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# Sulfate conjugating and transport functions of MDCK distal tubular cells

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**Sulfate conjugating and transport functions of MDCK distal tubular cells.**

*Background.* Transfected Madin-Darby canine kidney (MDCK) cells (of distal tubular origin) have been used to study transport of organic anions. These cells have not been shown to possess sulfate-conjugating activity. Neither has transport activity been demonstrated in nontransfected MDCK cells.

*Methods.* Polarized and monolayers of nontransfected MDCK type II cells were incubated with prototype substrates of phenolsulfotransferase (PST) and sodium sulfate in the absence or presence of known inhibitors of multidrug resistance protein (MRP): (3-3-(2-(7-chloro-2-quinionlinyl) ethenyl)phenyl)(3-dimethylamino-3-oxopropyl)thio)methyl)thio) propanoic acid (MK571), cyclosporin A (CsA), and probenecid. Effects of glutathione (GSH) and buthionine sulfoximine (BSO), potential modulators of the organic anion transporting protein/polypeptide (OATP) isoform, OATP1 were also examined. Sulfated conjugates were identified by high-performance liquid chromatography (HPLC)-radiometry or HPLC-fluorimetry.

*Results.* Uptake, sulfate conjugation, and efflux of the sulfated conjugates of harmol, *p*-nitrophenol, N-acetyldopamine and acetaminophen were demonstrated. Activities in MDCK type II cells were higher than those in HepG2, human fetal liver, and Chang liver cells. A significant decrease in extracellular with a reciprocal increase in intracellular harmol sulfate was observed with MK571, CsA, and probenecid and with preloading of glutathione. Depletion of intracellular glutathione by BSO had the opposite effects.

*Conclusions.* Normal (nontransfected) MDCK type II cells provide a suitable system for the study of the physiologic processes of uptake, sulfate conjugation, and transport of sulfated conjugates in kidney cells. Based on the action of specific inhibitors and modulators of MRP2 and OATP1, it was concluded that MRP2-like and OATP1-like transporters are possibly responsible for the transport of sulfated conjugates.

Sulfate and glucuronide conjugations are two major phase II reactions in humans. Quantitatively, glucuronide conjugation appears to predominate in both liver and kidney [1–3], with reported renal values of 2% to 20% compared to the liver [4–8]. This could account for the lack of interest in the study of renal sulfate conjugation [9]. The enzyme phenolsulfotransferase (PST) has been demonstrated in kidneys of various experimental animals from our laboratory [10, 11] and in human fetal and adult kidneys [12]. The only PST isoform reported to be expressed in human kidney is SULT1C1 [13].

On the other hand, all isoforms of organic anion transporter (OAT), organic cation transporter (OCT), and multidrug resistance protein (MRP) where tissue distribution has been examined, have been found in kidney [14–16]. These transporters play a crucial role in the phase III elimination of the anionic conjugates. This highlights their potential contribution to the urinary excretion of endobiotics and xenobiotics.

This paper is the first report to demonstrate sulfate conjugation and subsequent transport of a number of sulfated conjugates out of Madin-Darby canine kidney (MDCK) type II distal tubular cells. These activities were higher than those in HepG2, Chang liver, and human fetal liver cells. Hitherto, only proximal tubular cells of kidneys were thought to be endowed with such activities. Both MRP and organic anion transporting protein/polypeptide (OATP), reported to be present on the apical membranes of kidney cells [14–20] are tentatively identified as responsible for the transport of harmol sulfate and other sulfated conjugates. Our study with MDCK type II cells provides a model system for the study of uptake and sulfation cum transport, which reflects sequential physiologic metabolic processes in normal kidney cells.

## MATERIALS

Sodium $^{35}$sulfate (specific activity of 540.37 mCi/mmol) was purchased from DuPont, NEN, Boston, MA, USA. N-acetyldopamine, acetaminophen, *p*-nitrophenol, harmol, cyclosporin A (CsA), glutathione (GSH), glutathione ethyl ester (GEE), probenecid, and methotrexate were purchased from Sigma Chemical Co. (St. Louis, MO,

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USA). MK-571 was a gift from Merck-Frosst, Canada. MDCK type II cells and human fetal liver cells were gifts from Dr. Walter Hunziker of the Institute of Molecular Cell Biology and Professor K.H. Sit of the Department of Anatomy, National University of Singapore, respectively. Transwell inserts (P/N 3401) were purchased from Costar, Cambridge, MA, USA. Tissue culture flasks were obtained from Nunclon (Resklide, Denmark). Fetal bovine serum (FBS) was obtained from Trace Bioscience, Pty., Ltd., Castle Hill, New South Wales, Australia and the antibiotic/antimycotic mixture was purchased from Gibco (Grand Island, NY, USA).

## METHODS

### Cell cultures

MDCK type II, human fetal liver, HepG2, and Chang liver cells were purchased from American Type Culture Collection (ATCC) were cultured in monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS in a humidified incubator (5% $CO_2$, 37°C)

### Assay conditions

*Overall sulfation in cell lysate.* The cell lysate was prepared by harvesting confluent cells in 0.15 mol/L potassium chloride containing 3 mmol/L dithiothreitol (DTT); it was stored frozen at –80°C until ready to use. It was used for measuring the overall sulfate conjugation reactions (i, ii and iii) with harmol, *p*-nitrophenol, N-acetyldopamine and acetaminophen as substrates.

$$\text{(i) } SO_4^- + \text{ATP} \xrightarrow{\text{ATP sulfurylase}} \text{APS} + PP_i$$

$$\text{(ii) APS} + \text{ATP} \xrightarrow{\text{APS kinase}} \text{PAPS} + \text{ADP}$$

$$\text{(iii) PAPS} + \text{substrate} \xrightarrow{\text{PST}} \text{Sulfated substrate} + \text{PAP}$$

where ATP is adenosine 3′-triphosphate, APS is adenosine 3′-phosphosulfate, $PP_i$ is pyrophosphate, PAPS is 3′-phosphoadenosine 5′-polysulfate, and PST is phenolsulfotransferase.

All reactions were carried out in 50 mmol/L $KH_2PO_4$/NaOH buffer at pH 7.4 with the following substrates: 2.5 μmol/L *p*-nitrophenol, 20 μmol/L N-acetyldopamine, 2 mmol/L acetaminophen, and 1 mmol/L harmol. These optimal concentrations were based on kinetics obtained with liver cells (data not shown). Each of these substrates was co-incubated with 0.1 mmol/L sodium $^{35}$sulfate and 5 mmol/L each of ATP and $MgCl_2$ in a final volume of 200 μL; these concentrations were based on previous studies from our laboratory [21–23]. In the fluorimetric assay, harmol and unlabeled sodium sulfate, each at 1 mmol/L, were used. Residual harmol was removed by adding 120 μL sodium phosphate buffer (pH 9.5) to the reacted incubate, followed by extraction two times with 0.4 mL ethyl acetate. This step was introduced essentially to reduce the chromatographic time. The reaction was carried out at 37°C and terminated by precipitation of inorganic sulfate and proteins with 30 μL each of 0.3 mol/L zinc sulfate and 0.3 mol/L barium hydroxide. Following centrifugation, the supernatant was passed through a 0.4 μm filter and aliquots were analyzed for harmol sulfate by high-performance liquid chromatography (HPLC)-fluorimetry. For the radiolabeled sulfated conjugates, separation and quantification was by HPLC radiometry.

*Overall sulfate conjugation cum transport in monolayer cells.* The overall sulfation cum transport was studied with the substrates mentioned at concentrations given above. Approximately $1.5 \times 10^5$ cells were seeded into each well of a 12-well plate 1 day before use. The medium was removed and the cells were washed with 1 × phosphate-buffered saline (PBS) solution. One milliliter of loading buffer, containing the reaction mixture in Hank's balanced salt solution (HBSS)/glucose, was added to each well. The plate was then incubated at 37°C for 1 hour. The extraction step with ethyl acetate as described above was only applied to the assays with harmol as substrate. The external medium was removed quantitatively, precipitated as described above with zinc sulfate and barium hydroxide, and filtered. The cells remaining in the wells were washed and lysed with 1 mL distilled water and sonicated for 1 minute before adding 10 μL 70% perchloric acid, followed by centrifugation and filtration. Measurement of intracellular harmol sulfate in the cell lysates and harmol sulfate in the incubating medium was by HPLC fluorimetry while the radiolabeled sulfated conjugates formed from 0.1 mmol/L sodium $^{35}$sulfate were measured by HPLC-radiometry. The introduction of inhibitors and other chemicals are as specified in individual experiments.

*Transport studies of harmol sulfate using polarized MDCK type II cells.* Approximately $1.6 \times 10^5$ cells were seeded on Transwells covered with a polycarbonate membrane of 0.4 μm pore size (Costar P/N3401). These were inserted into a 12-well plate. The upper and lower compartments were filled, respectively, with 0.4 mL and 1.0 mL medium. The medium was changed on the second and fourth day followed by monitoring the tightness of gap junction for 4 hours after removing the medium from the lower compartment. The medium was then discarded and the cells were washed with 1 × PBS. Basolateral or apical loading of various chemicals was as stated in the individual sets of experiments described below. The plate was then incubated at 37°C for 1 hour. The external media from both compartments, as well as the cell lysates, processed as described above for monolayer cells, were analyzed in the same manner by HPLC fluorimetry.

**Table 1.** Separation of radiolabeled sulfated conjugates from sodium $^{35}$sulfate, by high-performance liquid chromatography (HPLC)-radiometry and harmol sulfate from harmol by HPLC-fluorimetry[a]

| Mobile phase | Sulfate conjugate of | | Retention time *minutes* | |
|---|---|---|---|---|
| | | | Sodium$^{35}$ sulfate | Harmol |
| A | *p*-Nitrophenol | 7.5 | 2.1 | — |
| B | N-acetyldopamine | 5.7 | 1.7 | — |
| C | Acetaminophen | 3.4 | 1.7 | — |
| D | Harmol | 5.9 | 2.4 | — |
| E* | Harmol | 2.6 | | 10.5 |

[a] A flow rate of 1 mL/min was employed using a column of 4.6 × 200 mm, except in mobile solvents C and E, where a 2.1 × 100 mm column was used with a flow rate of 0.5 and 0.3 mL/min, respectively.

A, 75 mmol/L $NaH_2PO_4$, 1 μmol/L EDTA and 0.35 mmol/L 1-octanesulfonic acid adjusted to pH 4.0, plus 4% acetonitrile and 11% methanol.

B, Same as A but with 2% methanol.

C, 20 mmol/L $KH_2PO_4$ and 1 mmol/L EDTA adjusted to pH 7.0 and 4% methanol.

D, 20 mmol/L $KH_2PO_4$ and 2.5 mmol/L octylsulfate adjusted to pH 3.5 and 35% methanol.

E, Same as D but with 50% methanol.

Harmol sulfate was synthesized in vitro in a similar reaction as described above using rat liver cytosol. An extended incubation of 2 hours was used. Harmol sulfate was separated from harmol by gel filtration with Sephadex G-25, following the procedure reported previously from our laboratory [24]. Quantification of the preformed harmol sulfate was by HPLC-fluorimetry and extrapolated from a standard of harmol. In two different sets of experiments, the basolateral compartment was loaded in a final volume of 1 mL HBSS/glucose, pH 7.4 containing (*a*) 0.01 mmol/L preformed harmol sulfate and (*b*) 1 mmol/L each of harmol and sodium sulfate. (3-3-(2-(7-chloro-2-quinionlinyl)ethenyl)phenyl)(3-dimethyl-amino-3-oxopropyl)thio)methyl)thio)propanoic acid (MK571) (25 μmol/L) was introduced into the apical compartment in both experiments. Incubation was carried out for 1 hour at 37°C and harmol sulfate was measured in the apical compartment for experiment (a) and in the apical and basolateral compartments for experiment (b) and intracellular compartments for both.

### HPLC radiometry and HPLC fluorimetry

The radiolabeled sulfated conjugates of *p*-nitrophenol, N-acetyldopamine, acetaminophen and harmol were detected and quantified by an HPLC system (Hewlett-Packard 1090, Hewlett-Packard, Waldbronn, Germany) connected to a radioactivity detector (Flo-one/Beta, Packard Instrument Co, Inc., Downers Grove, IL, USA). A solid cell of 250 μL volume packed with calcium fluoride was used. For ease of reference, the different solvent systems used in the separation of the sulfated conjugates from sodium $^{35}$sulfate are shown in Table 1 together with their retention times. Unless otherwise stated, the mobile phase was delivered at 1 mL/min through a C18 column (2.1 × 100 mm or 4.6 × 200 mm) packed with Hypersil-ODS (Hewlett-Packard) of 5 μm particle size. The amount of sulfate conjugate formed was determined by extrapolating from a standard curve of 1.6 pmol – 22.4 pmol sodium $^{35}$sulfate, which showed a linear detector response.

Harmol sulfate was also detected and quantified by HPLC-fluorimetry. The system consisted of a Hewlett Packard 1050 system attached to an Hewlett Packard 1046 fluorescence detector. Measurements were made at excitation wavelength of 310 nm and emission of 420 nm. As harmol sulfate is not commercially available, results are expressed as "harmol equivalents" from a standard curve of harmol of 20 pmol to 100 pmol.

### Comparative studies of sulfate conjugating and sulfate conjugating cum transport activities between kidney and liver cells

The overall sulfate conjugation was measured in cell lysates and sulfate conjugation cum transport was measured in monolayers of MDCK type II, HepG2, human fetal liver, and Chang liver cells. The protein content was determined by the Bradford method [25].

## RESULTS

### Sulfate conjugation in lysates of MDCK type II cells

Lysates prepared from MDCK type II cells were able to form sulfated conjugates of *p*-nitrophenol, N-acetyldopamine, acetaminophen, and harmol from sodium $^{35}$sulfate and ATP. Under the same condition of assay, the specific activities of the overall sulfate conjugation reaction for these substrates are shown in Figure 1. These values were calculated from a standard of sodium $^{35}$sulfate determined at the same time by HPLC-radiometry. The chromatographic conditions and retention times are shown in Table 1.

The sulfation of harmol with unlabeled sodium sulfate was also measured but by HPLC-fluorimetry. The retention times of harmol and harmol sulfate are also shown in Table 1. The overall sulfation of harmol in lysates of renal tubular cells of MDCK type II showed Michaelis-Menten kinetics measured with 0.02 mmol/L to 2 mmol/L sodium sulfate and 1.5 μmol/L to 200 μmol/L harmol and the overall rate of reaction was linear for cell lysate containing up to 0.4 μg protein/assay incubate (data not shown).

### Sulfate conjugation cum transport in MDCK type II monolayers

The overall sulfation of harmol, *p*-nitrophenol, N-acetyldopamine and acetaminophen, followed by efflux of the respective sulfated conjugates was also demonstrated in MDCK type II monolayer cells, without added ATP, indicating their ability to form "active sulfate" from sodium sulfate. A comparison of the combined activity of

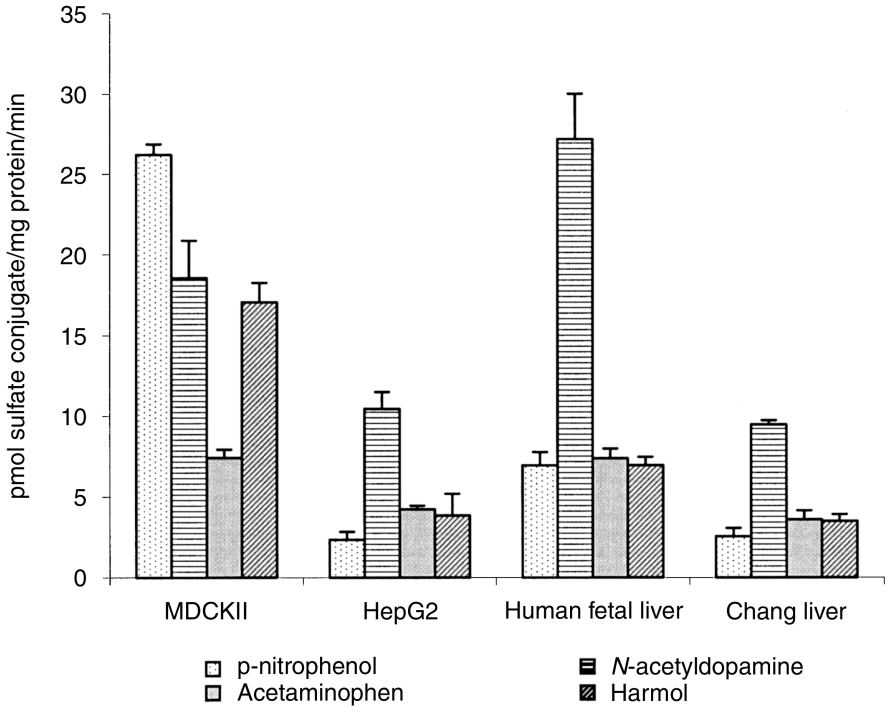


**Fig. 1. Overall sulfate-conjugating activities in cell lysates of Madin-Darby Canine Kidney type II (MDCKII) cells compared to HepG2, human fetal liver, and Chang liver cells measured with different substrates.** Values are means ± SD from triplicates and are expressed in picomole sulfate conjugates $min^{-1}$ mg $protein^{-1}$, extrapolated from a standard of 3 pmol to 22 pmol $sodium^{35}$ sulfate.

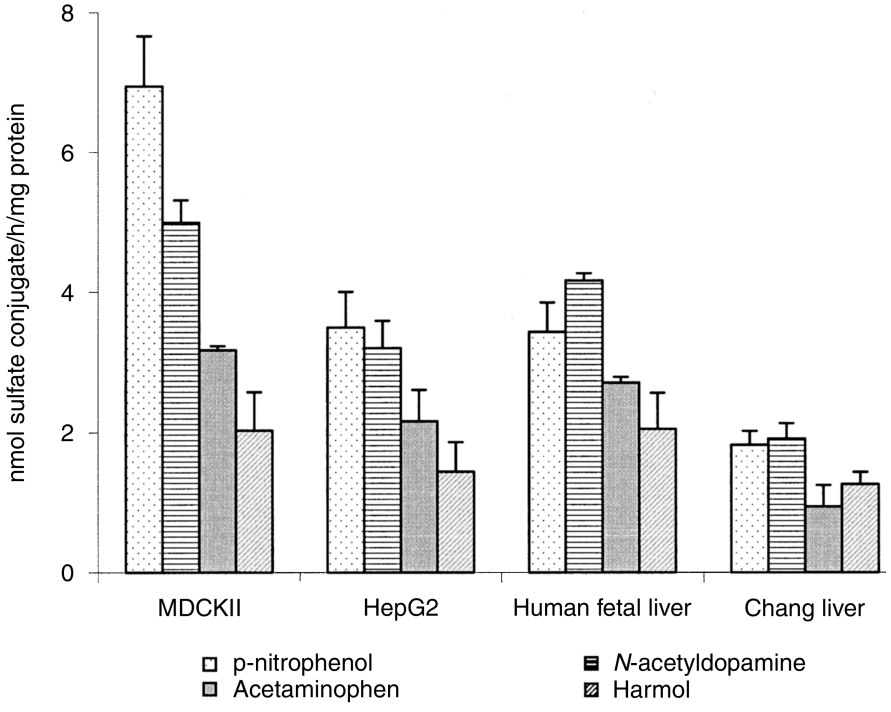


**Fig. 2. Measurement of uptake, sulfate conjugation and efflux of the sulfated conjugates in monolayers of Madin-Darby Canine Kidney type II (MDCKII), HepG2, human fetal liver, and Chang liver cells.** The sulfate conjugates formed from *p*-nitrophenol (2.5 μmol/L), N-acetyldopamine (20 μmol/L), acetaminophen (2 mmol/L), and harmol (1 mmol/L) with 0.1 mmol/L $sodium^{35}$ sulfate were separated from sodium $^{35}$sulfate by high-performance liquid chromatography (HPLC)-radiometry (see Table 1 for chromatographic conditions and retention times). A standard of 1.6 pmol to 12.8 pmol $sodium^{35}$ sulfate similarly measured at the same time was employed for the quantitative analysis. Values are expressed in means ± SD in nmol sulfated $^{35}$sulfated conjugate formed mg $protein^{-1}$ $h^{-1}$ ($N = 3$).

uptake of various substrates, followed by sulfate conjugation, and finally efflux of the sulfated products is shown in Figure 2. Experiments carried out with preincubation of substrates (an attempt to facilitate their uptake), followed by sodium sulfate, showed essentially similar results as co-incubation of both. This seemed to suggest that the uptake of the substrates did not influence the kinetics of reaction measured.

As the fluorimetric assay with harmol provides a simple and inexpensive assay procedure, it was preferred in subsequent experiments. In addition, nonradiolabeled sodium sulfate could be employed at a concentration 10 times higher in the fluorimetric assay. The kinetics of intracellular accumulation and efflux of harmol sulfate are shown in Figure 3. The rate of accumulation of the conjugate inside the cells was low compared to that of

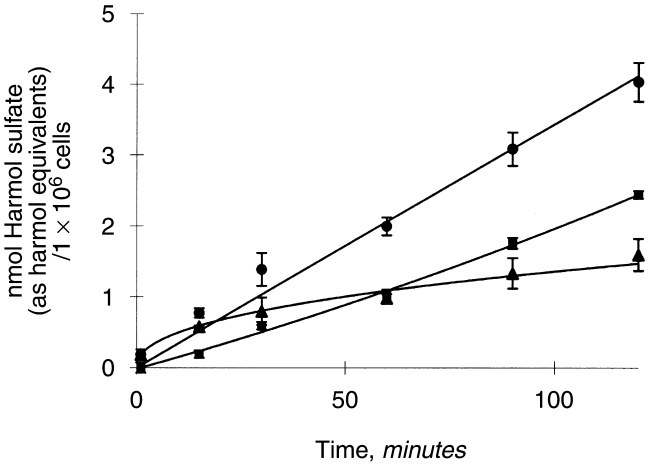


**Fig. 3. Time course of accumulation of intracellular (▲), efflux (■), and overall (●) of harmol sulfate by Madin-Darby Canine Kidney type II (MDCKII) monolayer cells following exposure to 1 mmol/L each of harmol and sodium$^{35}$ sulfate.** The "overall" represents the sum of accumulated and effluxed harmol sulfate measured at each time point. Values are average of duplicates. Harmol sulfate was quantified by high-performance liquid chromatography (HPLC) fluorimetry and values are expressed as nmol harmol equivalents/$10^6$ cells in means ± SD.

the efflux measured up to 2 hours. The sum of extracellular and intracellular harmol sulfate increased progressively from 1 hour to 2 hours, even though there was little further accumulation of harmol sulfate during this period (Fig. 3).

### Comparison between renal and hepatic cells

Interestingly, monolayer MDCK type II distal tubular cells showed higher sulfate conjugating cum transport activity compared to HepG2, human fetal liver, and Chang liver cells, measured with almost all the substrates studied (Fig. 2). This was also evident in the overall sulfate-conjugating activities determined in cell lysates (Fig. 1) with the exception of a higher activity for human fetal liver cells measured with N-acetyldopamine. The overall sulfate conjugation of harmol, measured in the lysate of MDCK type II cells by HPLC-radiometry (Fig. 1) and HPLC-fluorimetry (data not shown) was three to four times higher than that of HepG2 cells. This was an unexpected observation, as generally the liver is considered to be the main organ for detoxification.

### Effects of inhibitors/substrates/modulators of transporters on harmol sulfation cum transport

*MK571, CsA, and probenecid.* The effects of three known specific inhibitors of MRP, namely MK571, CsA, and probenecid [14, 15, 26, 27] were examined on MDCK type II cells grown in monolayers. On exposure to 2.5 μmol/L of MK571, there was a significant accumulation of harmol sulfate intracellularly, compared to the controls (Fig. 4A). This was accompanied by an apparent decrease (although not statistically significant) in the medium. However, at a higher concentration of 5 μmol/L, MK-571 elicited a more significant accumulation and a significant decrease of harmol sulfate in the efflux medium (Fig. 4B). Likewise, CsA at 20 μmol/L and probenecid at 1 mmol/L produced a significant increase in intracellular accumulation of harmol sulfate (Fig. 4 C and D). The observation of a significant increase in intracellular accumulation of harmol sulfate and its reciprocal decrease in the efflux medium on exposure to MK571, CsA, and probenecid suggests that MRP-like transporter/s are responsible for the efflux of harmol sulfate. In each instance, there was no change in the "total" (sum of intra- and extracellular) harmol sulfate, indicating that the overall sulfate conjugation reaction was not compromised.

*Glutathione-ethyl ester and buthionine sulfoximine (BSO).* Glutathione-ethyl ester (GEE) is a permeant form of glutathione [28]. Preloading with GEE at 20 mmol/L for 15 minutes prior to the assay of sulfation cum transport resulted in a significant decrease (40%) in extracellular harmol sulfate (Fig. 5A). Conversely, depletion of glutathione by preincubation with 50 μmol/L BSO for 16 hours produced a significant increase in the effluxed harmol sulfate and a reciprocal decrease in intracellular accumulation (Fig. 5B). The opposing effects of GEE and BSO on the transport and accumulation of harmol sulfate suggest that the glutathione/organic anion exchanger (or OATP1), located on the luminal membrane of renal cells, may also be involved in the transport of harmol sulfate.

*Combination of MK571 and glutathione-ethyl ester.* The combined exposure to MK571 and GEE produced an additive effect (Fig. 6), which was more significant than when each of these compounds was added alone (Figs. 4A and 5A). Both the decrease in harmol sulfate in the medium and the reciprocal increase in intracellular compartment were significantly different from the corresponding controls (Fig. 6). The total harmol sulfate (sum of extracellular and intracellular) remained relatively unchanged; these compounds therefore had no effect on the overall sulfation reactions.

*Methotrexate.* Besides the MRP2 and glutathione/organic anion exchanger, OATK1/K2, has been identified in the apical membrane of kidney tubules. This isoform has a narrow substrate specificity [29, 30]. Methotrexate is a known substrate of OATK1/K2 [31, 32]. Methotrexate at 5 μmol/L had no effect on sulfation cum transport of harmol (Fig. 7). It seems unlikely that OATK1/K2 is involved in the transport of harmol sulfate.

### Transport of harmol sulfate in polarized cells

Uptake of preformed harmol sulfate by polarized cells from the basolateral compartment was evident by its appearance in the apical compartment showing transcel-

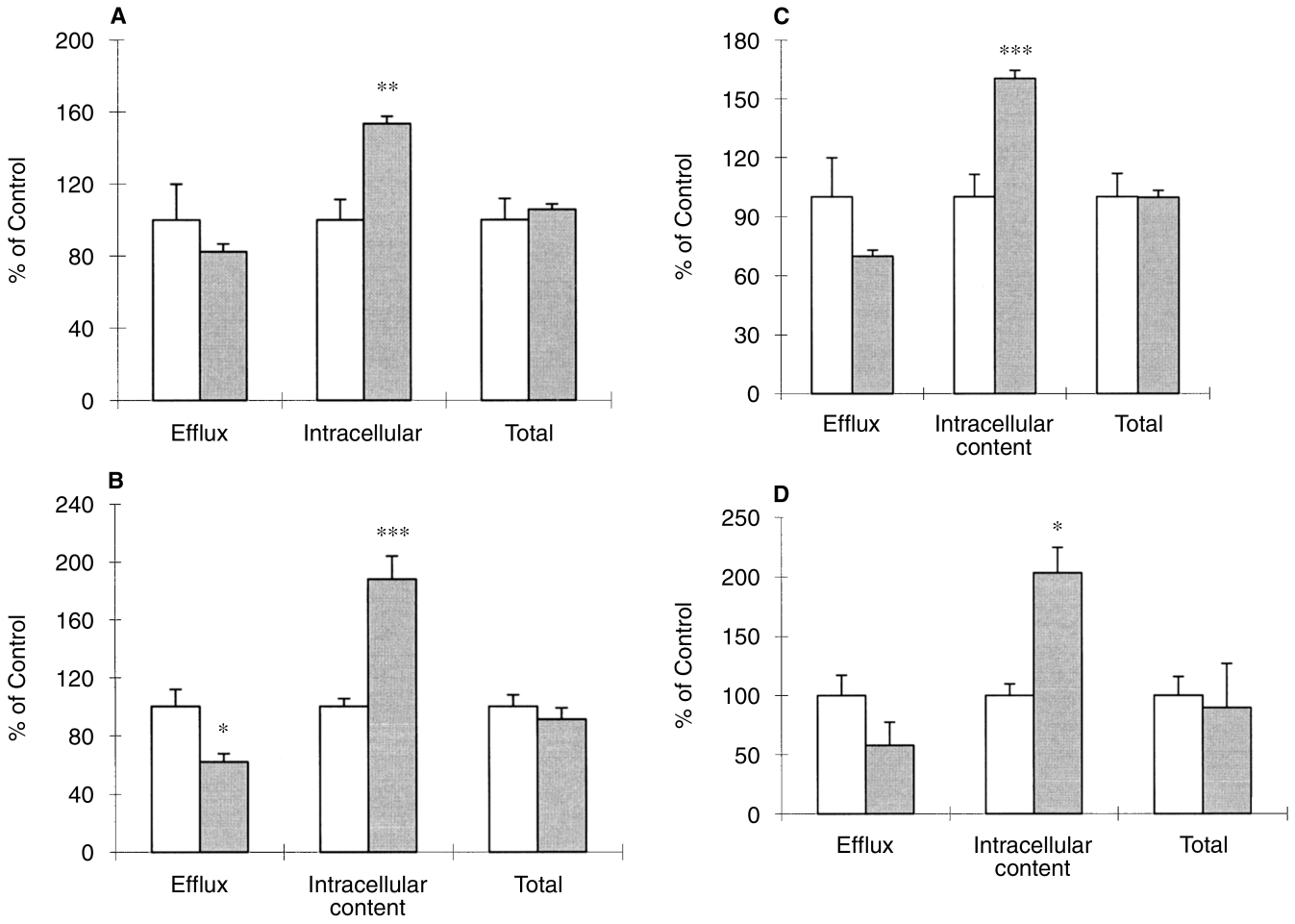


**Fig. 4. Effects of (*A*) 2.5 μmol/L MK571 (■) and control (□), (*B*) 5 μmol/L MK571 (■) and control (□), (*C*) 20 μmol/L cyclosporin A (■) and control (□), and (*D*) 1 mmol/L probenecid (■) and control (□), on sulfation cum transport of harmol sulfate.** Harmol sulfate was determined in the medium and in the cells following uptake of harmol and sodium sulfate, introduced at 1 mmol/L each to Madin-Darby Canine Kidney type II (MDCKII) monolayer cells. The "total" represents the sum of the two. Values are expressed in means ± SD ($N = 3$). *$P < 0.01$; **$P < 0.005$; ***$P < 0.001$

lular transport. The efflux through the apical membrane was inhibited by MK571 (Fig. 8A), resulting in an increased accumulation intracellularly. Harmol sulfate, formed from harmol and sulfate introduced in the basolateral compartment, was detectable in both the apical and basolateral compartments. The transport was again inhibited by MK571, leading to a significant accumulation of intracellular harmol sulfate (Fig. 8B).

## DISCUSSION

The proximal tubules of the kidney are thought to be more metabolically active compared to the distal tubules [8]. Thus, most studies to date had been carried out with proximal tubular cells. However, our study has shown that, besides the proximal tubule, other nephron segments such as the distal tubules of MDCK cells could possess substantial sulfation and transport activities. Other drug-metabolizing enzymes reported in the distal tubules include glutathione-S-transferase [33], steroid 21-hydroxylase [34], phenylethanolamine N-methyltransferase [35], catechol-O-methyltransferase [36] and N-acetyltransferase [37]. Interestingly, all glutathione-S-transferase enzymes showed intense expression in the distal tubules [38]. Our preliminary studies on glutathione conjugation with monochlorobimane, using MDCK type II cells corroborated this last observation. Because of the strategic location of the proximal tubule in the nephron, it is conceivable that it participates more actively in detoxification as it is exposed to the initial filtrate of the glomerulus. However, the distal tubule could provide a backup system. This is particularly relevant as sulfate conjugation is a system of low capacity [39], being limited by the bioavailability of the "active sulfate" or 3′-phosphoadenosine 5′-phosphosulfate. Second, with the reabsorption of water in the distal tubule, any molecule that has escaped biotransformation in the proximal tubule

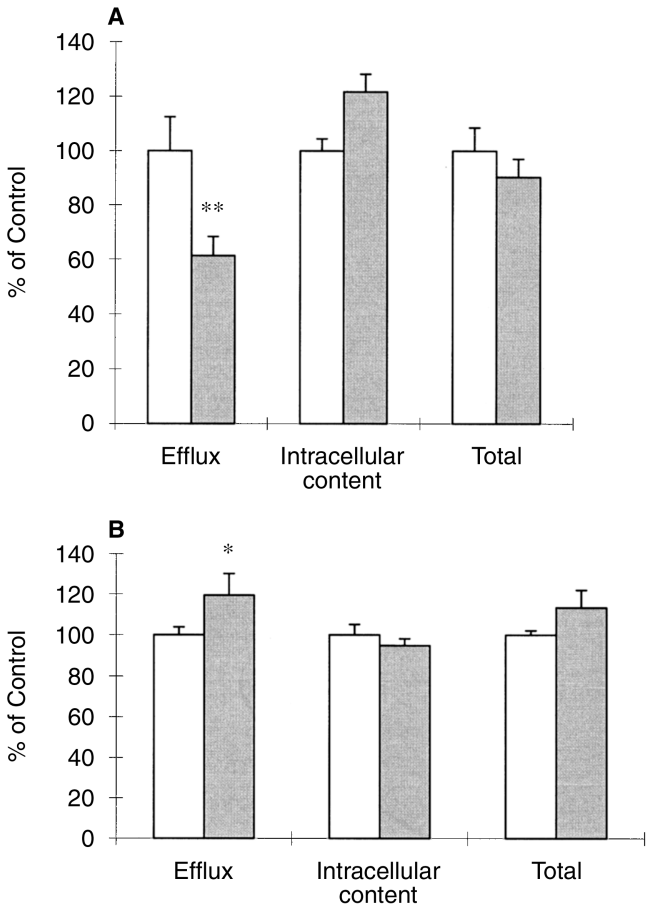


**Fig. 5. Effects of increase and depletion of intracellular glutathione, (GSH), respectively, by preexposure to (*A*) 20 mmol/L glutathione ethyl ester (GEE) (■) and control (□) for 15 minutes and (*B*) 50 μmol/L buthionine sulfoximine (BSO) (■) and control (□) overnight (16 hours) on the sulfation and transport of harmol sulfate by Madin-Darby Canine Kidney type II (MDCKII) monolayer cells**. Values are means ± SD ($N = 3$). *$P < 0.05$; **$P < 0.01$

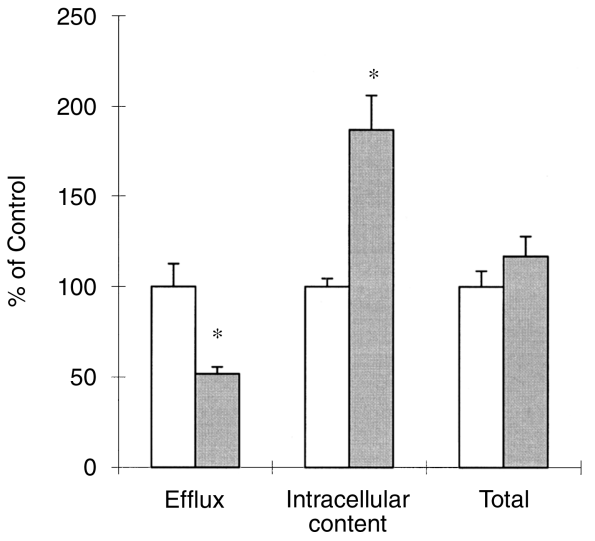


**Fig. 6. Combined effects of preexposure to 20 mmol/L glutathione ethyl ester (GEE) and control (□) for 15 minutes followed by co-exposure of 2.5 μmol/L MK571 (■) and 1 mmol/L each of harmol and sodium sulfate.** Harmol sulfate was measured in the external medium and intracellularly in treated and untreated Madin-Darby Canine Kidney type II (MDCKII) monolayer cells. Values are means ± SD ($N = 3$). *$P < 0.005$.

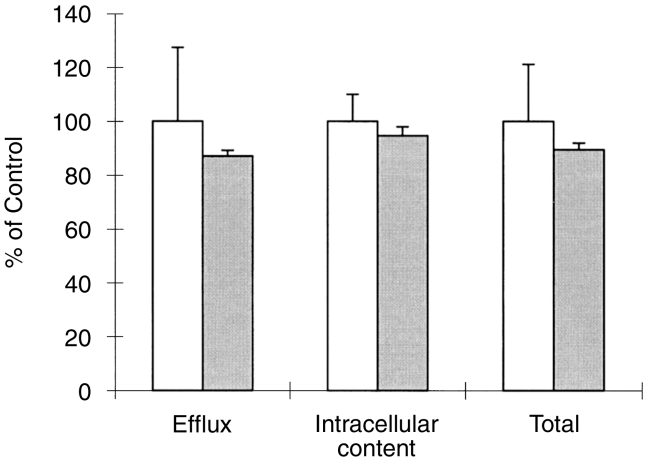


**Fig. 7. Effects of 5 μmol/L methotrexate (■) and control (□) on sulfation cum transport with harmol as substrate.** No significant difference was observed in harmol sulfate in the extra- and intracellular compartments between control and treated Madin-Darby Canine Kidney type II (MDCKII) cells. Values are means ± SD ($N = 3$).

would be diluted. A high affinity system such as sulfate conjugation [39] would be particularly advantageous.

Our study with MDCK type II cells, using a few substrates, has demonstrated the sulfate conjugating and transport functions of the distal tubular epithelia. The contribution of the distal tubules to the metabolic function of the kidneys has, therefore, been overlooked both with respect to sulfation (a major phase II detoxification pathway in humans) and the subsequent transport or excretion of the sulfated conjugates (representing the phase III elimination function of the kidney). Interestingly, both the sulfate conjugating and sulfate conjugating cum transport activities measured, respectively, in the lysates and monolayer cells were significantly higher in MDCK type II cells compared to three other types of liver cells (HepG2, human fetal liver, and Chang liver cells) in culture. In addition to uptake of harmol followed by sulfation and efflux of harmol sulfate formed, polarized MDCK type II cells could also take up preformed harmol sulfate through the basolateral membrane and its transport through the apical membrane could be inhibited by MK571. Previous studies from our laboratory had shown that kidneys from monkey, mouse, and dog exhibited higher activity in sulfation of isoprenaline and harmol compared to the corresponding hepatic tissues [10, 11]. Appreciable sulfation had also been reported in isolated perfused kidneys [1, 40].

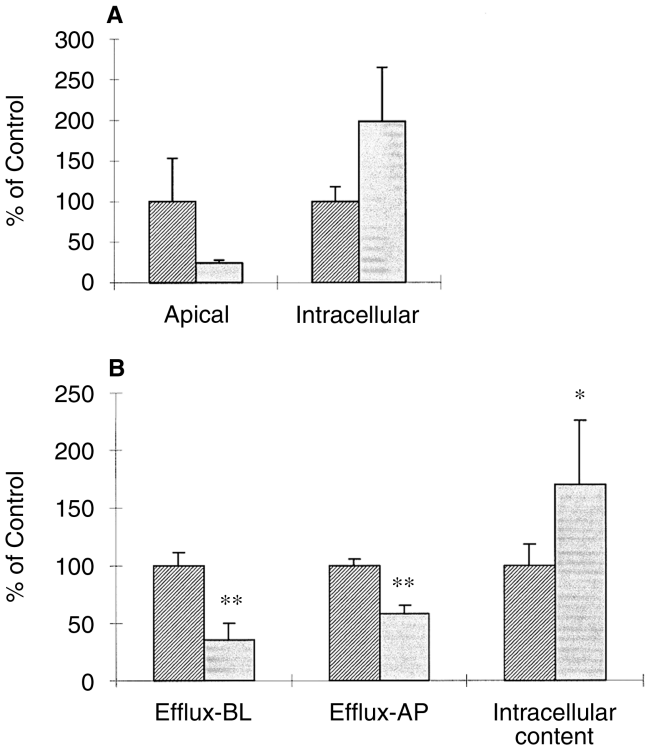


**Fig. 8. Transcellular transport of harmol sulfate.** (*A*) Preformed harmol sulfate was introduced in the lower compartment of polarized Madin-Darby Canine Kidney type II (MDCKII) cells grown on Transwell inserts. Harmol sulfate was measured in the upper (apical) compartment and intracellularly by high-performance liquid chromatography (HPLC)-fluorimetry. (*B*) Inhibition of transport of harmol sulfate synthesized intracellularly from 1 mmol/L each of harmol and sodium sulfate in polarized Madin-Darby Canine Kidney type II (MDCKII) cells. When 25 μmol/L MK571 (■) was introduced in the upper compartment there was intracellular accumulation of harmol sulfate in both (A) and (B) as compared to controls (▨). Values are means ± SD ($N = 3$). *$P < 0.01$; **$P < 0.005$. Abbreviations are: AP, apical; BL, basolateral.

Harmol had been used as a substrate for both sulfate and glucuronide conjugation [24, 41–43]. From pharmacokinetic studies, 70% of an administered dose of harmol was excreted in the urine as harmol sulfate [44]. Extrahepatic clearance of harmol appeared to contribute to 77% of total body clearance, suggesting that extrahepatic conjugation of harmol, possibly by the kidneys, could be substantial [44]. Indeed, harmol was found to be a suitable substrate for sulfate conjugation measured in MDCK type II cell lysate (Fig. 1). The activity was three to four times higher than that of HepG2 cells (Fig. 1). Our aim was to identify candidate transporters localized in the apical membranes of MDCK type II cells, using harmol as a substrate. The transporters, MRP2, OATP1 and OATK1/K2 [15–17], are directly responsible for the secretion and excretion of organic anions in the urine because of their luminal location. Localization of MRP2 and mrp2 was demonstrated in apical membrane of MDCK-transfected cells using MRP2-specific antibody and with human MRP2 fused to green fluorescent protein (GFP) to its C-terminus [18, 20, 45]. However, endogenous expression was not shown in nontransfected MDCK and human embryonic kidney (HEK) cells [20], suggesting that native expression may be too low for detection. We had attempted to demonstrate transport activity in the apical membrane by using MDCK polarized cells and our results showed that the efflux activity could be inhibited by MK571, a specific inhibitor of MRP2 (Fig. 8 A and B).

Three inhibitors of MRP, specifically MK571, CsA at micromolar concentration, and probenecid at millimolar concentration, inhibited the efflux of harmol sulfate with accompanying intracellular accumulation, suggesting that MRP2 or MRP-like transporter/s may be responsible for the transport of harmol sulfate. However, this does not exclude other MRP isoforms present on the basolateral membrane such as MRP1 [46]; this isoform shares a broad substrate specificity with MRP2 [47]. Thus, MK571 and CsA have also been shown to inhibit transport of *p*-aminohippurate (PAH) in membrane vesicles prepared from MRP1- and MRP2- transfected cells [48]. Another basolateral isoform, MRP3, is expressed at a very low level in kidney [49]. The localization of other MRP isoforms are not well characterized [14]. Thus, of the known MRP isoforms, MRP2, with its localization at the apical membrane [17–20], would more likely contribute to the excretion of anionic conjugates in the urine. Interestingly, chronic renal failure resulted in a specific overexpression of MRP2 in both kidney and liver. while the expression of the other apical multidrug transporter P-glycoprotein was unchanged [50]. Verapamil, a specific inhibitor of MDR, when introduced at 0.1 mmol/L did not affect the kinetics of harmol sulfation cum transport in our assay system with MDCK type II monolayers (data not shown), suggesting that the MDR transporter(s) may not be involved.

In addition to the MRPs discussed above, the OATPs form another family of multispecific organic anion transporters. Some OATP-related transporters include OATP1, OATP2, and OATP3 [51–53] and the kidney-specific OAT-K1/K2 [29–32]. Although OATP was characterized as a basolateral transporter in liver [54], it was localized to the apical membrane of the proximal tubules of rat kidney [55]. The first evidence of the involvement of glutathione in the transport function of OATP came from the observation that glutathione efflux might drive the uptake of organic anions such as the glutathione conjugate of bromosulfophthalein (BSP), a known substrate of OATP [56]. Further reports on the trans-stimulation between glutathione and certain organic solutes supported a role of glutathione [57, 58]. Glutathione efflux provides the driving force for uptake of taurocholate into hepatocytes by OATP1 with a stoichiometry of 1:1 for the glutathione:taurocholate exchange [59]. Our

results on the transport of harmol sufate suggested that such an exchange system between harmol sulfate and glutathione may exist in MDCK type II cells. Preloading the cells with GEE to increase intracellular glutathione demonstrated a significant intracellular accumulation of harmol sulfate, with a corresponding decrease in the medium. This was possibly due to an enhanced reuptake of harmol sulfate aided by the higher glutathione content in the cells that drives the OATP exchanger. Conversely, BSO, which inhibits glutathione biosynthesis and thereby depletes cellular glutathione, showed the opposite effect. The enhanced action of GEE and MK571 was probably the result of the combined effects of these compounds on the two transporters. As the effect of MK571 was partial (Fig. 4 A and B), the fraction of harmol sulfate transported out was taken back into the cells by the glutathione-organic anion exchanger, OATP1. This reuptake of harmol sulfate was facilitated by GEE.

Two recent reports showed that parental non-transfected MDCK type II cells had negligible endogenous expression of OATP8 and OATP2 compared to the corresponding single or double-transfected counterparts [60, 61]. The transcellular transport of BSP was negligible in the control and single transfectants [60]. Likewise, transcellular transport of all the substrates studied, specifically, 17β-estradiol-D-17β-glucuronide, pravastatin, estrone-3-sulfate, dehydro- epiandrosterone sulfate, taurolithocholate sulfate, and leukotriene $C_4$, were barely detectable in nontransfected MDCK type II cells except at 2 hours' incubation [61]. Our studies with native MDCK type II cells demonstrated appreciable transport of harmol sulfate and this activity was modulated by changes in intracellular glutathione, suggesting that an OATP-like transporter could be involved. Other transporters whose function appears to be influenced by intracellular glutathione concentrations cannot be excluded [62–64].

A third apical anionic transporter in kidney epithelia is OATK1/K2. It is involved in the transport of the anticancer drug, methotrexate, among other substrates [29–32]. Methotrexate had no effect on the transport or accumulation of harmol sulfate. Similar studies in our laboratory showed that methotrexate from 5 μmol/L to 20 μmol/L also had no effect on glutathione conjugation of monochlorobimane cum transport (unpublished data). Thus the OATK1/K2 isoform is probably not involved in the transport of anionic sulfated and glutathione conjugates.

MDCK is a fast growing cell line and has been shown to be suitable for study of membrane permeability [65]. When transfected with the *mdr1* gene, it provides a good model for screening of Pgp substrates and inhibitors [66]. Our study shows that MDCK cells, though of a distal tubular origin, are metabolically active in sulfate conjugation and transport. The parental (nontransfected) cells provide a convenient and robust cell system for the study of normal physiologic functions of renal cells in terms of uptake of substrates, followed by intracellular sulfate conjugation, and the eventual efflux of the sulfated conjugates. This normal system can also be used to examine the action of potential substrates, inhibitors, and modulators of transporter of sulfate conjugates. The ability of MDCK cells to differentiate into columnar epithelia to form tight junctions when cultured on semipermeable membranes is an added attractive feature for study of directional transport. Polarized MDCK type II cells grown for 4 days on Transwell inserts showed apical transport of harmol sulfate (both preformed and biosynthesized from precurors harmol and inorganic sulfate); this efflux could be inhibited by MK571 introduced at the apical compartment. Recent reports had cautioned that the loss of polarity in transfected cells could compromise their use in the study of vectorial transport [66, 67]. Our studies, however, were performed on normal nontransfected MDCK type II cells.

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