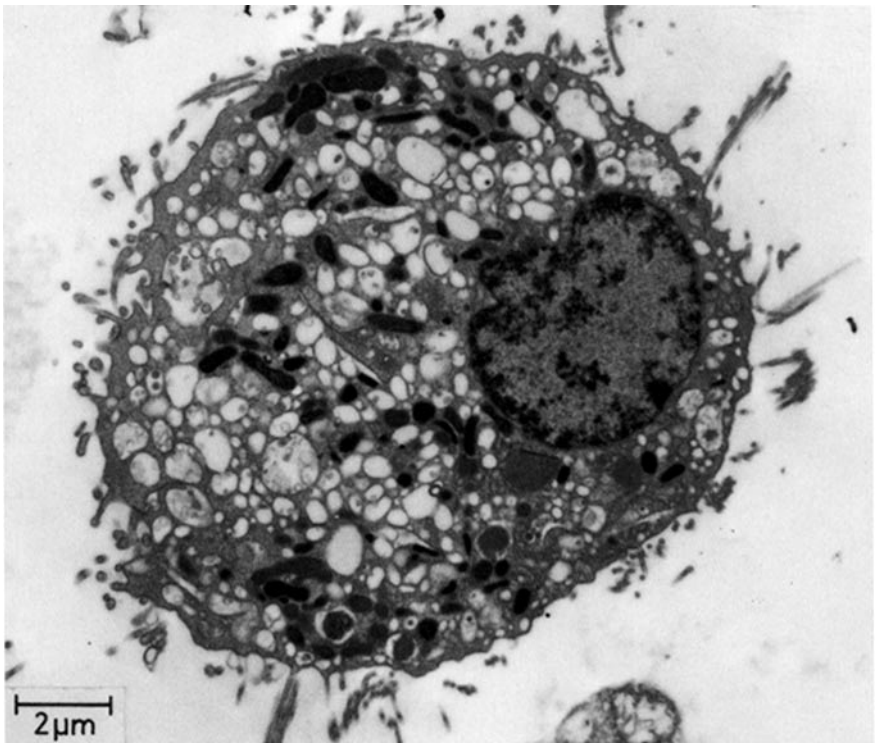

FIGURE 5 Electron micrograph of a proximal tubule cell from the total cell population. × 6,200.

In an electron micrograph of the cells from the dense fraction 10 (Fig. 8), the majority of cells are morphologically identifiable as TALH cells. Occasionally, a proximal tubule cell or principal cell of the collecting duct is seen. The former is especially evident if, in the first step of isolation procedure, the kidney medulla is not very carefully dissected from the cortical tissue. The TALH cells (Fig. 9) have intact triple-layered plasma membranes and their polarity is apparently maintained. Short microvilli are evident on one side of the cell, the former apical membrane, whereas the other cell pole is characterized by deep infolding of the plasma membrane, the former basal-lateral membrane.

Enzymatic Identification

The enzymatic criteria used to identify the medullary TALH cells were the enrichment of calcitonin-sensitive adenylate cyclase activity and Na, K-ATPase activity. Both enzyme activities have been shown by microdissection to be highly concentrated in the TALH (7, 13, 22). As shown in Fig. 10, in a linear Ficoll gradient ranging in density from 0.22 to 1.145 gm/ml, the protein was distributed approximately evenly. The calcitonin-sensitive adenylate cyclase activity was, however, concentrated in the densest portion of the gradient (fraction 10), the maximum enzyme activity being 700 pmol cAMP

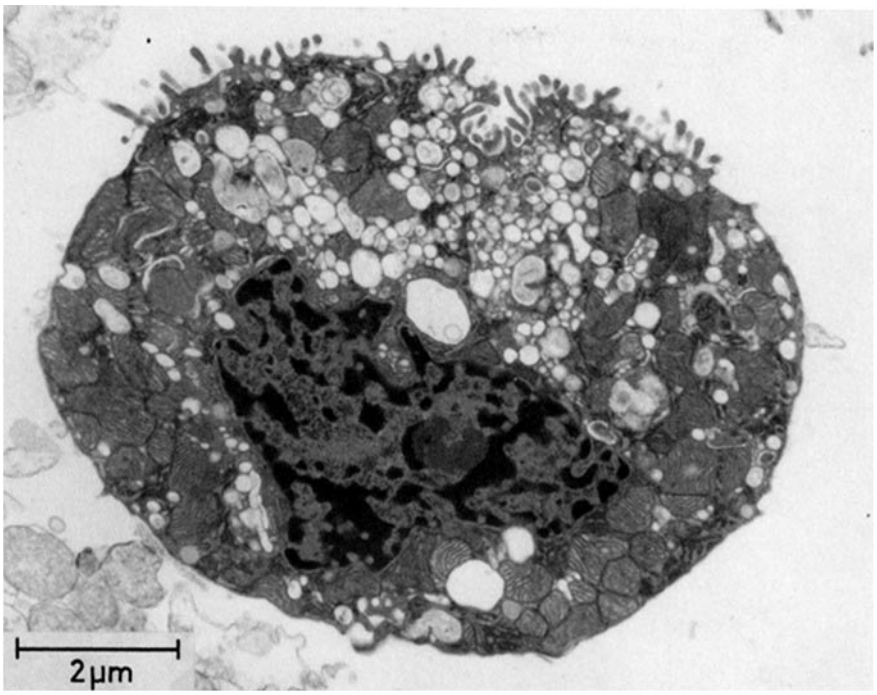

FIGURE 6 Electron micrograph of a thick ascending limb of Henle's loop cell from the total medullary cell population. × 10,800.

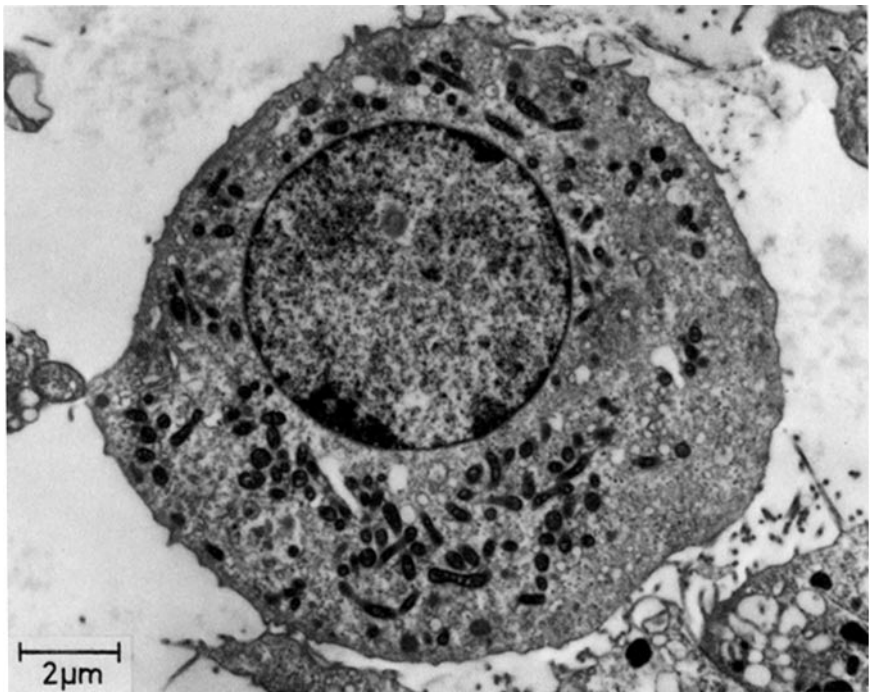

FIGURE 7 Electron micrograph of a light or principal collecting duct cell from the total medullary cell population. × 6,400.

formed per milligram of protein per 15 min. The Na, K-ATPase activity was also highest in fraction 10 of the gradient, reaching an activity of 250 mU/mg protein. In contrast, the highest activities of alkaline phosphatase, an enzyme found in high concentrations in proximal tubule cell membranes (3), was found in the top portion of the gradient (fraction 2) and decreased throughout the gradient to an activity of 32 mU/mg protein in fraction 10 (Fig. 11). The distribution of AVP-sensitive adenylate cyclase activity, present in high amounts in the collecting duct cells (18), was similar to, but not identical with, the distribution of alkaline phosphatase (Fig. 11). Maximum activity was achieved in the lighter portions of the gradient (fraction 2), the specific activity being 890 pmol cAMP formed per milligram of protein per 15 min. The enzymatic activity decreased throughout the gradient so that an activity of 98 pmol cAMP formed per milligram of protein per 15 min was found in the densest fraction 10.

In accordance with the high activities of the marker enzymes alkaline phosphatase and AVP-sensitive adenylate cyclase in the lighter fractions of the gradients, these fractions morpho-

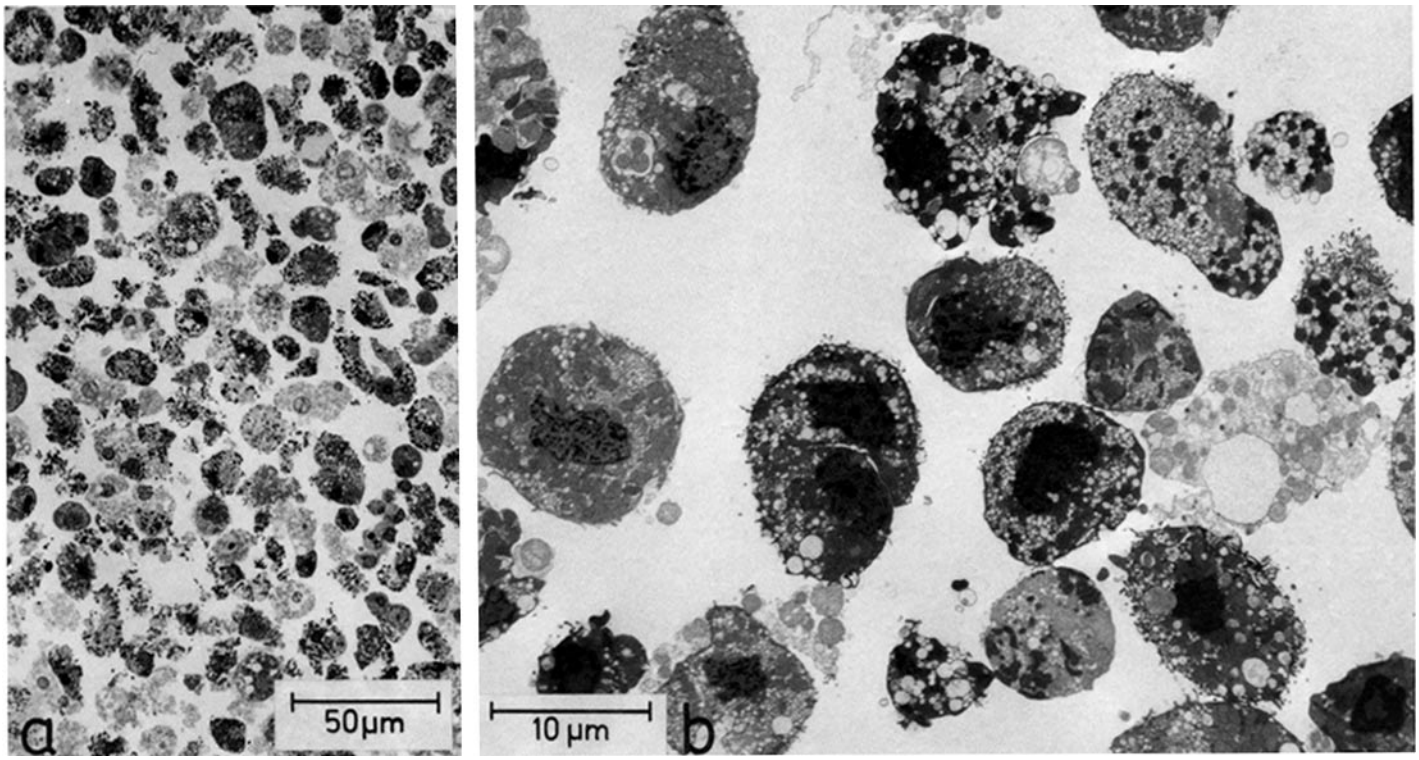

FIGURE 8 Electron micrograph of the cells found in the fraction 10 of the Ficoll gradient. *a*, × 325; *b*, × 2,000.

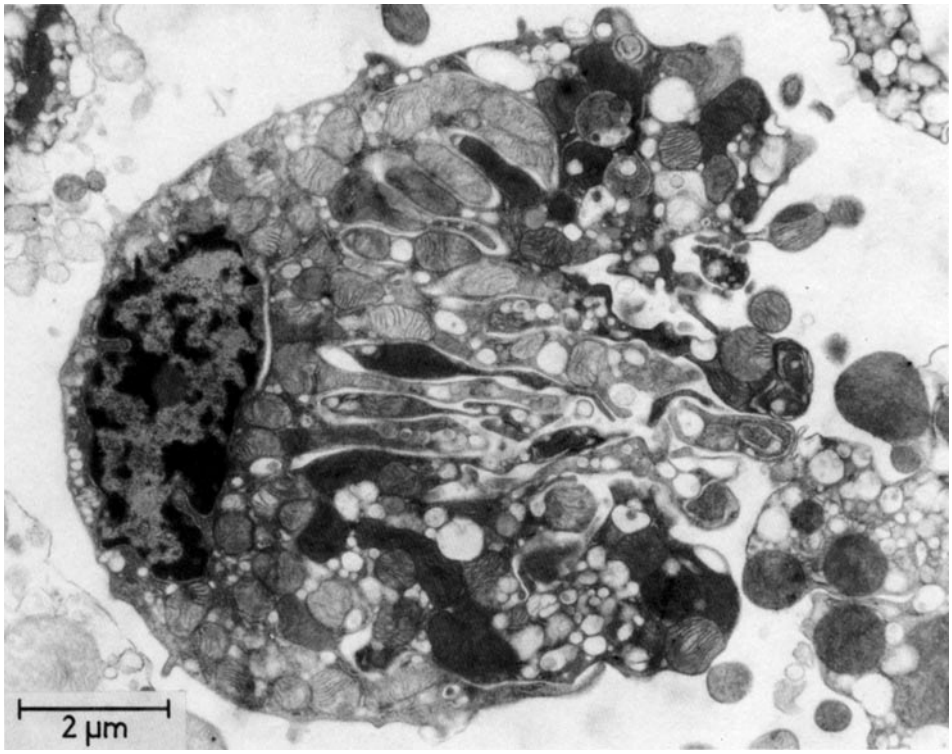

FIGURE 9 Electron micrograph of a TALH cell found in the fraction 10 of the Ficoll gradient. × 9,800.

logically contained mostly proximal tubule and collecting duct cells (J. Eveloff et al., unpublished observation).

In Table II, the enzyme activities of fraction 10 of the gradient are compared with the enzyme activities in the medullary cell population before density gradient centrifugation. The calcitonin-sensitive adenylate cyclase and Na, K-ATPase activities in the dense fraction 10 are enriched 5.8 and 4.2 times, respectively, over the activities in the total medullary cell population. The AVP-sensitive adenylate cyclase and alkaline phosphatase activities, however, were lower than found in the total medullary cell population. Thus, also according to the enzymatic characteristics, the cells found in the dense

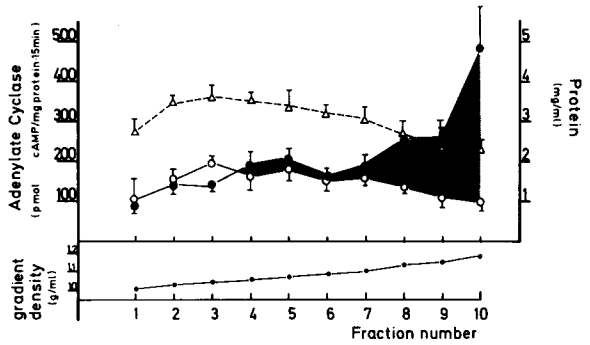

FIGURE 10 The distribution of calcitonin-sensitive adenylate cyclase and protein after separation of the total medullary cell population on a Ficoll gradient (2.6–30.7% wt/wt). Cells were removed from the gradient in 4-ml fractions. The distribution of calcitonin-stimulated adenylate cyclase is depicted by the shaded area. Calcitonin-stimulated adenylate cyclase was determined in the absence (○) and presence (●) of 10^{-6} M calcitonin. Protein distribution is depicted by the open triangles (△). Points are the means of six experiments ± SEM. The lower half of the graph indicates the gradient density, in grams per milliliter, for the above determined 4-ml fractions. The recovery of calcitonin-stimulated adenylate cyclase and protein from the medullary cells put on the gradient was 94% and 110%, respectively.

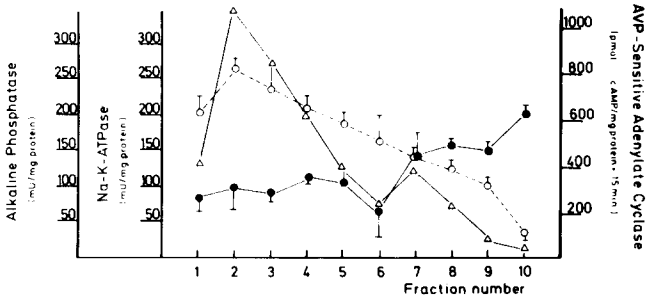

FIGURE 11 The distribution of Na, K-ATPase, alkaline phosphatase, and AVP-sensitive adenylate cyclase after separation of the total medullary cell population on a Ficoll gradient. Cells were removed from the gradient in 4-ml fractions. The distribution of Na, K-ATPase (●), alkaline phosphatase (○), and AVP-sensitive adenylate cyclase (△) are given. Points represent the mean values of six experiments ± SEM. The recovery of Na, K-ATPase, alkaline phosphatase, and AVP-sensitive adenylate cyclase activities from the gradient was 93, 102, and 92%, respectively.

fraction of the Ficoll gradient are derived from the medullary TALH.

Functional Characteristics and Viability of the Isolated TALH Cells

To further verify the identity of the TALH cells, furosemide inhibition of respiration was measured. The fraction 10 cells had an oxygen consumption of 33.6 μl O_2/mg protein × h, which fell by 40% to 20.4 μl O_2/mg protein × h upon the addition of 10^{-5} M furosemide to the incubation medium (Table III). The oxygen consumption response to furosemide was dose dependent; further additions of furosemide to final concentrations of 10^{-4} and 10^{-3} M reduced oxygen consumption by 48% and 54% of the control (to 17.4 and 15.6 μl O_2/mg protein × h). This effect was not seen in the less dense fractions: fraction 2 showed only 20% inhibition of respiration with 10^{-3} M furosemide; the respiration decreased from a control value of 10.7 μmol O_2/mg protein × h to 8.5 with 10^{-3} M furosemide. In contrast, amiloride, a diuretic that acts mainly in the distal tubule (24) inhibiting sodium reabsorption and potassium secretion, had little effect on oxygen consumption of fraction 10 cells. Oxygen consumption was inhibited 11% (to 29.4 μl O_2 mg protein × h) by 10^{-4} M amiloride (E. Bayerdörffer et al., unpublished observations). The dose-dependent furosemide inhibition of respiration and the small effect of amiloride support further the assumption that the cells present in fraction 10 are derived from the TALH of the medulla.

The isolation procedure takes ~6 h with exposure of the cells to collagenase, hyaluronidase, and trypsin. Therefore, the viability of the isolated cells was measured using the following criteria: the metabolic state of the cells was estimated by the effect of uncouplers on oxygen consumption and by measuring the ATP levels of the cell; and the state of the plasma membrane was assessed by measuring the permeability to trypan blue, succinate, sodium, and potassium. As can be seen in Table IV, the addition of 0.05 mM 2,4 dinitrophenol stimulated oxygen consumption by 88% and the ATP levels were 3.7 μmol/g wet weight. Furthermore, the addition of 10^{-4} ouabain inhibited oxygen consumption by 51%, indicating a tight coupling between respiration and the energy-requiring active sodium transport via the Na, K-ATPase. Trypan blue exclusion from the cells, a test for changes in plasma membrane permeability, was >99%. Respiration was minimally stimulated, 1% by 1 mM succinate, indicating that the permeability of the cell membrane was unaltered. It has been shown in isolated liver cells that when the plasma membrane is damaged, succinate

TABLE II

Enzyme Activities and Enrichments in TALH Cells Prepared from the Rabbit Kidney

Enzyme	Specific activity*		Enrichment‡
	Total medullary cell population	TALH cells	
Calcitonin-sensitive adenylate cyclase	120.7 ± 25.6	700 ± 58.7	5.8
Na, K-ATPase	49.2 ± 5.1	204.5 ± 35.6	4.2
AVP-sensitive adenylate cyclase	326.7 ± 13.8	98.2 ± 26.7	0.3
Alkaline phosphatase	325 ± 49	32 ± 4	0.1

Values are means ± S.E. The number of experiments was six.

* Specific activity of the adenylate cyclases is expressed as picomoles cAMP formed per milligram of protein × 15 min. Na, K-ATPase and alkaline phosphatase specific activity is expressed as picomoles per milligram of protein × hours.

‡ Enrichment is the enzyme specific activity in the TALH cells compared with the specific activity in the total medullary cell population.

TABLE III

Furosemide Inhibition of Oxygen Consumption in Isolated TALH Cells

	Oxygen consumption*	Inhibition
		%
Control	33.6 ± 6.1	—
Furosemide, 10^{-5} M	20.6 ± 6.2	38.8
Furosemide, 10^{-4} M	17.6 ± 4.1	47.7
Furosemide, 10^{-3} M	15.8 ± 5.0	53.0

Values are means ± SEM. The number of experiments is eight.

* Oxygen consumption is expressed as microliter per milligram of protein × hour.

TABLE IV

Viability and Characteristics of TALH Cells

Criteria of viability	TALH cells
Oxygen consumption (QO_2) (μl O_2/mg protein × h)	33.6 ± 0.8 (8)
QO_2 stimulation by 0.05 mM 2,4 dinitrophenol, %	88.4 ± 28.3 (7)
QO_2 inhibition by 10^{-4} M ouabain, %	50.6 ± 4.2 (4)
Intracellular Na, *meq/liter*	9.4 ± 3.9 (3)
Intracellular K, *meq/liter*	139.2 ± 10.6 (3)
Intracellular ATP (μmoles/gm wet weight)	3.7 ± 0.5 (4)
Stainability by trypan blue, %	1 ± 0.2 (3)

Values are means ± SEM. The number of experiments is given in parentheses.

enters the cell at a rate sufficient to stimulate respiration (2). This was confirmed in the TALH cells. After freeze-thawing the cells for 5 min, succinate stimulated oxygen consumption by 200%. Further confirming the viability of the TALH cells, an intracellular Na^+ concentration of 9.4 meq/liter and an intracellular K^+ concentration of 139.2 meq/liter were found in the isolated TALH cells. Thus, the plasma membranes seem to be tight to trypan blue, succinate, sodium, and potassium. In addition, intracellular metabolism and the Na, K-ATPase are coupled tightly enough to maintain the ion gradients across the plasma membrane.

DISCUSSION

We have described a method to isolate a population of cells highly enriched in cells of the TALH from the rabbit kidney medulla. It consists of a dissociation of the tissue into tubules by collagenase-hyaluronidase followed by the digestion of the tubules into cells by a series of trypsin digestions. The use of collagenase-hyaluronidase alone did not produce single cells from the medullary tissue. Similarly, treatment with trypsin alone produced extremely few cells from the intact tissue. Other procedures investigated to obtain cells from the tubules or directly from the intact tissue included treatment with dispase and elastase, low calcium media, perfusion of the kidney with citrate, hypoosmotic shocks, or the above in various combinations. Under all circumstances, either too few cells were obtained or the cells were not viable. The release of tubules with collagenase-hyaluronidase from the intact tissue is relatively fast and easy, but the separation of tubules into cells repeatedly resisted experimental manipulation. This finding is in contrast to the results obtained with proximal tubule cells from rabbit kidney, in which the preparation of cells, through perfusion of the kidney with a citrate buffer or incubation with trypsin, was accomplished relatively easily (9, 15, 16, 20). Some notable properties of the TALH tubules may be responsible for keeping the cells within their tubular matrix; (*a*) the cellular tight junctions appear to be broader than in the proximal tubule and (*b*) the cellular interdigitations between cells appear stronger and more complex than in the proximal tubule cells. Additionally, both the tubules and cells in their isolated state remained viable only in a buffer of normal osmolality (~300 mosmol). Higher osmolalities resulted in marked cellular dehydration.

That the isolated cell population, prepared as described, is derived from the TALH is supported by three independent, specific criteria. One, morphologically, the majority of the isolated cells were identifiable as TALH cells. This was actually the most difficult criterion to assess because when single cells were released from their tubular structure, they rounded up and, outside of their neighboring landmarks, became difficult to distinguish. A series of studies was made following the structure of the medullary cells within the tissue to their structure in isolated tubules, to single cells, and to their structure after separation on the gradient. This investigation led to the definition of the morphological criteria compiled in Table I. With these criteria, isolated single proximal tubule cells, collecting duct cells, and TALH cells can be identified. The thin descending and thin ascending limbs of Henle's loop, intercalated cells, the interstitial cells, and the blood vessel cells are, however, still difficult to distinguish in the isolated cell state.

Two, the enzyme calcitonin-sensitive adenylate cyclase, which is found only in the TALH cells in the medulla (7), was greatly enriched in the isolated TALH cells. Further, Na, K-ATPase, a less specific enzyme marker, because it is present in all cells, was also found in very high concentrations in the TALH, in agreement with the microdissection studies (13, 22). Alkaline phosphatase and AVP-sensitive adenylate cyclase, enzymes found mainly in the proximal tubule and collecting duct (3, 11, 18), were used as negative markers to follow the separation of these cells from the TALH cells. The concentration of alkaline phosphatase and AVP-stimulated adenylate cyclase decreased during preparation of the TALH cells. Theoretically, parathyroid hormone-sensitive adenylate cyclase should have represented another enzyme marker for the pars recta of the proximal tubule of the medulla (18), but the enzyme activity was destroyed during the enzymatic digestions in accordance with observations of the high sensitivity of the parathormone receptor to tryptic digestion. In contrast, the recovery of the other marker enzymes, Na, K-ATPase, alkaline phosphatase, and calcitonin-sensitive adenylate cyclase, was ~100%, indicating no inactivation during cell separation. In our hands, the use of enzymatic markers to assess purification of the cells was more accurate than the use of morphological criteria. Cell counts performed on the medullary cell populations and fraction 10 definitely showed an enrichment of TALH cells similar to the enzymatic enrichments. Quantitative data were, however, difficult to obtain because of the uncertainties in identifying all of the cell types.

Three, the respiration of the TALH cells was inhibited in a dose-dependent manner by furosemide, a diuretic which inhibits salt transport in the TALH (6, 10). It was found that other cell fractions within the Ficoll gradient did not respond to furosemide. Thus, at low concentrations, furosemide inhibition of respiration appears to be a quite specific indicator for cells derived from TALH.

In the isolation of a homogeneous population of single cells, which are ultimately intended to be used for physiological and biochemical investigations, it is important not only that the cells are homogeneous and identifiable but also that they are viable. Otherwise, the biochemical and physiological parameters measured in vitro may not be identical with those in vivo. Therefore, not only one but several viability parameters were measured. One of the most sensitive criteria of cellular viability that we used was the response of oxygen consumption to 1 mM succinate. Compared with the large molecule trypan blue,

succinate, with a molecular weight of 162, has a small molecular radius and, therefore, small changes in membrane permeability can be more easily detected. Similarly, the sodium and potassium gradients across the cell membrane are very sensitive indicators of membrane integrity and the functional coupling of the cell.

The lack of effect of succinate on the respiration of TALH is an interesting experimental observation because the respiration of renal cortical slices is stimulated normally by succinate (8). This discrepancy is probably caused by the presence of succinate transport systems in the plasma membranes of the cells in the proximal tubules but not in the TALH (14).

As a last point, the present method yields ~4 mg of protein from two rabbit kidneys in ~6 h. If a calculation is made to compare this method with the teased tubule technique, the following results are obtained. A TALH tubule of ~1.5 mm in length can be dissected by microdissection in 5 min. The tubule has a protein content of 0.06 μg/mm (11); thus, a maximal yield of ~6.8 μg protein could be maximally expected in an equivalent 6-h period. The present method, therefore, yields at least 1,000 times more protein, allowing for flexibility in the application of many biochemical and physiological techniques in the study of the mechanisms of ion transport to this nephron segment.

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