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Oxalate transport in cultured porcine renal epithelial cells

T. R. Wandzilak[1], L. Calo[2], S. D'Andre[1], A. Borsatti[2], and H. E. Williams[1]

[1]University of California, Davis, Calif., USA
[2]Institute of Internal Medicine, Division of Nephrology University of Padova, Padova, Italy

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Summary. Oxalate-containing kidney stones are the most common type (75%) of renal stones. In order to control oxalate excretion in the urine, a basic understanding of the cellular transport of oxalate is imperative. We have utilized the technique of continuous cell culture to establish and characterize a model system to study renal epithelial cell (LLCPK1) oxalate transport. Our data demonstrate that oxalate uptake in these cells is dependent on time, concentration and energy. The K_m for oxalate uptake was 200 μM. Oxalate uptake was decreased at lower temperatures and elevated in an acidic extracellular environment. Both anion exchange inhibitors DIDS and SITS inhibited oxalate uptake. Sulfate, chloride, and bicarbonate decreased oxalate uptake, as did the diuretics bumetanide and furosemide. There was no evidence for the co-transport of oxalate with sodium. Our data show that monolayers of cultured kidney epithelial cells are a valuable model system for study of the basic cellular mechanisms of oxalate transport.

Key words: LLCPK1 – Cell culture – Anion transport – Kidney stones – DIDS

Calcium oxalate is the major constituent of the majority of kidney stones obtained from patients with nephrolithiasis and urinary oxalic acid plays a dominant role in the formation of calcium oxalate kidney stones. Therefore, studies examining oxalate transport may prove important in the diagnosis and treatment of stone disease.

Oxalic acid transport studies have been conducted using a variety of preparations including gastrointestinal tract [2, 4, 6, 9, 14, 19, 22], kidney [12, 16, 27], erythrocytes [1], and mitochondria [17, 25]. A medley of methodological approaches have also been utilized to study oxalic acid transport including mucosal tissue accumulation [2], everted gut sac [2, 4, 22], the Ussing chamber short-circuit [6, 9], cell suspension [1, 19], microperfusion [26], microPuncture [28] and membrane vesicle preparations [12, 14, 16, 27]. The present study was undertaken to characterize the basic mechanism of oxalate transport in the renal epithelial cell line LLCPK1 [10], and to establish a model system to study oxalate transport using the technique of cell culture.

Methods and materials

Culture conditions

LLCPK1 cells (American Type Culture Collection ATCC CRL 1392) were maintained in Dulbecco's modified Eagle's medium/F12 supplement (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum with antibiotics, by serial passages in 75 cm^2 tissue culture flasks (Falcon) in an incubator at 37°C. The culture media was later changed to alpha-minimal essential medium supplemented with FCS and antibiotics (Gibco). For experiments, cells were grown on 35 × 10 mm tissue culture dishes until confluent. All experiments were carried out at 37°C, under 95% air and 5% CO_2. Cells were washed twice in buffer A consisting of 265 mM mannitol, 5 mM Na^+, 5 mM K^+, 10 mM Ca-EGTA, 25 mM Hepes/Tris, pH = 7.4. Using a Ca-EGTA buffer system enabled us to set and maintain the extracellular free calcium concentration and calculate the extracellular free oxalate concentration. Cells were preincubated for 10 min in buffer A containing experimental drugs after which time the experiment was started. ^{14}C labeled oxalate (specific activity 103 mCi/mmol; Amersham UK) was used to determine cellular uptake. At the end of the incubations, the cells were washed 5 times with ice-cold phosphate-buffered saline, and 3 ml 1 *N* NaOH was added to solubilize the cells. The amount of ^{14}C-oxalate taken up by the cells was determined using liquid scintillation. Another aliquot was assayed for protein (Lowry). Oxalate uptake is expressed as picomoles per milligram of protein, unless otherwise noted.

Total cell counts and viable cell number

Total cell counts and cell viability in mannitol buffer was assessed using a trypan blue dye exclusion procedure (Sigma Technical Information, Sigma Chemical Co., St. Louis, MO 63178, USA). Cell viability was 97.5% in DMEM/F12 media, 93.2% after 30 min, 92.5% after 1 h, 87.9% after 2 h and 86.6% after 3 h of incubation in mannitol buffer. The average total cell number per tissue culture dish was 1.6×10^6 cells.

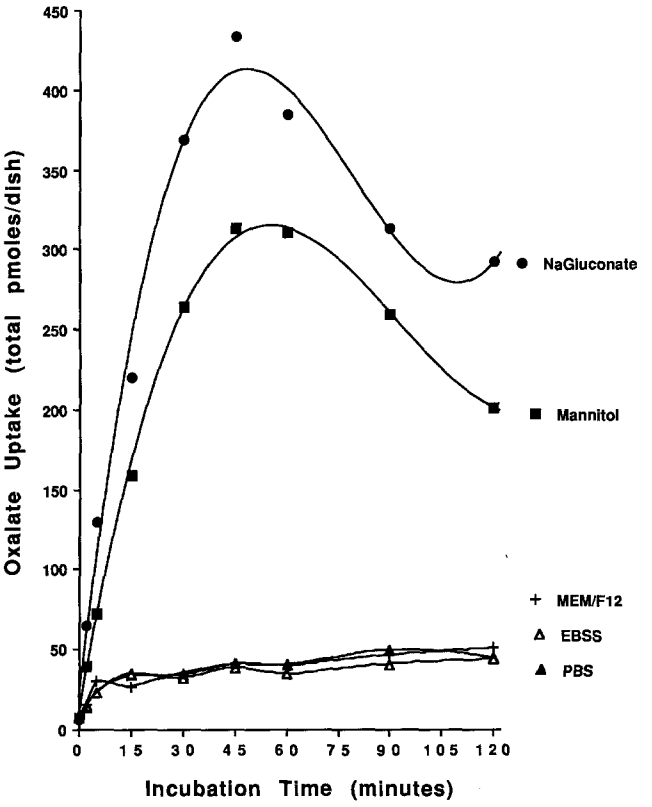

Fig. 1. The effect of different buffer types on the uptake of oxalate. 140 mM Na-Gluconate, 20 mM Mes/Tris; 280 mM Mannitol 20 mM Mes/Tris. Cells were preincubated in the different buffer types (pH = 7.4) for 60 min prior to the addition of [^{14}C] oxalate

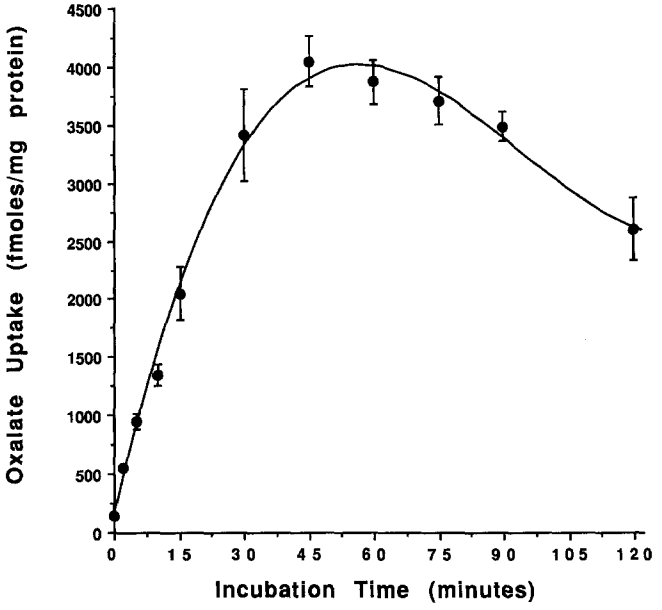

Fig. 2. Time curve for oxalate uptake by LLC-PK$_1$ monolayer. Values are means ± SEM; $n = 3$. Oxalate uptake was determined using buffer A

Intracellular volume determination

To determine whether cells were able to concentrate oxalate, the intracellular volume of the cells was measured indirectly. This was accomplished by determining both the total water (extracellular plus intracellular) and separately the extracellular water, and then the intracellular water by subtraction. [^{14}C]Urea, which partitions equally in both extracellular water, was used to determine total water volume. [^{3}H]Inulin partitions only in the extracellular water and was used to determine the extracellular water space.

Statistics

Results are expressed as mean ± SEM. Data were statistically evaluated utilizing an unpaired, two-tailed Student's *t*-test as appropriate, and performed using the Statview program (Brain Power, Calabasas, Calif.) on a Macintosh IIcx computer. Any *P*-values smaller than 0.05 (95% confidence) were considered to indicate significant differences.

Results

Effect of extracellular media type on oxalate transport

Figure 1 shows the effect of different buffer types on oxalate transport. Oxalate transport was diminished in buffers (phosphate-buffered saline (PBS), Earl's balanced salt solution (EBBS), minimal essential media with F12 nutrient additive (MEM/F12), containing other permeant anions such as Cl, SO_4, PO_4, and HCO_3. Oxalate transport was higher in the Na-gluconate and mannitol buffers, which do not contain permeant anions other than oxalate, which might be competitive.

Time course of oxalate transport

Figure 2 shows the time-course of oxalate uptake at 37°C. Oxalate uptake was maximal between 45 and 60 min. Uptake slowly decreased by 2 h and remained level for up to 4 h.

Effect of temperature and pH

Oxalate transport was substantially lower at decreased temperature (4° < 25° < 37°C). Acidification of the external media (pH = 6) increased oxalate uptake, while alkalinization (pH = 8) decreased oxalate uptake.

Effect of anion transport inhibitors on oxalate transport

Figure 3 shows the effect of 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) on the uptake of oxalate. DIDS produced a concentration dependent inhibition of oxalate uptake in the cells. Oxalate transport was decreased 85–90% at 10^{-5} M DIDS. 4-Acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) also produced a concentration dependent inhibition of oxalate transport.

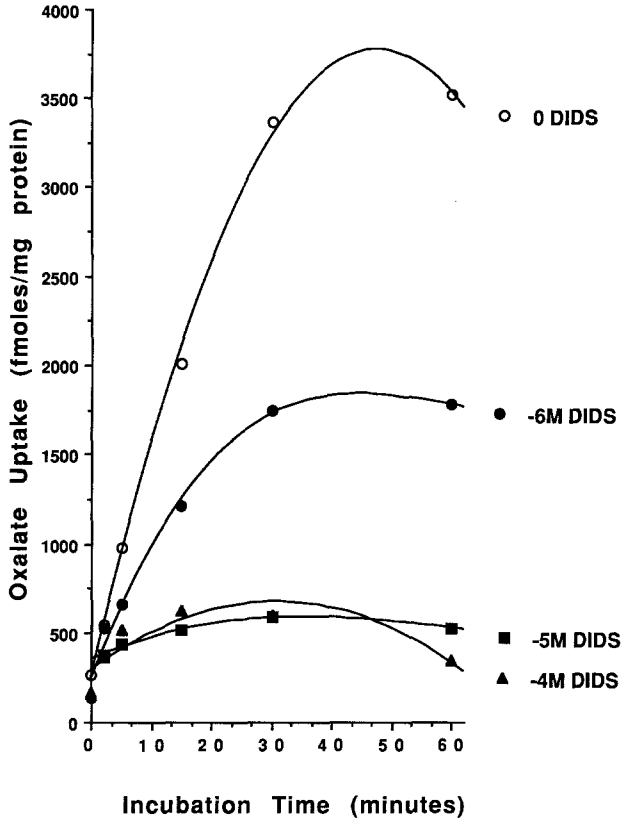

Fig. 3. Concentration-dependent inhibitory influence of DIDS on oxalate transport. Cells were preincubated with DIDS for 30 min in buffer A prior to addition of [^{14}C] oxalate

Determination of intracellular oxalate concentration

The total intracellular water volume per dish for LLCPK1 cells was 2.17 μL, or 1.35 pL per cell. Cells concentrated oxalate approximately 2.5-fold to give an intracellular concentration of 26 μM after 45 min of incubation, assuming no endogenous intracellular oxalate.

Effect of metabolic inhibitors on oxalate transport

Oxalate transport at 25 min was decreased by lodoacetamide (37%; $P = 0.0012$), dinitrophenol (DNP) (46%; $P = 0.008$) and dithio-nitrobenzoic acid (DTNB) 35%; $P = 0.018$) (Table 1).

Kinetic parameters of oxalate uptake in LLCKP1 cells

All K_m studies were performed at 10 min in buffer A. The concentration of free calcium varied from 1000 to 50 μM and the free oxalate concentrations ranged from 600 to 10 μM. Significant saturation of transport (without precipitation of calcium oxalate) was achieved at a calcium concentration below 200 μM. Figures 4A–C plots data performed with 50-μM free calcium. The K_m for oxalate transport determined under these conditions was 200 μM.

Table 1. Effect of different test substances on oxalate transport

Substance	Concentration	Decrease (%)
DIDS	10 μM	90
SITS	1 mM	90
Iodoacetamide	5 mM	37
Dinitrophenol	5 mM	46
DTNB	5 mM	35
Sulfate	2 mM	40
Chloride	2 mM	25
Bicarbonate	2 mM	24
Phosphate	2 mM	0
Amiloride	5 mM	0
Bumetanide	1 mM	57
Furosemide	5 mM	60
Vitamin D	10 nM	0

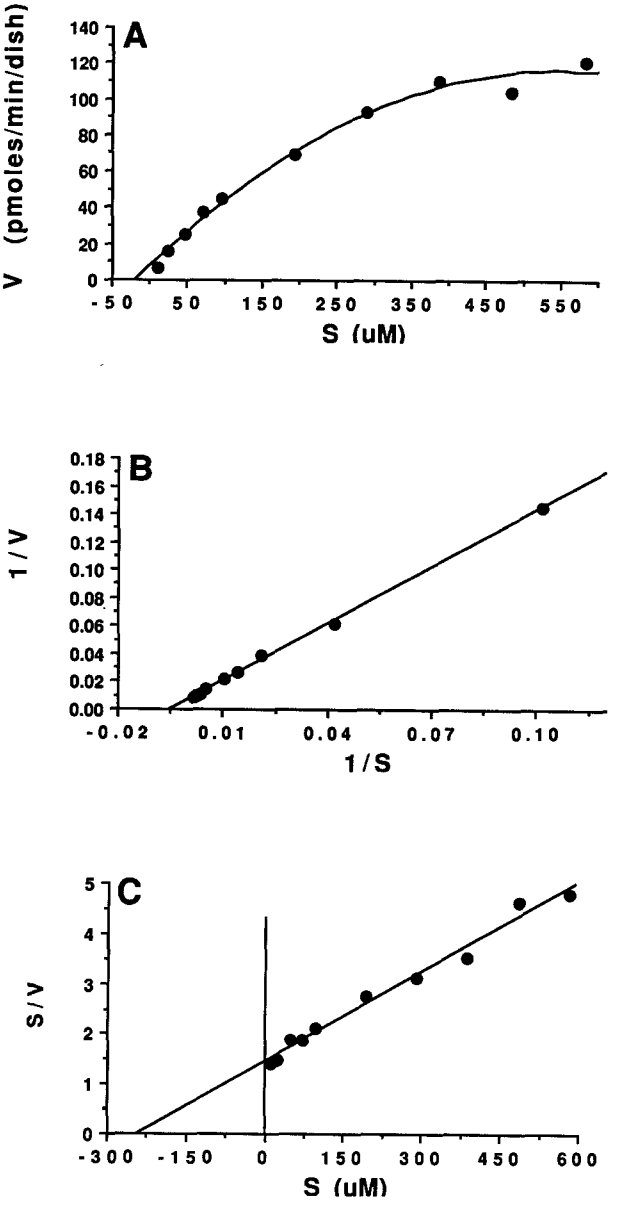

Fig. 4. A Curve obtained by plotting initial velocity versus substrate concentration; B double reciprocal Lineweaver-Burke plot; C Hanes-Woolf plot

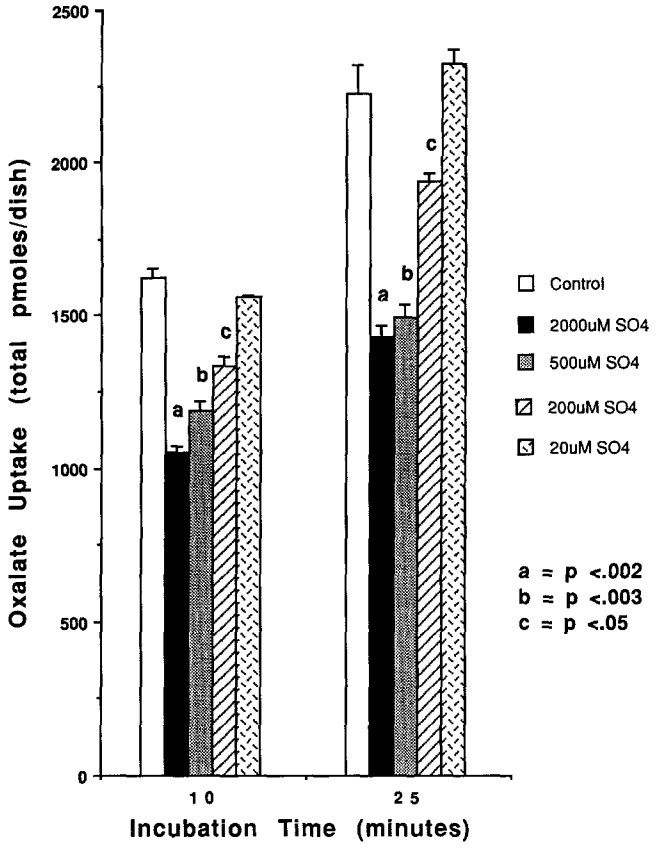

Fig. 5. Inhibitory effect of sulfate on oxalate uptake. Cells were preincubated for 10 min in buffer A. Oxalate uptake was determined in the presence of various concentrations of sulfate

Oxalate uptake in different cell lines

The uptake of oxalate is not unique to the LLCPK1 cell line, which has a mixture of proximal and distal tubule characteristics [10]. Oxalate uptake also occurred in three other kidney epithelial cell lines, which exhibit distinct proximal and distal tubule characteristics. These include the OK line (proximal) [15], the MDCK line (distal) [21], and a new human renal cancer cell line G2101 (characteristics not yet identified) [7]. Oxalate uptake was comparable in the LLCPK1, OK, and MDCK cells, however, uptake in the G2101 cell line was approximately six-fold higher than in the other three. Oxalate uptake in OK, MDCK, and G2101 cell lines was also inhibitable by DIDS (85–90% at 10^{-4} M), suggesting that oxalate uptake in all these cell lines is mediated through an anion exchanger.

Influence of other anions on oxalate transport

The concentration of free oxalate used in all competition studies was 200 μM. Sulfate significantly decreased the cellular uptake of oxalate (Fig. 5). Chloride decreased both 10-min ($P<0.005$) and 25-min ($P<0.04$) oxalate uptake at a concentration of 2 mM. Bicarbonate also decreased 10, 25- and 45-min uptake at 20 mM ($P<0.04$), 10 mM ($P<0.03$) and 2 mM ($P<0.05$). There was no effect of phosphate up to 2 mM on oxalate uptake.

Effect of drugs on oxalate uptake

The oxalate uptake at 10, 25, and 45 min was decreased by the diuretics bumetanide (1 mM; $P<0.02$) and furosemide (5 mM; $P<0.01$). Neither the Na/H exchange inhibitor amiloride (5 mM), nor vitamin D (1,25-dihydroxycholecalciferol; 10 nM) significantly altered oxalate transport (Table 1).

Effect of Na and K on oxalate uptake

Varying the extracellular Na^+ concentration had no effect on oxalate uptake as oxalate uptake was similar at 5,70 and 135 mM Na^+. These data imply that oxalate is not co-transported with Na^+. However, oxalate uptake was increased 55% ($P<0.01$) at 70 mM, and 64% ($P<0.01$) at 135 mM K^+. This effect is likely the result of a K^+-induced membrane depolarization and the subsequent electrogenic movement of oxalate.

Discussion

The function of kidney epithelial cells in vivo is the transepithelial transport of an array of solutes. LLCPK1 cells in culture (in vitro) have been shown to retain this ability for a number of solutes including sugars [18], amino acids [24], phosphate [20], chloride/bicarbonate [5], and Na^+/H^+ [3]. The present study demonstrates the ability of monolayers of cultured kidney cells to also transport oxalic acid.

In our system, oxalate uptake was time, energy and concentration dependent. Oxalate transport was diminished at lower temperatures and reduced by lodoacetamide, DNP and DTNB, all of which lessen or deplete available energy stores. This implies an energy-dependent transport process for oxalate.

Oxalate uptake was also inhibited by the classic anion transport inhibitors DIDS and SITS indicating that oxalate uptake in these cells is mediated through one of the family of anion transporter proteins, possibly analogous to the erythrocyte anion transport protein band 3 [11].

Oxalate uptake was also demonstrated in other cell lines exhibiting distinct proximal and distal tubule characteristics, as well as a human renal cell line. Oxalate uptake in all cell lines was inhibited by DIDS.

The K_m for oxalate uptake in the LLCPK1 cell line was determined to be 200 μM. Knickelbein et al. [13] reported a K_m value of 86 μM for oxalate:OH exchange and 566 μM for oxalate:Cl exchange in rabbit ileal brush border membrane vesicles. Strzelecki and Menon [25] reported a K_m value of 13.4 mM for oxalate uptake in kidney mitochondria preparations. To relate this to the vivo situation, plasma oxalate levels are estimated to be approximately 1–2 μM [23, 30], and normal 24-h urinary levels range from 100 to 500 μM [29].

Cultured kidney cells grow in monolayer with a specific polarized orientation. Attachment to the tissue culture plate is from the basolateral surface [8]. In our present system, it was not possible to determine whether both surfaces (apical and basolateral) have equal access to the experimental media and therefore not possible to delineate whether oxalate uptake is occurring at only one or both sites. Without being able to verify transport at only one site, we can only speculate at this time that the uptake of oxalate in this system is mediated by both the apical and basolateral surfaces. Support for both apical and basolateral uptake comes from our inhibition data using sulfate, bicarbonate and chloride anions. Both sulfate and bicarbonate, which utilize the basolateral exchange mechanism [16, 27], and chloride, which utilizes the apical exchange mechanism [12], were able to decrease oxalate uptake in these cells. Future studies using cells grown on porous membranes, which permit differential access and sampling from either surface, should allow confirmation of this point.

The technique of cell culture has proven to be a useful tool for investigations into kidney cell physiology and particularly transport processes. We feel that the continued application of this technique to the investigation of oxalic acid transport will further substantiate it as a useful tool in the study of the mechanisms of kidney stone formation.

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Theodore R. Wandzilak, PhD
University of California, Davis
Office of the Dean
Medical Sciences Building 1C, Room 104
Davis, CA 95616, USA