Sodium-Sensitive, Probenecid-Insensitive *p*-Aminohippuric Acid Uptake in Cultured Renal Proximal Tubule Cells of the Rabbit

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Abstract. **In the intact kidney, renal proximal tubule cells accumulate *p*-aminohippurate (PAH) via a basolateral, probenecid- and sodium-sensitive transport system. Primary cultures of rabbit proximal tubule cells retain sodium-glucose co-transport in culture, but little is known about PAH transport in this system. Purified proximal tubule cells from a rabbit were grown in culture and assessed for PAH and α-methyl-D-glucoside uptake capacities as well as proximal tubule marker enzyme activities. Control PAH uptake on collagen-coated filters (20 ± 3 pmol/mg protein·min; $n = 8$) was not significantly different from uptake in the presence of 1 m*M* probenecid (19 ± 4 pmol/mg protein·min; $n = 8$). Uptake from the basal side of the cell was 3.9 ± 0.7 times greater than that from the apical side. In multi-well plate studies, the uptake was significantly reduced by removing sodium from the medium and stimulated by coating the wells with collagen. Glutarate (10 m*M*) had no effect on the uptake of PAH. Other differentiated proximal tubule characteristics were retained in culture, including the ability to form domes and to transport glucose by a phlorizin-sensitive system. Phlorizin-sensitive 1 m*M* α-methyl-D-glucoside uptake was 134 ± 42 pmol/mg protein·min ($n = 7$; $P < 0.02$). The proximal tubule marker enzymes alkaline phosphatase and γ-glutamyltranspeptidase, increased in activity in the cultures after confluence. It was concluded that whereas some differentiated properties were retained during primary culture of rabbit proximal tubule cells, the PAH transport system was selectively lost or modified from that present in the intact kidney.** [P.S.E.B.M. 1992, Vol 199]

Through the use of defined media which limit fibroblast growth, primary cultures of rabbit proximal tubule cells can be produced that retain apical, Na-glucose co-transport (1–3) and have proximal tubule marker enzyme activities (1). An established renal cell line, LLC-PK_1, while retaining the apical Na-glucose co-transport system (4), has lost the *p*-aminohippuric acid (PAH) transport system (5, 6). Yang *et al.* (7) have shown that plasma membrane vesicles prepared from basolateral membranes of rabbit cells in primary culture carry out probenecid (PB)-sensitive PAH uptake. This uptake, however, is not concentrative in either the vesicles or in whole cells grown in plastic culture dishes. Since differentiation in culture may require basolateral access of the medium to the cells (8–11), the present study was undertaken to assess PAH uptake in rabbit cells and LLC-PK_1 cells grown on plastic surfaces as well as on porous membrane filters. Since the transport system is known to be Na dependent and coupled to dicarboxylic acid transport (12–14), and since collagen has been shown to stimulate attachment, growth, and differentiation in culture (8), the Na sensitivity of PAH uptake was determined, as well as the effect of glutarate and collagen on uptake.

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Materials and Methods

Proximal Tubule Purification. Proximal tubules from a single New Zealand White rabbit were purified from a collagenase-DNase digest of renal cortex, as described previously (15). By microscopic assessment, the final purified proximal tubule preparation consisted of 93% proximal tubules, 4% nonidentifiable tubules or vessels, and 3% glomeruli.

Cell Culture. The purified proximal tubules from the rabbit were plated in plastic culture dishes and grown at 37°C in a humidified atmosphere of 5% CO_2 in air in defined medium RK-1 as described by Chung *et al.* (2). RK-1 medium consisted of a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (Sigma Chemical Co., St. Louis, MO) containing 15 m*M* HEPES, 14.3 m*M* $NaHCO_3$, 5×10^{-8} *M* selenium, 100 μg/ml of penicillin G, 50 μg/ml of streptomycin sulfate, 5 μg/ml of bovine insulin, 5 μg/ml of human transferrin, and 5×10^{-8} *M* hydrocortisone. At Passage 1, cell stocks were frozen in liquid nitrogen for later use. In some cases, 10% fetal calf serum (FCS; Commonwealth Serum Laboratories, UK) was included during the last day of culture before measuring organic solute uptake in order to facilitate cell adhesions to the plastic of 96-well plates during washing of the monolayers. Epidermal growth factor was included in the medium at a concentration of 5×10^{-8} *M* to stimulate growth (16); however, epidermal growth factor was always removed from the medium at least 3 days prior to uptake or enzyme measurements and was never included in the medium when cells were seeded at superconfluent density. During routine passaging and cell dissociation with trypsin-EDTA, 0.05% (w/v) trypsin inhibitor (chicken egg white) was added to the first wash following trypsinization and to the final cell suspension used for plating.

The established pig kidney cell line LLC-PK_1 (17) was obtained from Flow Laboratories (Scotland) at Passage 188 and was maintained in culture and passaged as described previously (18). The culture medium, DM medium, consisted of Dulbecco's modified Eagle's medium containing 15 m*M* HEPES, 44 m*M* $NaHCO_3$, 100 μg/ml of penicillin G, 50 μg/ml of streptomycin sulfate, and 10% FCS.

PAH and Sugar Uptake. *Collagen-coated filters.* Rabbit cells at Passage 1 were grown in standard culture dishes or flasks in RK-1 medium until confluent. The specific procedures used for measuring uptake of radioactive solutes into cells grown on porous filter supports have been described previously (18). In brief, the cells were dissociated by trypsin-EDTA and washed, and 1 ml of cell suspension was seeded at a superconfluent density (3×10^5 cells/cm^2) into a 14.5-mm (i.d.) filter cup. The porous bottom of the filter cup consisted of a polycarbonate Nuclepore filter (3-μm pore size; Nuclepore Corp., CA) that had been precoated with 20 μg of basement membrane collagen from human placenta (Type VI; Sigma Chemical Co.). After adding cells to the filter cups, the culture medium was changed daily for 3 days, at which time the uptake assay was performed. For measuring uptake of radioactive solutes, a filter cup with its accompanying cell monolayer was washed in three changes of balanced salt solution (BSS) consisting of (in m*M*) 148 NaCl, 5 KCl, 1.3 $CaCl_2$, 4.2 $NaHCO_3$, 0.9 $MgSO_4$, 0.3 Na_2HPO_4, and 0.4 KH_2PO_4 pH 7.4). The filter cup was placed in a 35-mm culture dish, and radioactive uptake solution was added to both sides of the monolayer. Incubations were performed for 1 hr at room temperature (25°C). All uptakes were expressed as rates and were assumed to be below equilibrium based on previous studies with PAH in rabbit primary cultures by Yang *et al.* (7). Two radioactive uptake solutions were used consisting of BSS with 6.6 m*M* acetate, to which had been added 1 m*M* D-[3-*o*-*methyl*-^{3}H]glucose (OMG) and either 0.1 m*M* *p*-[^{14}C]-aminohippuric acid or 1 m*M* D-[α-*methyl*-^{14}C]glucoside (MDG). Each isotope was present at a concentration of 0.5 μCi/ml. In some cases, either 1 m*M* probenecid for PAH uptake or 100 μ*M* phlorizin (PHZ) for MDG uptake was added to the incubation medium to determine the inhibitor sensitivity of uptake. Since stock PB was dissolved in diluted NaOH, the pH of the final solution was adjusted after PB addition. Following incubation, the cup and attached monolayer were washed in three successive changes of ice-cold, nonradioactive BSS containing 1 m*M* probenecid and 100 μ*M* phlorizin and phloretin. The Nucleopore membrane, with its attached cells, was then removed from the plastic cup and placed in a centrifuge tube containing 0.6 ml of 0.5 *M* perchloric acid (PCA). The cells were freeze-thawed once and centrifuged, and an aliquot of supernatant (0.5 ml) was removed for determination of radioactivity in a three-channel liquid scintillation counter (Beckman model LS3801). Actual counts per min ranged from 100 to 400 above background for 14.5-mm collagen-coated filters. The remaining cell-Nucleopore membrane pellet was washed with 1 ml of cold 0.5 *M* PCA, recentrifuged, and resuspended in 0.25 ml of 0.3 *M* NaOH for protein estimation by the method of Lowry *et al.* (19) using bovine serum albumin as a standard. Intracellular water space was estimated from the uptake of [^{3}H]OMG as described by Kletzien *et al.* (20).

Multiwell plates. Rabbit cells at Passage 1 or 2 were seeded at superconfluent density (4×10^5 cells/cm^2) into wells of a 96-well plate (Nunc, Denmark). The culture medium was changed on Day 1 and Day 3 after seeding. FCS (10%) was added to the wells on Day 3, and organic solute uptake was measured on Day 4. The uptake procedure was as follows. The media in the wells was aspirated off and the monolayers were washed twice with BSS at room temperature. For Na-free uptake measurements, the monolayers were washed two additional times with 0.2 ml of Na-free salt solution consisting of (in m*M*) 153 choline Cl, 1.3 $CaCl_2$, 4.2 $KHCO_3$, 0.9 $MgSO_4$, 0.3 K_2HPO_4, and 0.4 KH_2PO_4 at pH 7.4. Uptake solution (50 μl, without acetate) containing either 0.1 m*M* [^{14}C]PAH or 0.1 m*M* [^{14}C]MDG (0.5 μCi/ml) was added to each well. After a 30-min incubation at room temperature, the radioactive uptake

solution was rapidly aspirated off, and the plate was dipped into 500 ml of ice-cold wash buffer consisting of BSS containing 1 m*M* PB and 100 μM PHZ. The plate was inverted and tapped onto absorbant paper and the wash procedure was repeated once. The plate was then frozen and thawed before the addition of 0.2 ml of 0.3 *M* NaOH to dissolve the cells. After incubation at 37°C for 1 hr, two 80-μl aliquots were removed from each well for determination of radioactivity and protein, respectively, as described above. Radioactivity was very low in these samples due to the low uptake rates and the small number of cells.

LLC-PK$_1$ cell cultures. The procedures used for LLC-PK$_1$ cell uptakes differed slightly from those for the rabbit cell cultures. Cells at Passage 192 were seeded at superconfluent density into either 60-mm plastic culture dishes or large filter cups (44 mm i.d.) with a Nucleopore filter bottom as described above. Where indicated, the filters were coated with 100 μg of basement membrane collagen. For uptake measurements, each filter cup was incubated in a 60-mm dish in BSS with acetate and containing 1 m*M* [^{3}H]OMG and 0.1 m*M* [^{14}C]PAH. For determining PB-sensitive uptake, 1 m*M* PB was added to the incubation medium of selected assays. Incubation was carried out for 1 hr at room temperature, and the filter cups and dishes were gently shaken during the uptake period on a rotary shaker (IKA-VIBRAX-VXR; Janke and Kunkel, Germany). After incubation, the filter cups were washed as described above in ice-cold BSS containing 1 m*M* PB and 100 μM phloretin. Radioactivity and PCA-insoluble protein were determined as before. For cultures grown directly on plastic in 60-mm culture dishes, the cell monolayers were scraped off the plastic with a rubber scraper following the wash procedure and transferred to centrifuge tubes for similar treatment as outlined for the filter cup assays. For multiwell plate uptakes, LLC-PK$_1$ cells were seeded at 2×10^5 cells/cm^2 and treated as described above for rabbit cell cultures grown in multiwell plates.

Marker Enzyme Assays. Rabbit cells at Passage 1 were seeded into 60-mm and 35-mm culture dishes at subconfluent and confluent densities, respectively. The culture medium was changed on Day 1 for subconfluent cultures and Days 1 and 4 for confluent cultures. Ten percent FCS was added to some of the cultures in both groups on the day before harvesting. Cell monolayers were washed with BSS and scraped from the surface of the culture dish with a rubber scraper. The cells were sonicated in 1.5 ml of ice-cold 0.01 *M* Tris-HCl at pH 7.4 using a Heat Systems Sonicator at setting 4, 50% cycle, for 1 min (model W-380; Heat Systems-Ultrasonics, Inc., Plainview, NY). The sonicates were clarified by centrifugation at 150*g* for 5 min, and the supernatants were stored at −20°C. Protein in the sonicates was assayed as total Lowry-positive material. Protein concentrations averaged 574 ± 41 μg/ml for subconfluent culture sonicates (n = 7) and 570 ± 52 μg/ml for confluent culture sonicates (n = 7). Alkaline phosphatase (AP) and γ-glutamyltranspeptidase (GT) were assayed as described previously (18). In brief, for the AP assay, 100 μl of homogenate were incubated at 37°C in a 1-ml reaction solution containing 1 m*M* *p*-nitrophenylphosphate, 2.5 m*M* $MgCl_2$, and 0.1 *M* glycine (pH 10.2). The reaction was stopped with 2 ml of 0.25 *M* NaOH and the absorbance of the solution was read at 420 nm in a Schmadzu spectrophotometer (model UV-265FS; Schmadzu Corp., Kyoto, Japan). For the GT assay, 100 μl of homogenate was incubated at 37°C in a 1-ml reaction solution containing 2.2 m*M* γ-glutamyl-*p*-nitroanilide, 20 m*M* glycylglycine, 0.1 *M* Tris-HCl (pH 8.2). The reaction was stopped with 2 ml of 10% acetic acid and the absorbance was read at 410 nm.

Statistical Analysis. All data are presented as the mean ± SE. Statistical significance of the data at the 95% confidence level was determined by the Student's t test or by analysis of variance using the Statview 512+ program (Brainpower Inc., Calabasas, CA) for the Apple Macintosh computer (Apple Computer, Inc., Cupertino, CA).

Materials. All chemicals used were of analytical grade. Culture media, drugs, and hormone supplements were obtained from Sigma Chemical Co. Hormones and drugs were stored frozen at −20°C in stock solutions for addition to culture media as required. Insulin was dissolved in water at pH 4 and hydrocortisone was dissolved in ethanol. *p*-[*glycyl*-1-^{14}C]-Aminohippuric acid at a sp act of 48 mCi/mmol was obtained from New England Nuclear (DuPont Co., Wilmington, DE). Methyl(α-D-[U-^{14}C]gluco)pyranoside at 279 mCi/mmol and 3-o-methyl-D-[1-^{3}H]glucose at 4.5 Ci/mmol were obtained from Amersham (Buckinghamshire, UK).

Results

Rabbit Proximal Tubule Cell PAH and MDG Uptakes. Confluent rabbit proximal tubule cell cultures grown on plastic surfaces formed domes (or hemicysts) after 1 day in culture. The number of domes was somewhat less than that seen in cultures of LLC-PK$_1$ cells. Although the rabbit cells were able to accumulate low levels of PAH, no significant PB-sensitive PAH uptake was found in the cultures grown on porous membranes coated with basement membrane collagen or in cells grown on plastic (Table I). Attempts to induce development of a PB-sensitive PAH transport system by inclusion of 5 m*M* PAH in the culture medium during cell growth were unsuccessful. Using an estimated value of 10.2 ± 0.6 μl of cell water per mg PCA-insoluble protein (n = 54 assays from three culture experiments), the cell water to medium concentration

Table I. PAH Uptake by Rabbit Proximal Tubule Cells

	Incubation time (min)	PAH uptake (pmol/mg prot·min)	
		Control	+Probenecid
Collagen-coated filters[a]			
Apical and basal	60	20.3 ± 3.3 (8)	18.9 ± 3.6 (8)
Apical only	15	14.3 ± 2.5 (9)	15.3 ± 3.8 (9)
Basal only	15	42.4 ± 4.8 (9)[b]	42.0 ± 4.6 (9)[b]
96-Well plates[c]			
Plastic	30	0.73 ± 0.06 (12)	0.96 ± 0.10 (12)
10 m*M* glutarate	30	0.88 ± 0.14 (12)	0.80 ± 0.09 (12)
Na-free	30	0.22 ± 0.05 (12)[d]	0.41 ± 0.09 (12)
Collagen-coated	30	1.31 ± 0.08 (12)[d]	1.66 ± 0.12 (12)

[a] For collagen-coated filters, rabbit cells were plated at confluence and maintained for 3 days in RK-1 medium. On the third day, 0.1 m*M* ^{14}C-PAH, with or without 1 m*M* probenecid, was added, and uptakes were carried out at room temperature. Data were collected from three culture preparations, each preparation beginning from frozen cell stocks at Passage 1. Uptake values were corrected for zero time uptakes, which were approximately 10–15% of the 1-hr uptakes. Protein values were corrected for noncellular Lowry-reactive material using control filters without cells.

[b] $P < 0.0005$ relative to apical.

[c] For 96-well plates, rabbit cells were seeded at confluent density in RK-1 medium. After 3 days in culture, 10% fetal calf serum was added to the culture medium to enhance cell attachment. After one more day in culture, 30-min uptakes of 0.1 m*M* ^{14}C-PAH, with or without 1 m*M* PB, were measured at room temperature as described in Materials and Methods. Values have not been corrected for zero time because zero time uptakes were not carried out for all experimental conditions. Controls, however, had zero time uptakes that were approximately 50% of total uptake. Protein values were corrected for noncellular Lowry-reactive material using appropriate control wells without cells. Values are presented as the mean ± SE, and the number of samples (n) is given in parentheses.

[d] $P < 0.0001$ relative to plastic.

ratio (C:M) for PAH in the cells grown on filters (60-min incubation) was not significantly different from 1 (1.3 ± 0.2, $n = 8$). Addition of [^{14}C]PAH to one side only of the monolayer during a 15-min incubation (Table I) gave a basal to apical uptake ratio of 3.9 ± 0.7 ($n = 9$, $P < 0.005$ relative to one); however, neither polarized uptake was PB sensitive. The 15-min basolateral uptake rate was greater than one quarter of the 60-min rate, suggesting that uptake was not linear with time, thus confirming the results of Yang *et al.* (7), who reported an initial rapid uptake over the first minute followed by an extended period of slower uptake. Transcellular fluxes ($n = 7$) were measured simultaneously for 0.1 m*M* [^{14}C]PAH and 1 m*M* [^{3}H]OMG after a 15-min incubation at room temperature. Fluxes in nmol/cm^2·min for PAH were 0.117 ± 0.032 (basal to apical [ba]) and 0.109 ± 0.048 (apical to basal [ab]). Fluxes for OMG were 4.43 ± 0.33 (ba) and 1.75 ± 0.43 (ab) nmol/cm^2·min. The respective flux ratios (ba:ab) were 1.1 ± 0.7 for PAH and 2.5 ± 0.8 for OMG ($P < 0.02$ for OMG relative to 1).

Due to differences in the culture and uptake conditions, the PAH uptake by cells grown on plastic was significantly less than that of cells grown on porous filters (Table I). C:M values could not be calculated because the cell water to protein ratio was not known. PB-insensitive PAH uptake was significantly inhibited by removal of sodium from the medium and was stimulated by coating the plastic surface of the wells with collagen. Addition of 10 m*M* sodium glutarate to the uptake medium had no effect on PAH uptake.

Using collagen-coated membranes, PHZ-sensitive, 1 m*M* MDG uptake (Table II) was found to be 134 ± 42 pmol/mg prot·min ($n = 7$; $P < 0.02$). The MDG C:M ratio for these cultures was significantly greater than 1 (1.48 ± 0.05, $n = 7$, $P < 0.001$). In the multiwell plate preparation, a PHZ-sensitive MDG uptake system was also found to be present, and PHZ-sensitive uptake of 0.1 m*M* MDG was calculated to be 3.7 ± 0.4 pmol/mg prot·min ($n = 12$).

LLC-PK$_1$ PAH Uptake. PAH uptakes by an established pig kidney cell line, LLC-PK$_1$, under different culture conditions are presented in Table III. The PAH uptake by cells grown on porous membranes without collagen was approximately half that found in the rabbit proximal tubule cell cultures described in Table I and showed a similar lack of sensitivity to PB. Longer postconfluent maintenance of the cells (10 days com-

Table II. MDG Uptake by Rabbit Proximal Tubule Cells[a]

	α-Methyl-D-glucoside uptake (pmol/mg prot·min)	
	Control	+Phlorizin
Collagen-coated filters	272 ± 37 (7)	137 ± 29 (7)[b]
96-Well plate	6.8 ± 0.4 (12)	3.1 ± 0.2 (10)[c]

[a] Culture and assay conditions are as given in Table I. The concentration of D-[α-*methyl*-^{14}C]glucoside in the uptake solution was 1 m*M* for collagen-coated membranes and 0.1 m*M* for the 96-well plate. Phlorizin was present at 100 μ*M* where indicated. Values are presented as the mean ± SE, and the number of samples (n) is given in parentheses.

[b] $P < 0.02$ relative to control without PHZ.

[c] $P < 0.0001$ relative to control without PHZ.

Table III. PAH Uptake by LLC-PK$_1$ Cells[a]

	PAH uptake (pmol/mg prot·min)	
	Control	+Probenecid
Membrane filters		
No collagen—3 days	11.3 (2)	11.1 (2)
No collagen—10 days	10.3 (2)	12.3 (2)
BM collagen—3 days	12.8 (2)	12.8 (2)
Plastic dishes		
Plastic—3 days	26.6 (2)	23.4 (2)
96-Well plate		
Plastic—4 days	4.2 ± 0.2 (8)	4.5 ± 0.4 (12)

[a] LLC-PK$_1$ cells were grown as described in Materials and Methods. All values for membrane filters and plastic dishes were from a single preparation, and uptakes were measured for 1 hr at room temperature. The number of filter cups or plastic dishes tested (n) is given in parentheses. Days refers to the number of days in confluent culture before uptake was assessed. Uptakes from a single preparation of a 96-well plate were carried out for 30 min at room temperature. The number of wells tested (n) is given in parentheses. Values are presented as the mean ± SE. For all three groups, ^{14}C-PAH was present at 0.1 m*M* and probenecid at 1 m*M*.

pared to 3 days) failed to increase the uptake in the presence or absence of PB, and the growth of cells on filters coated with basement membrane collagen or on nonporous plastic surfaces had no significant effect on PB-sensitive uptake. The mean C:M ratio for LLC-PK$_1$ cells grown on filters with collagen was only 0.38 (n = 2), based on a value of 6.6 μl of cell water/mg acid-insoluble protein (18). Cells grown in wells of a multi-well plate again showed markedly reduced uptakes relative to cells grown on filters or in 60-mm culture dishes.

Proximal Tubule Marker Enzyme Activities. AP and GT enzyme activities in subconfluent and confluent rabbit cell cultures are presented in Table IV. AP activity was consistently below the limit of detection of the assay in growing cells, but increased to measurable values after confluence. GT activity was increased 2.2-fold in confluent cultures relative to subconfluent cultures. Since it was necessary to add serum to the culture media in the 96-well plate during the final day in culture to prevent detachment of the rabbit cell monolayers during radioactive uptake measurements, cultures used for enzyme assay measurements were tested for the effect of 1 day's exposure to FCS (Table IV). No significant difference was found between control enzyme activities and activities in FCS-treated cultures.

Discussion

PAH secretion in the intact renal proximal tubule involves a secondarily active, PB-sensitive transport system at the basolateral border and a PB-sensitive, facilitated diffusion system at the apical border (21). In the rabbit, Martinez *et al.* (22) reported that the apical transport system was not an exchange system, but was driven by membrane potential. Recent *in vivo* studies by Ullrich and Rumrich (14) and studies in membrane vesicles by Pritchard (12) and Shimada *et al.* (13) have demonstrated a role for Na-coupled dicarboxylic acid uptake in the mechanism of PAH transport into the cell across the basolateral border. In the present study, addition of 10 m*M* glutarate to rabbit proximal tubule cell cultures during uptake measurements had no effect on the rate of PAH transport (Table I), causing neither a *cis*-inhibition as reported by Shimada *et al.* (13) using 10 m*M* glutarate in membrane vesicle studies, nor a *trans*-stimulation as a result of uptake of the glutarate into the cells during the 30-min incubation. Removal of Na, however, significantly reduced uptake in these cultures (Table I), confirming the Na dependence of PAH uptake. Further elucidation of the molecular mechanism of transport would be helped by the availability of an *in vitro* model, such as that offered by primary or established renal cell cultures. Unfortunately, one of the most commonly used proximal tubule-like cell lines, LLC-PK$_1$, although retaining a Na-glucose co-transport system (4) and an organic cation transport system (6, 23), has lost the PB-sensitive PAH transport system characteristic of intact tubule cells (5, 6). In the present study, it was confirmed that LLC-PK$_1$ lacks a PB-sensitive PAH transport system even

Table IV. Rabbit Cell Proximal Tubule Marker Enzyme Activities[a]

	Alkaline phosphatase (nmol/mg prot·min)	γ-Glutamyltranspeptidase (nmol/mg prot·min)
Subconfluent		
Without FCS	0 (5)	42.9 ± 7.3 (5)
With FCS	0 (2)	39.6 ± 4.9 (2)
Confluent		
Without FCS	1.12 ± 0.12 (5)	92.2 ± 7.6 (5)*
With FCS	1.76 ± 0.69 (2)	83.8 ± 5.7 (2)

[a] Rabbit cells at Passage 1 were cultured in plastic dishes as described in Materials and Methods. Fetal calf serum (10%) was added to some of the cultures the day before assay. Subconfluent cultures were harvested 2 days after seeding while still subconfluent, and confluent cultures were harvested 4 days after seeding at confluent density. The number of cultures tested (n) is given in parentheses. Values are presented as the mean ± SE.
[b] $P < 0.002$ relative to subconfluent.

when cells are grown on porous supports coated with basement membrane collagen, conditions that favor differentiation in other culture systems (8–11).

Using a similar rabbit primary cell culture system as in the present study, Yang *et al.* (7) reported that PB-sensitive PAH uptake was present in basolateral membrane vesicles isolated from these cultures. These workers also reported PB-sensitive uptake in intact cultured cells; however, little experimental detail was given and nonspecific uptake or binding was greater than 50% of total uptake. In their study, PB-sensitive uptake was rapid over the first minute, then slowed, but continued to increase for at least 60 min. In addition, the PAH concentration inside the cell was well below the medium concentration. *In vivo* PAH uptake is generally concentrative, giving a C:M ratio greater than 1. Rabbit proximal tubule primary cultures have been shown to retain other transport systems, including apical Na-glucose co-transport (1, 2) and apical Na-phosphate co-transport (24). In the present study, using the same culture system as Yang *et al.* (7) but growing the cells on porous membranes coated with basement membrane collagen as well as on plastic, the findings of Yang *et al.* (7) could not be confirmed (Table I), even though culture conditions were optimized for differentiation by promoting access of the medium to the basolateral side of cells and by attachment of cells to collagen surfaces (8–11). In the study by Yang *et al.* (7), total PAH uptake into intact cells in the absence of PB was 15 pmol/mg prot·min, a value very similar to that found here in cultures grown on membrane filters (20 pmol/mg prot·min). Extrapolating to 1 hr and assuming uptake to be linear over this time, a similar C:M ratio of 1.3 may exist in their study as well. Although this value is greater than unity and is four times higher than that found in LLC-PK_1 cells, it may not represent active uptake, since PAH may bind to intracellular components after passive entry into the cell. In membrane vesicle preparations from both rabbit cells (7) and rat renal cortical cells (12), high nonspecific binding of PAH was reported. Shimada *et al.* (13) reduced the nonspecific binding to the filters used for collection of membrane vesicles by pretreatment of the filters with PB and albumin or by passage of the incubation medium through high pressure liquid chromatography membrane filters. Nonspecific binding may obscure low levels of PB-sensitive transport in cultures. In the present study, although nonspecific PAH binding to collagen-coated filters was only about 10% of total uptake, it was closer to 50% in the multiwell plate studies (data not presented). The PAH transport system in primary cultures of rabbit cells appears to be modified from that found in the intact tubule. Whereas a Na-dependence is maintained as well as polarity (basal uptake greater than apical), the PB sensitivity and interaction with dicarboxylic acid appears to be absent. These results are difficult to explain if the Na dependence derives from a link between PAH uptake and dicarboxylic acid efflux (12–14). It seems unlikely that the loss of PAH uptake in Na-free medium is due to cell death, since the cells remain attached to the plastic surface. The possibility also exists that the particular rabbit proximal tubule segments from which the cultures in this study originated may have come predominantly from a segment of the tubule having a low or altered PAH uptake capacity. Shimomura *et al.* (25) have reported that segments S_1 and S_3 of rabbit proximal tubules have only 15–20% of the capacity of PAH uptake of segment S_2.

Various different lines of evidence suggest that the rabbit cell primary cultures in the present study were functioning as expected. For example, domes were present when the cells were grown on plastic surfaces, indicating retention of the capacity for transepithelial salt and water transport. In addition, the fact that PHZ-sensitive MDG uptake was present in these cultures (Table II) provides direct evidence that at least one proximal tubule-specific differentiated transport function was maintained in culture. Although the actual PHZ-sensitive uptake rate for MDG of 15 pmol/mg prot·min found in this study was similar to that of 140 pmol/mg prot·min originally reported by Chung *et al.* (1) and Sakhrani *et al.* (2), the C:M concentration ratio for MDG was considerably lower than that reported by Chung *et al.*, who calculated a value of 11 after 1 hr of incubation. There is a major discrepancy, however, in Chung's study, which used an unusually low cell water to protein ratio of only 0.75 μl/mg of protein to calculate the cell water concentration of isotope. This low value compares to 10 μl/mg measured by similar techniques in rabbit proximal tubule cells in both the present study and in the study by Yang *et al.* (7). The C:M ratio of 2.8 for MDG reported by Yang *et al.* (7) was, however, still twice that found in the present study.

Another line of evidence that the cultures used were viable and expressing normal differentiated function was the appearance of proximal tubule enzyme activities at confluence (Table IV). Alkaline phosphatase and γ-glutamyltranspeptidase activities are present in confluent cultures of rabbit proximal tubule cells (1) as well as in LLC-PK_1 cells (18, 26). Although changes in activity with cell density have not been measured previously in rabbit primary cell cultures, in LLC-PK_1 cells, the activities of these proximal tubule marker enzymes are very low in growing cells and increase dramatically at confluence (18, 26). In the present study with rabbit cell cultures (Table IV), it is evident that both AP and GT activities increase markedly after confluence at a time when other differentiated functions appear, such as domes or Na-glucose co-transport. The low enzyme activities reported in rabbit cells by Chung *et al.* (1) relative to the present study are presum-

ably due to their being measured in intact monolayers in Chung's study rather than in sonicates of cells as in the present study. The fact that adding serum to the cultures for the last day before enzyme assay had no significant effect on the enzyme activities (Table IV) suggests that a brief serum treatment does not adversely affect differentiated function in confluent cultures of these cells. For this reason, it was assumed that the PAH uptake experiments reported in Table I were not compromised by 1 day of serum exposure needed to enhance cell attachment in multi-well plates.

It can be concluded from the results of this study and others that whereas numerous differentiated functions, such as Na-glucose co-transport, dome formation, and marker enzyme activities, are qualitatively retained by rabbit proximal tubule cells in culture, the PAH transport system may be lost or modified. These changes to a major renal transport system in culture suggest that caution should be followed in using cell culture systems to investigate detailed transport mechanisms. Although attempts have been made to measure PAH transport in cultured cells and to develop a culture system for investigation of PAH transport mechanisms (5–7), little success has been achieved. Intact tubules (14) and membrane vesicle preparations from intact tubules (12, 13) remain the best systems for studying organic acid transport.

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