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## ABSENCE OF A TRANSCELLULAR OXALATE TRANSPORT MECHANISM IN LLC-PK<sub>1</sub> AND MDCK CELLS CULTURED ON POROUS SUPPORTS

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### Abstract

Transepithelial oxalate transport across polarized monolayers of LLC-PK<sub>1</sub> cells, grown on collagen-coated microporous membranes in Transwell culture chambers, was studied in double-label experiments using [<sup>14</sup>C]-oxalate together with [<sup>3</sup>H]-D-mannitol as an extracellular marker. The [<sup>14</sup>C]-labeled glucose analog  $\alpha$ -methyl-glucoside ( $\alpha$ -MG) was used as functional marker for active proximal tubular sugar transport. Cellular uptake of oxalate and  $\alpha$ -MG at both the apical and basolateral plasma membrane was determined. When added to the upper compartment,  $\alpha$ -MG was actively taken up at the apical membrane, directed through the cells to the basolateral membrane and transported to the lower compartment, indicating functional epithelial sugar transport by LLC-PK<sub>1</sub> cells. In LLC-PK<sub>1</sub> cells, the uptake of  $\alpha$ -MG at the apical membrane was approximately 50 times higher than that at the basolateral membrane. In contrast to this active transport of sugar, LLC-PK<sub>1</sub> cells did not demonstrate oxalate uptake either at the apical or basolateral plasma membrane. The apical-to-basolateral (A->B) flux of oxalate in LLC-PK<sub>1</sub> cells was identical to the basolateral-to-apical (B->A) oxalate flux in these cells. Moreover these flux characteristics were similar to those found for D-mannitol, indicating paracellular movement for both compounds. From these data, it is concluded that, under the experimental conditions used, LLC-PK<sub>1</sub> cells do not exhibit a specific transcellular transport system for oxalate.

**Key Words:** Calcium oxalate stone disease, epithelial transport, renal tubular cell lines, LLC-PK<sub>1</sub>, MDCK, Transwells, oxalate,  $\alpha$ -methyl-glucoside, D-mannitol.

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### Introduction

Hyperoxaluria is considered a major risk factor in calcium oxalate (CaOx) urolithiasis [1, 2, 11, 20, 21, 30]. Amongst idiopathic CaOx stone formers, mild hyperoxaluria is frequently found. It has been postulated that cellular transport defects, located in the epithelium of the gastrointestinal tract and/or in the epithelium of the renal proximal tubule, could lead to increased urinary oxalate concentrations as a result of hyperabsorption from the normal diet and/or enhanced tubular secretion [1, 2, 10, 11, 30]. Oxalate is an apparently useless end-product of metabolism. Excretion in the urine seems to be the sole route for its elimination [10, 30]. In man [16] as well as in several animal species [23, 30], it was demonstrated that tubular secretion is an important pathway in oxalate excretion. Clearance studies in rats [28] and rabbits [24] indicated that tubular secretion of oxalate takes place in the proximal convoluted tubule but not further down the nephron.

The objective of this paper is to present a tissue culture model system for studying renal tubular oxalate transport and its possible regulation. The system employs a two-compartment culture chamber in which established renal tubular cell lines are grown on polycarbonate collagen-coated permeable supports. Under such conditions, the cells form differentiated polarized monolayers with apical microvilli, apical tight junctions, desmosomes, and basolateral infoldings and retain the morphological and functional features of transporting renal epithelia [6, 8, 9]. Although the same renal epithelial cell lines have been widely used during the last decade to study epithelial transport systems [5], oxalate transport across monolayers cultured on porous supports, has not been published before. The functional integrity of LLC-PK<sub>1</sub> monolayers was assessed in parallel by studying vectorial transport of  $\alpha$ -MG, a non-metabolizable probe for the proximal tubular apical sodium-glucose co-transport carrier [12, 14, 15].

## Materials and Methods

### Cell lines

Two established renal tubular cell lines, LLC-PK<sub>1</sub> (CRL 1392) and MDCK (strain II), obtained from the American Type Culture Collection (Rockville, MD, USA), were used in this study, at passage numbers 201-230 and 20-30, respectively. The porcine kidney cell line LLC-PK<sub>1</sub> [7], was used as a model for renal proximal tubular epithelium [5, 8, 9], whereas the Madin-Darby Canine Kidney cell line MDCK [4] is assumed to originate from the cortical collecting duct of the nephron [5, 9]. Both cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (SANBIO, Uden, The Netherlands), 20 mM Hepes buffer (GIBCO-BRL, UK), 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were grown in plastic tissue culture flasks at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Subculturing was performed weekly by use of 0.05% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA; GIBCO-BRL, UK).

### Transmission electron microscopy

For morphological inspection at the ultrastructural level, cells were fixed in 1.5% (v/v) glutaraldehyde in 0.1 M cacodylate/HCl buffer for 1 hour at 37°C. After aldehyde fixation, cells were postfixed in 1% (w/v) OsO<sub>4</sub> in the same buffer system, to which K<sub>4</sub>Fe(CN)<sub>6</sub>·3H<sub>2</sub>O was added to a final concentration of 0.05 M, for 1 hour at room temperature. Subsequently the cells were routinely dehydrated and embedded in Epon. Ultrathin sections (30-50 nm) were sectioned with diamond knives collected on unfilmed 400 mesh copper grids and inspected in a Zeiss EM 902 (Zeiss, Oberkochen, FRG) transmission electron microscope operated at 80 kV.

### Growth on porous filter supports

Transwell cell culture chambers (Costar Transwell-COL, Badhoevedorp, The Netherlands, diameter 24.5 mm, 0.45 µm pore size), containing a collagen-coated transparent microporous membrane were used to obtain monolayers of polarized cells. When placed in a six-well cluster plate, such monolayers separate the culture chamber into two individually accessible compartments, which makes it possible to study directed transcellular solute fluxes and uptake via the apical or basolateral plasma membrane. Cells were seeded at subconfluent density (1-2 × 10<sup>6</sup> cells per insert of 4.71 cm<sup>2</sup>), rinsed with phosphate-buffered saline (PBS) the following day, and re-fed with fresh culture medium twice a week. Transport and uptake studies were usually performed 9 days after cell seeding.

### Transport and uptake studies

Transepithelial fluxes and cellular uptake of [<sup>14</sup>C]-oxalate or α-[<sup>14</sup>C]-methyl-glucoside were measured using Transwell cell culture chambers. Such measurements were carried out as dual-label experi-

ments in combination with D-[<sup>3</sup>H]-mannitol. As this compound is not transported transcellularly, it is generally used as a marker of paracellular leakage in transport flux studies, and a marker for extracellular trapping of radioactive tracers in uptake studies. Prior to use, monolayers were rinsed three times and preincubated for 15 minutes with MOPS-buffer (pH 7.3), containing 119 mM NaCl, 5.3 mM KCl, 6.6 mM Na acetate·3H<sub>2</sub>O, 0.25 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 1.3 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 2.0 mM KH<sub>2</sub>PO<sub>4</sub>, 6.6 mM NaHCO<sub>3</sub> and 9.2 mM Na-morpholinopropane sulfonic acid (MOPS). Incubation buffer volumes were 2600 µl in the lower compartment (facing the basolateral cell membrane) and 1500 µl in the upper compartment (facing the apical cell membrane), as recommended by the supplier in order to avoid differences in hydrostatic pressure. To study transepithelial transport, the following combinations of radiolabelled compounds (final concentration: 50 µM in MOPS-buffer each) were added to either the upper or the lower compartment of Transwell culture chambers: [<sup>14</sup>C]-oxalate (3.7 KBq/ml) plus D-[<sup>3</sup>H]-mannitol (14.8 KBq/ml), or α-[<sup>14</sup>C]-methyl-glucoside (3.7 KBq/ml) plus D-[<sup>3</sup>H]-mannitol (14.8 KBq/ml). Tracer-free MOPS-buffer was supplemented to the opposite compartment. Appearance and disappearance of radioactive tracers in both compartments was determined by counting radioactivity in 50 µl aliquots from both compartments in a β-scintillation counter (Packard) 0, 1, 2, 3 and 4 hours after the start of incubation. Fifty µl aliquots of tracer-free MOPS-buffer were added to keep the hydrostatic pressure unaffected. Prior to performing cellular uptake studies, monolayers were rinsed three times and preincubated for 15 minutes with MOPS-buffer at 37°C. To start the uptake, the buffer was aspirated from either the lower or the upper compartment and replaced by MOPS-buffer containing radioactive tracers. To discontinue uptake at 0, 15, 30 and 60 minutes, the filter insert with monolayer was rapidly (a few seconds) dipped in three successive ice-cold PBS baths. The porous support was cut out from the inserts by a scalpel and transferred to an Eppendorf tube containing 500 µl 1 M perchloric acid (PCA) to extract the radioactive compounds. After two cycles of freezing and thawing, the tubes were centrifuged for 2 minutes at maximum speed in an Eppendorf centrifuge. In this way, more than 99% of the radioactivity was extracted from the pellet. From the supernatant 400 µl was used to determine <sup>3</sup>H- and <sup>14</sup>C-dpms by scintillation counting. The pellet was washed twice with 1 ml 1 M PCA and the dry pellet dissolved in 100 µl 1 N NaOH to determine the amount of protein per filter. Protein concentrations were measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA, USA).

### Calculation of transepithelial fluxes and cellular uptake

Clearance of solutes from the apical (upper) to the basolateral (lower) compartment (A->B) was

calculated by:

$$C_{A \rightarrow B} (\mu l) = V_L \cdot \frac{L}{U} \quad (1)$$

where  $V_L$  is the volume of the lower compartment (2600  $\mu l$ ).  $L$  and  $U$  are amounts of radioactivity (dpm/ $\mu l$ ) detected after 0, 1, 2, 3 and 4 hours in the lower and upper compartment respectively. Similarly the B- $\rightarrow$ A clearance was calculated by:

$$C_{B \rightarrow A} (\mu l) = V_U \cdot \frac{U}{L} \quad (2)$$

where  $V_U$  is the volume in upper compartment (1500  $\mu l$ ).

Assuming that the area of the monolayer is equal to the area of the filter, solute transport fluxes (in pmol/ $cm^2 \cdot min.$ ) could be calculated from equation (1) and (2), using 4.71  $cm^2$  filter inserts and solute concentrations of 50  $\mu M$ , according to the formula:

$$Flux = \frac{C (\mu l) \cdot 50 (pmol / \mu l)}{t (minute) \cdot 4.71 (cm^2)} \quad (3)$$

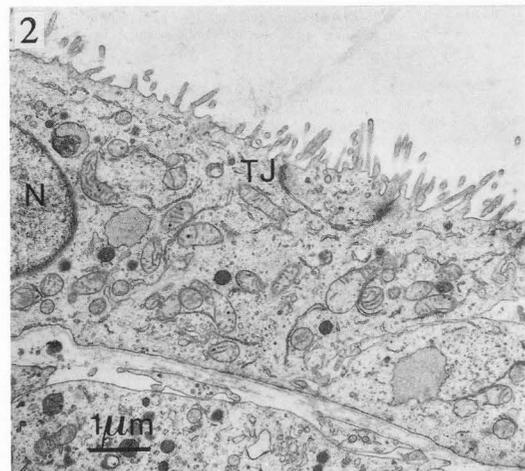
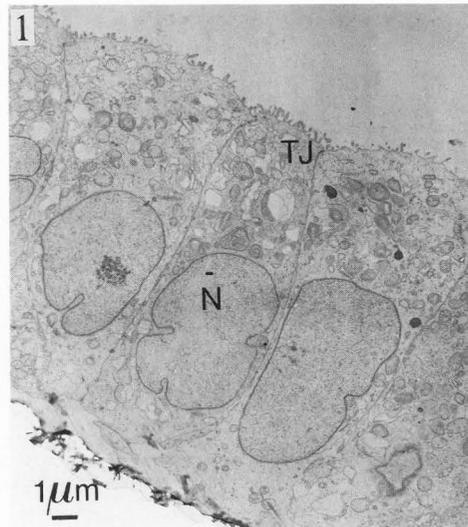
Solute uptake (in fmol/ $\mu g$  protein. $min.$ ) by the monolayers, after 0, 15, 30 and 60 minutes incubation, was calculated from the measured radioactivity (dpm per filter), the specific activity (dpm/fmol) and the amount of protein ( $\mu g$ ) per filter, using the formula:

$$Uptake = \frac{dpm}{(dpm / fmol) \cdot (\mu g \text{ protein}) \cdot t (minute)} \quad (4)$$

## Results

### Cell morphological studies

In some experiments, filter membranes were selected for cellular ultrastructural inspection. In Figure 1, a cross-section is shown of MDCK cells, eight days after confluency, as a neat row of unicellular columnar epithelial cells. At the apex, short villi were present. In between the cells, prominent tight junctions and rather small intercellular clefts and some wider clefts at the basal portion of the cells towards the membrane material were visible. Inside the cell, cytoplasm was well preserved with numerous lysosomal structures. In Figure 2, a similar cross-section is shown from the LLC-PK<sub>1</sub> cells eight days after confluency. The cells lost their strict unicellular aspect and formed a pseudo-stratified epithelial layer, with apical microvilli which are much longer than those present at the MDCK cell apex. Also in this case, patent tight junctions were observed, though the intercellular clefts were much wider. The cytoplasm was well preserved. Basement membrane material was seen outside the basal cell membrane.

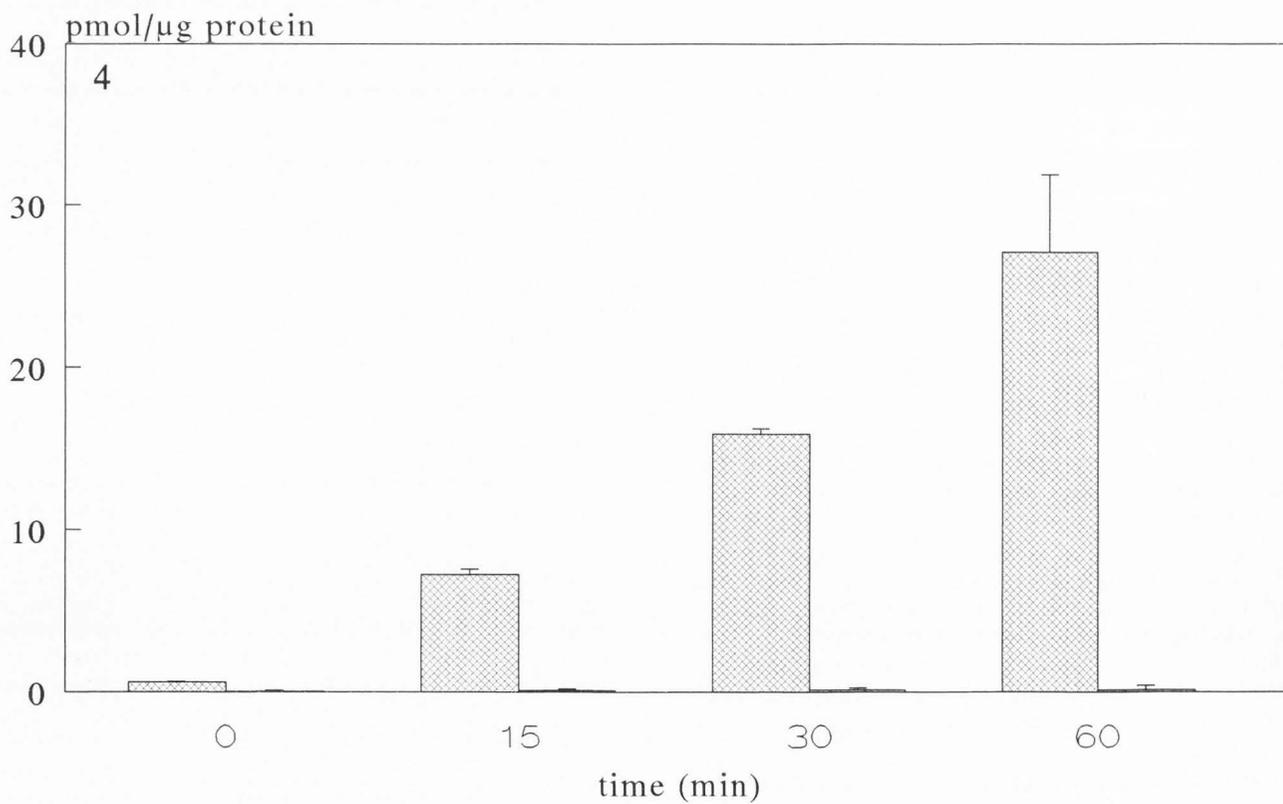
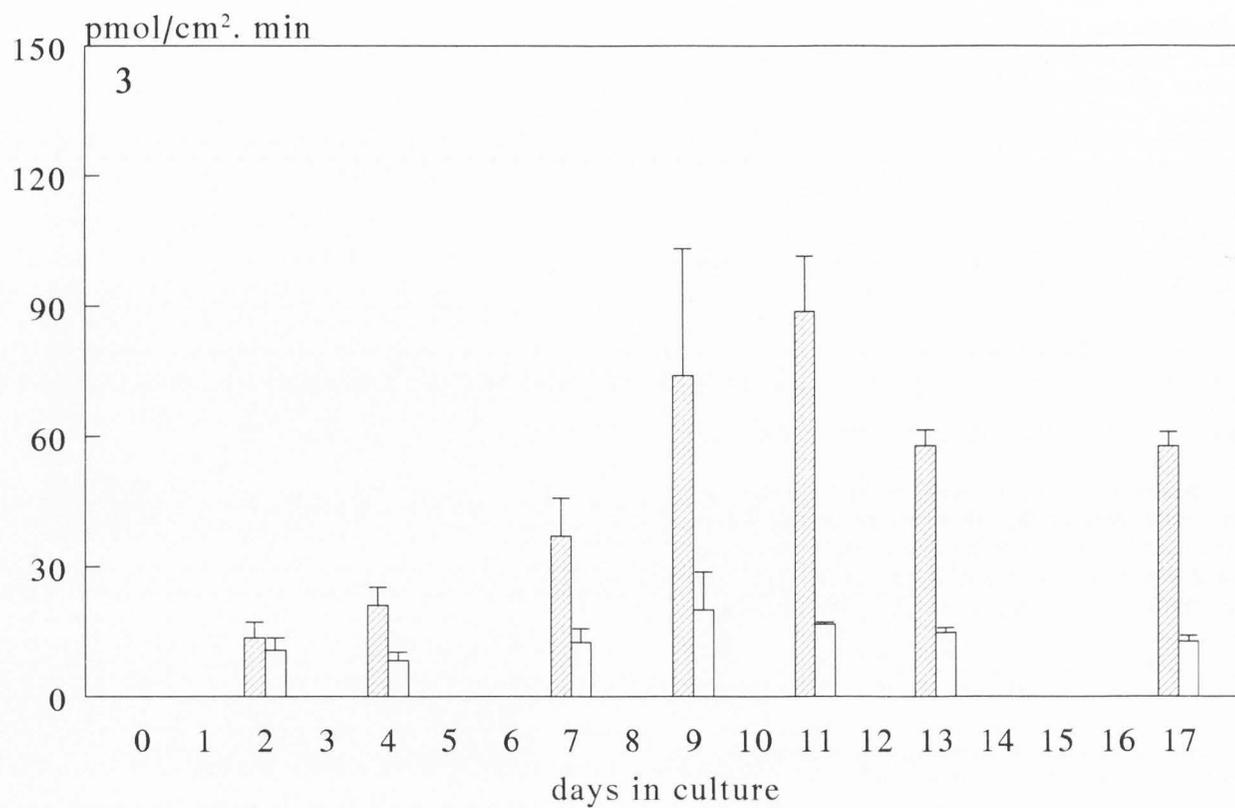


**Figure 1.** MDCK cells cultured on porous supports, showing columnar arrangement (N = nucleus, TJ = tight junction).

**Figure 2.** LLC-PK<sub>1</sub> cells cultured on porous supports (N = nucleus, TJ = tight junction).

### Effect of culture time on transport characteristics of LLC-PK<sub>1</sub> monolayers

Mannitol fluxes, measured daily in four hour flux experiments, reached a stable level within two days after seeding the cells at subconfluent density ( $1-2 \times 10^6$  cells/filter). In contrast, the A- $\rightarrow$ B transport of  $\alpha$ -MG increased with days in culture, attaining a maximal value after 9-11 days (Figure 3). Apparently this time in culture is needed by these monolayers to fully develop their transport capacity. Based on this observation, all further transport and uptake studies were performed 9-11 days after seeding the cells at subconfluent density. The integrity of the monolayer was monitored morphologically by phase-contrast light



Oxalate transport studies in renal tubular cell lines

**Figure 3 (at left, top).** A → B transcellular  $\alpha$ -methyl-glucoside transport capacity across LLC-PK<sub>1</sub> monolayers in time. The cells were plated on microporous membranes at subconfluent densities on day "0". Solute flux experiments were performed using  $\alpha$ -[<sup>14</sup>C]-methyl-glucoside (hatched bars) and D-[<sup>3</sup>H]-mannitol (open bars), added to the apical compartment. After each transport experiment, the monolayers were rinsed extensively with PBS to remove radiolabeled tracers, re-fed with fresh DMEM, and the experiments were repeated with the same monolayers every two or three days during a time period of 17 days. Bars represent means  $\pm$  standard deviation (S.D.) from three individual cell culture chambers.

**Table 1.** Transport fluxes of  $\alpha$ -methyl-glucoside and mannitol in pmol/cm<sup>2</sup>.min. across 9 days cultured polarized monolayers of LLC-PK<sub>1</sub> and MDCK cells. Each compound added at a final concentration of 50  $\mu$ M. Mean  $\pm$  standard deviation (S.D.) from three independent measurements.

Transport Flux (pmol/cm <sup>2</sup> .min.)	LLC-PK <sub>1</sub>		MDCK	
	$\alpha$ -MG	mannitol	$\alpha$ -MG	mannitol
apical-to-basolateral	38.6 $\pm$ 5.1	9.6 $\pm$ 3.4	1.2 $\pm$ 0.1	1.6 $\pm$ 0.1
basolateral-to-apical	5.5 $\pm$ 1.0	9.4 $\pm$ 2.5	1.5 $\pm$ 0.1	1.7 $\pm$ 0.2

**Table 2.** Transport fluxes of oxalate and mannitol in pmol/cm<sup>2</sup>.min. across 9 days cultured polarized monolayers of LLC-PK<sub>1</sub> and MDCK cells. Each compound added at a final concentration of 50  $\mu$ M. Mean  $\pm$  S.D. from three independent measurements.

Transport Flux (pmol/cm <sup>2</sup> .min.)	LLC-PK <sub>1</sub>		MDCK	
	oxalate	mannitol	oxalate	mannitol
apical-to-basolateral	8.3 $\pm$ 2.3	8.0 $\pm$ 2.2	2.1 $\pm$ 0.5	1.7 $\pm$ 0.5
basolateral-to-apical	12.1 $\pm$ 2.8	10.2 $\pm$ 2.3	1.9 $\pm$ 0.2	1.2 $\pm$ 0.1

**Table 3.** Uptake of  $\alpha$ -methyl-glucoside compared to the extracellular trapping of mannitol in fmol/ $\mu$ g protein.min. in 9 days cultured polarized monolayers of LLC-PK<sub>1</sub> and MDCK cells. Each compound added at a final concentration of 50  $\mu$ M. Mean  $\pm$  S.D. of three independent measurements.

Uptake (fmol/ $\mu$ g protein.min.)	LLC-PK <sub>1</sub>		MDCK	
	$\alpha$ -MG	mannitol	$\alpha$ -MG	mannitol
apical	452 $\pm$ 80	2.1 $\pm$ 0.50	0.93 $\pm$ 0.03	0.63 $\pm$ 0.12
basolateral	9.3 $\pm$ 0.90	1.3 $\pm$ 0.10	0.48 $\pm$ 0.05	1.15 $\pm$ 0.10

**Table 4.** Uptake of oxalate compared to the extracellular trapping of mannitol in fmol/ $\mu$ g protein.min. in 9 days cultured polarized monolayers of LLC-PK<sub>1</sub> and MDCK cells. Each compound added at a final concentration of 50  $\mu$ M. Mean  $\pm$  S.D. of three independent measurements.

Uptake (fmol/ $\mu$ g protein.min.)	LLC-PK <sub>1</sub>		MDCK	
	oxalate	mannitol	oxalate	mannitol
apical	1.01 $\pm$ 0.07	0.87 $\pm$ 0.12	1.21 $\pm$ 0.18	0.69 $\pm$ 0.13
basolateral	1.31 $\pm$ 0.30	1.75 $\pm$ 0.13	1.18 $\pm$ 0.12	1.57 $\pm$ 0.41

**Figure 4 (at left, bottom).** Apical uptake of  $\alpha$ -[<sup>14</sup>C]-methyl-glucoside (hatched bars) and D-[<sup>3</sup>H]-mannitol in LLC-PK<sub>1</sub> cells grown as monolayers on microporous membranes, after 9 days in culture. Each time point represents the mean  $\pm$  S.D. of three independent determinations in pmol/ $\mu$ g protein.

microscopy and functionally by determining the D- $[^3\text{H}]$ -mannitol flux.

#### Transport and uptake of $\alpha$ -methyl-glucoside

Monolayers of LLC-PK<sub>1</sub> cells exhibited vectorial transport for  $\alpha$ -MG: the mean A->B  $\alpha$ -MG flux (38.6 pmol/cm<sup>2</sup>.min), as measured in the four-hour flux experiments, was 7 times higher (Table 1) than the mean B->A flux (5.5 pmol/cm<sup>2</sup>.min.). In contrast, MDCK cells did not demonstrate vectorial  $\alpha$ -MG transport in either direction (Table 1). Similar results were obtained with respect to intracellular uptake of  $\alpha$ -MG. The uptake of  $\alpha$ -MG at the apical membrane proceeded linearly with time for at least 60 minutes (Figure 4). Although the uptake at the basolateral membrane also increased with time, the level of uptake (9.3 fmol/ $\mu$ g protein.min.) was approximately 50 times lower than the uptake at the apical membrane (452 fmol/ $\mu$ g protein.min.) and may represent B->A leakage followed by apical uptake (Table 3). Fluxes of mannitol across LLC-PK<sub>1</sub> monolayers, presumed to be paracellular, were low and similar in both directions (Table 1). The apparent uptake of mannitol was very low, non-directional (Tables 3 and 4) and independent of time (Figure 4) reflecting non-specific trapping of tracer. These results show that LLC-PK<sub>1</sub> cells, under the experimental conditions used, retained the capacity for directed  $\alpha$ -MG transport. In contrast, experiments performed with MDCK cells demonstrated that this cell line did not possess a transport system for  $\alpha$ -MG (Tables 1 and 3).

#### Transport and uptake of oxalate

Particle size measurements in a Coulter Multi-sizer II (Coulter Electronic Ltd, Luton, UK) demonstrated that using 50  $\mu$ M oxalate, which was the final oxalate concentration during the transport experiments, the solubility product of calcium oxalate was not exceeded in the presence of 0.25 mM CaCl<sub>2</sub>, a constituent of the incubation buffer. This implies that ionized oxalate was present under such conditions. Transport fluxes of oxalate across monolayers of LLC-PK<sub>1</sub> cells in either direction (A->B: 8.3 and B->A: 12.1 pmol/cm<sup>2</sup>.min), were not significantly different from transport fluxes of mannitol (A->B: 8.0 and B->A: 10.2 pmol/cm<sup>2</sup>.min) within the same experiment (Table 2). Flux measurements using filters without cells showed that radioactively labelled compounds were evenly distributed over both compartments after about four hours following addition to one of the compartments. When filters were covered with LLC-PK<sub>1</sub> monolayers, equal distribution of oxalate and mannitol was achieved after approximately 20 hours. No accumulation of oxalate in any of the compartments was observed upon longer incubation, in contrast to  $\alpha$ -MG that was transported against its concentration gradient. Hence, the equilibration of oxalate over the two compartments was retarded by the cellular barrier compared to the equilibration rate observed through filters without cells, but reflected passive diffusion. More-

over, the uptake of oxalate by LLC-PK<sub>1</sub> cells from either compartment (apical = 1.01 and basolateral = 1.31 fmol/ $\mu$ g protein.min.), was in the same low range as values found for the extracellular marker D-mannitol (apical = 0.87 and basolateral = 1.75 fmol/ $\mu$ g protein.min., Table 4). Experiments performed with higher concentrations of oxalate (up to 500  $\mu$ M) together with lower concentrations of calcium (down to 100  $\mu$ M) in order not to exceed the solubility product, did not result in significant oxalate uptake by these cells either. Uptake experiments performed with MDCK cells resulted in uptake patterns for oxalate and mannitol that were similar to those found for LLC-PK<sub>1</sub> cells (Table 3 and 4). Although the level of paracellular leakage among both cell lines differed, the extracellular trapping of mannitol, as determined in the uptake experiments, was of the same order of magnitude in both cell lines.

#### Discussion

The present data demonstrate that LLC-PK<sub>1</sub> monolayers were unable to take up oxalate from either the apical or the basolateral membrane and, as a consequence, to mediate active transcellular oxalate transport. In contrast, similar monolayers developed active sugar uptake at the apical plasma membrane within the same time period. Since A->B directed sugar transport (reabsorption) is a well documented characteristic of renal proximal tubular cells [12, 15], this result indicates the functional integrity of LLC-PK<sub>1</sub> monolayers under the conditions used. In accordance with their renal cortical collecting duct origin, monolayers of MDCK cells did not show to possess transcellular  $\alpha$ -MG or oxalate transport capacity in either direction.

The absence of oxalate transport in the LLC-PK<sub>1</sub> cell line is in contrast to the findings of Wandzilak *et al.* [27], who recently reported uptake of oxalate by this cell line. These authors claim that oxalate uptake was dependent on the buffers used. Uptake of oxalate was found to occur in Na-gluconate or mannitol buffers to a much greater extent than in PBS or Earl's salt solution. We repeated some uptake experiments with Na-gluconate buffer and found that the presence of this buffer resulted in disruption of tight junctions and subsequent loss of epithelial polarity. The amount of radioactive tracers in the monolayer was slightly increased in time not only for oxalate but also for mannitol, indicating non-specific uptake in this buffer. Na-gluconate buffer is often used to study isolated cell membranes, which in contrast to viable cells [3, 9, 12-15, 17-19] or tissues [22, 24, 28], do not require physiological conditions. In addition, Sigmon *et al.* [25] demonstrated uptake of oxalate in suspensions of rat renal cortical and papillary cells, using a buffer (Krebs-Ringer bicarbonate) very similar to our buffer, indicating that cellular oxalate uptake is not necessarily prevented in the presence of this buffer.

According to our experiments, the absence of a transcellular oxalate transport system at the basolateral plasma membrane of LLC-PK<sub>1</sub> cells can be explained in several ways. Firstly, dedifferentiation processes as a result of genetic drift during culture of immortal cell lines may have led to loss of certain cellular properties [9], including the ability to transport specific compounds. Despite the fact that the LLC-PK<sub>1</sub> cell line exhibits various transport systems, it appears to lack others [12, 19]. Another possibility is that oxalic acid is transported by the paracellular shunt pathway. This permeation pathway, controlled by a number of determinants such as pH, the passage of current, osmotic loads, calcium concentration, cytoskeletal function [23] and surface charge [29], selectively regulates the passive diffusion of ions and small water-soluble solutes. In so-called "leaky" epithelia, like the epithelium of the renal proximal tubule, passive movement of ions is predominantly via the paracellular route [23]. It is conceivable that alterations in determinants that control the paracellular flux could result in a renal leak for oxalate and consequently lead to hyperoxaluria. Interestingly, LLC-PK<sub>1</sub> monolayers showed a six times higher mannitol flux than MDCK monolayers (Table 1 and 2). This observation is in agreement with the more leaky, low-resistance paracellular pathway in proximal tubular epithelium (LLC-PK<sub>1</sub>) compared to tighter epithelium of distal parts of the nephron (MDCK).

Finally, the possibility exists that the cell lines used in this investigation are the descendants of tubular cells devoid of any specific oxalate transport system. Oxalate secretion is most probably restricted to a specific portion of the renal proximal tubule. It is difficult to pinpoint the exact original location of established renal cell lines and assignment of their origin is principally based on the observed properties. LLC-PK<sub>1</sub> cells exhibit apical sodium-dependent transport systems for sugars, amino acids and phosphate, and basolateral sodium-dependent neutral amino acid and inorganic phosphate transport systems [5] as well as a sodium-independent 3-O-methylglucose transport mechanism [13]. Such transport systems are indicative of renal proximal tubular epithelium. More specifically LLC-PK<sub>1</sub> cells demonstrate properties, like anion-selective occluding junctions [3, 17, 18] and the bioenergetics of Na<sup>+</sup>-coupled glucose transport [5], that correspond to those observed in the pars recta (S3-segment) of the proximal tubule [26]. Using intratubular microinjection, free-flow micropuncture, and droplet precession techniques, Weinman *et al.* [28] demonstrated that oxalate secretion is located predominantly in the early portions (S1, S2-segments) of the proximal convoluted tubule of the rat. This location of oxalate secretion was confirmed by clearance and microperfusion studies in isolated segments of renal proximal tubule of the rabbit [24]. No significant oxalate transport seemed to occur in segments of the nephron beyond the proximal convoluted tubule in these species.

Micropuncture studies of tubular transport in the pig kidney demonstrated that para-amino hippurate (PAH), the more traditional marker for renal proximal tubular organic acid transport, is secreted in the S2-segment of the proximal convoluted tubule and not in the pars recta [22]. Pig kidney derived LLC-PK<sub>1</sub> cells lack the probenecid sensitive Na<sup>+</sup>-dependent PAH transport system, suggesting that LLC-PK<sub>1</sub> originates from the pars recta, a segment that is possibly not involved in oxalate secretion.

In conclusion, the results of this study demonstrate that a specific oxalate transport system is missing in LLC-PK<sub>1</sub> cells. Unless it appears that oxalate transport is predominantly regulated by the paracellular shunt pathway, the characterization of renal oxalate handling will require other sources of renal proximal tubular cells, capable of transcellular oxalate transport. Irrespective of species (rats, rabbits and possibly pigs), the early proximal convoluted tubular part of the nephron (S1, S2-segments) seems to be the most promising region to find epithelial cells that are involved in the secretion of oxalate. Therefore, we will continue our efforts to determine and characterize renal oxalate secretory transport mechanisms by focusing our attention on the development of techniques to isolate and establish renal cell types, originating from specific segments of the nephron [9].

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#### Discussion with Reviewers

**A.P. Soler:** The clinical importance of the paper requires some comments on the doses of oxalate used in the assays. Also, it will be necessary to add data and references in relation to normal and pathological plasma and urinary values of oxalate. Furthermore, I would like to know if it is possible that some oxalate transport might occur in the presence of very high urinary levels of the molecule? Could there exist a very low affinity cellular transport system?

**Authors:** The average serum oxalate concentration is 1-2  $\mu$ M [30, 32]. Mean values of 24-hour excretion of oxalate are  $350 \pm 10 \mu$ mol (n = 60) in normal subjects and  $430 \pm 20 \mu$ mol (n = 65) in recurrent stone-formers [21]. The concentration profile of oxalate along the nephron as determined by Hautman and Oswald [32] showed a gradual increase from 1  $\mu$ M in Bowman space up to 300  $\mu$ M in the final urine. Routinely, our transport studies were performed with 50  $\mu$ M oxalate. Experiments performed with much higher oxalate concentrations up to 500  $\mu$ M, or much lower oxalate concentrations down to 1  $\mu$ M, never resulted in cellular uptake of radioactive labeled oxalate, which makes the existence of any oxalate transport system in these cells unlikely.

**R.L. Hackett:** Oxalate, 500  $\mu$ M, when incubated with DMEM with or without 10% fetal calf serum in the presence or absence of cells will form calcium oxalate crystals. Did you check your medium for presence of crystals when using oxalate at that concentration? Alternatively, what is the effect on cell function when cells are incubated with the reduced levels of calcium you utilized to avoid crystal formation?

**Authors:** The transport studies were performed in MOPS-buffer and not in DMEM. To study possible oxalate uptake under extreme conditions, we performed a small number of experiments using varying amounts of oxalate and calcium. In these experiments, care was taken that the solubility product for CaOx was not exceeded. Short-term incubations in the presence of the lowest calcium concentration used in this study (100  $\mu$ M) did not lead to morphological or functional changes of the monolayers.

**Reviewer V:** This paper presents evidence that LLC-PK1 cells in culture do not transport oxalate, which appears to be in opposition to a 1992 study from Dr. Williams' research group [27]. Explanation is offered as to why the current data is different, but the arguments are not compelling. The primary concern about the manuscript is the observation that these cells do not transport oxalate. Although many negative observations form the basis for significant publications, the current data should be part of a larger investigation defining exactly why oxalate transport was not observed and in what cells in this segment of the nephron oxalate transport is observed, i.e., a comparative study with some positive as well as negative observations.

**Authors:** To our opinion, this manuscript provides a constructive contribution to study oxalate transport in functional renal tubule epithelia in culture. As indicated in the discussion, this study will be part of a larger investigation and hopefully, we will be able to present more positive observations in the near future.

**J. Kavanagh:** Even if the structural integrity of the monolayer is not well preserved in gluconate buffer, Wandzilak *et al.* [27] seem to have demonstrated active uptake of oxalate by LLC-PK<sub>1</sub> (and MDCK) cells. Could the differences in the buffers used explain the absence of transport in your system and was the pH controlled by MOPS or MES/Tris when you tested the gluconate buffer?

**Authors:** The use of (MES/Tris buffered) Na-gluconate buffer not only resulted in the loss of epithelial polarity but also led to the apparent intracellular uptake of the extracellular marker mannitol. This indicates gluconate-buffer induced cell damage, which was confirmed by microscopical studies.

**J. Kavanagh:** Were other salts present which could affect the ionized oxalate concentration or inhibit active transport?

**Authors:** The transport experiments in this study were performed in MOPS-buffer containing physiological salt concentrations. It is known that anion exchange mechanisms exist in the proximal tubule [31]. Recently it has been suggested that the presence of chloride ions may negatively influence oxalate transport in proximal tubular cells (H Koul, personal communication). However, the biological significance of

oxalate transport inhibited by physiological concentrations of other anions remains to be determined. When we succeed to establish primary cultures of renal tubular cells exhibiting oxalate transport, we will study stimulating and inhibiting effects of anion gradients ( $\text{Cl}^-$ ,  $\text{HCO}_3^-$ ).

**R.L. Hackett:** Your evidence, that oxalate flux did not involve a transcellular pathway, was based only on a comparison with mannitol flux. Did you poison anion transport systems, as did Sigmon *et al.* [25], in order to demonstrate the absence of an active transporter?

**Authors:** Since the values measured for oxalate "transport flux" and "uptake" did not significantly exceed background levels (assessed with radiolabelled mannitol) in any of our experiments, it was not considered relevant to apply anion-exchange inhibitors such as disulfonic stilbenes (SITS, DIDS).

**R.L. Hackett:** MDCK cells at 9-10 days post-seeding begin to demonstrate morphological evidence of regressive changes. Would you comment on that in relation to your transport studies?

**Authors:** Compared to monolayers cultured on tissue culture plastics, monolayers cultured on microporous membranes can be maintained much longer without loss of morphological or functional integrity. LLC-PK<sub>1</sub> cells cultured on microporous membranes have been maintained in culture for up to 28 days without loss of glucose transport capacity [8].

**A.P. Soler:** The data in Table 3 showing the apical and basolateral uptake of  $\alpha$ MG in LLC-PK<sub>1</sub> cells, contradict the results of previous works, demonstrating the absence of basolateral  $\alpha$ MG uptake in this cell line. One possible explanation for this could be that the relatively high values observed do not represent the basolateral uptake of  $\alpha$ MG by LLC-PK<sub>1</sub> cells, but rather a "re-uptake" of  $\alpha$ MG from the apical surface of the cells after a paracellular diffusion of the molecule.

**Authors:** We also assumed that the basolateral uptake of  $\alpha$ MG in LLC-PK<sub>1</sub> cells was the result of initial apical-to-basolateral diffusion and referred to this possibility in the **Results** section when describing Table 3.

**C.A. Rabito:** I think there is a conceptual error in the role of the paracellular pathway in the transepithelial transport of oxalate as presented in the discussion. In the absence of active secretion, there should be a net reabsorption and no secretion of oxalate along the proximal tubule. The isotonic fluid reabsorption that occurs along the proximal tubule (and that amount to 85% of the total glomerular filtrate) will increase the concentration of oxalate in the tubular fluid and allow its diffusion (reabsorption) to the blood side of the epithelium. It is only a large reduction and not

increase in the permeability of the occluding junctions to oxalate that may explain the presence of hyperoxaluria in absence of active oxalate secretion.

**Authors:** The expression "renal leak" as depicted in the **Discussion**, emphasizes increased urinary excretion of oxalate. We subscribe to the postulation that only a decrease in the permeability of the occluding junctions of proximal tubular epithelium could result in enhanced amounts of oxalate excreted into the urine.

**C.A. Rabito:** The absence of transepithelial oxalate transport in LLC-PK<sub>1</sub> cells comes not as a surprise since most if not all the characteristics of this cell line correspond to the S3 segment of the renal proximal tubule and this nephron segments lack of an specific oxalate secretion system. The significance of this meticulous paper, however, resides in the fact that it contradicts the results obtained by Wandzilak *et al.* [27] in the same cell line with a less rigorous technique. Whereas the difference may merely represent technical differences (as discussed in this paper) other possibilities, such as, difference in cell lineage, difference in passage number, etc. should also be considered. For instance, we have isolated, from the original LLC-PK<sub>1</sub> cell provided by ATCC, at least two different clones designated LLC-PK<sub>1A</sub> and LLC-PK<sub>1B4</sub>. These two clones show totally different properties with reference to their Na<sup>+</sup>-dependent sugar transport system and Na<sup>+</sup>-H<sup>+</sup> exchange system. Also it is well known that the transport characteristics of different renal epithelial cell lines change with the number of passages in culture.

**Authors:** We cannot exclude the possibility that some of the characteristics of the LLC-PK<sub>1</sub> cells used in our studies are not identical to those used by Wandzilak *et al.* [27]. However, as discussed, we consider it more likely that the segmental origin of the LLC-PK<sub>1</sub> cells accounts for the observed lack of oxalate transport. The recognition that these cells probably descend from the S3 segment of the renal proximal tubule encouraged us to develop techniques to establish *in vitro* primary cultures of defined proximal cells. As a matter of fact, attempts to isolate and culture S1, S2 cells of rat renal proximal tubuli were recently initiated in collaboration with Prof. M.F. Horster from the Physiological Institute of the University of Munich [33].

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