# Effects of Deoxycholate on the Transepithelial Transport of Sucrose and Horseradish Peroxidase in Filter-Grown Madin–Darby Canine Kidney (MDCK) Cells

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Madin–Darby canine kidney (MDCK) epithelial cells grown on microporous polycarbonate filters were used as a model system to investigate the mechanisms of enhancement by deoxycholate in the transepithelial transport of horseradish peroxidase (HRP) and $^{14}$C-sucrose. Deoxycholate at 0.025% had no effect on the transepithelial electrical resistance (TEER); a fivefold enhancement on the transepithelial transport of HRP, but not on that of $^{14}$C-sucrose, was observed. Deoxycholate at 0.05% induced a reversible decrease of TEER; a 2- and 50-fold enhancement on the transepithelial transport of $^{14}$C-sucrose and HRP, respectively, was observed. At 0.1%, deoxycholate induced an irreversible decrease in TEER and the epithelial barrier in the cell monolayer was completely eliminated. A 3.3-fold increase in cellular uptake in HRP, but not in $^{14}$C-sucrose, was also observed in the presence of 0.025% deoxycholate. The increase in cellular uptake was abolished when HRP was conjugated to polylysine. These results suggest that deoxycholate can increase the transepithelial transport by at least two different mechanisms, i.e., a transcellular pathway, possibly due to the enhancement of cellular uptake of selective molecules, and a nonselective paracellular pathway, due to the loosening of tight junctions by deoxycholate at higher concentrations.

KEY WORDS: deoxycholate; transepithelial transport enhancement; Madin–Darby canine kidney (MDCK) cells.

## INTRODUCTION

Bile salts are a class of penetration enhancers which have been widely used to promote the absorption of drugs, particularly peptides or proteins, across nasal (1), buccal (2), or rectal (3) epithelia. Even though enhancement by bile salts has been demonstrated in the *in vivo* absorption of insulin (4), interferon (5), or enkaphlin (6), the exact mechanism of the action at the molecular and cellular level has not been explicitly elucidated. Effects of bile salts on epithelia are difficult to investigate in animal models, because of the complexity of the *in situ* measurement of various parameters, such as the tightness of the junction between the cells, and the rates of the cellular uptake and the transepithelial transport of macromolecules.

Recent developments of the epithelial cell cultures have provided us with a useful *in vitro* model system for the detection of the response of epithelial cells to various treatments. For example, chelators, such as citrate, have been shown to lower the transepithelial electrical resistance (TEER) on MDCK cells, possibly due to the removal of $Ca^{2+}$ ions involved in the tight junction formation (7). Similarly, both dimethyl sulfoxide and ethanol can cause a reversible decrease in TEER which correlates with an increase in the transepithelial transport of both $^{14}$C-sucrose and horseradish peroxidase (HRP) (8). Most recently, a decrease in TEER has also been demonstrated in cultured kidney epithelial cells upon the treatment of tumor necrosis factor, a cytokine which has been shown to cause epithelial and endothelial leakage (9).

In this paper, we describe the effects of one of the most commonly used bile salts, deoxycholate, on the filter-grown Madin–Darby canine kidney (MDCK) epithelial cell monolayers. We also demonstrate that MDCK cell monolayers can be useful as an *in vitro* epithelial model system for the elucidation of the mechanism of the transepithelial transport, particularly in the presence of penetration enhancers.

## MATERIALS AND METHODS

Eagle's minimum essential medium (MEM), fetal bovine serum (FBS), and trypsin/EDTA were obtained from GIBCO laboratories (Grand Island, NY). Transwells with 24.5-mm diameter and 0.4-μm pore size were obtained from Costar (Cambridge, MA). Sodium deoxycholate and horseradish peroxidase (HRP) were purchased from Sigma Chemical Co (St. Louis, MO). $^{14}$C-Sucrose was purchased from ICN Radiochemicals (Irvine, CA). Polylysine conjugates of HRP (HRP-PLL) were prepared by a thioether coupling reaction as described in our previous report (11).

Strain 1 MDCK cells (a gift from Dr. M. J. Cho, The Upjohn Co.) were routinely cultured at 37°C in a T-25 flask with 5% $CO_2$ and Eagle's MEM supplemented with 10% FBS as previously described (10,11). Cells ($2.2 \times 10^4/cm^2$) were seeded on each of the wells in Transwells. The amount of FBS in the medium was reduced to 2.5% when the transepithelial electrical resistance (TEER), measured with an Epithelial Voltohmmeter, EVOM (World Precision Instruments, West Haven, CT), reached $1000\ \Omega \cdot cm^2$. Generally, confluent monolayers were obtained in 5–6 days with a final TEER of approximately $2000\ \Omega \cdot cm^2$ and were maintained in medium containing 1% FBS. All experiments were carried out in the presence of 1% FBS in the medium.

### Effects of Deoxycholate on the Transepithelial Electrical Resistance (TEER) of MDCK Cell Monolayers

Confluent MDCK cell monolayers in Transwells were incubated at first with medium containing 0.01, 0.05, 0.1, and 0.5% of deoxycholate in the apical compartments. After 1 hr at 37°C, deoxycholate-containing medium was replaced by fresh medium without deoxycholate and the incubation was continued for another 5 hr. TEER across the cell monolayers was measured with EVOM electrodes at various time intervals. Results are expressed as ohms per square centimeter.

### Transepithelial Transport of HRP and $^{14}$C-Sucrose in MDCK Cell Monolayers

Confluent MDCK cell monolayers in the Transwells

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were incubated with 0.025% deoxycholate in the apical medium. Two fluid phase markers, i.e., $^{14}$C-sucrose and HRP, were added together to the apical medium to give final concentrations of 1 μCi/ml and 1 mg/ml, respectively. The cell monolayers were then incubated at 37°C, and at various time intervals, TEER values across the monolayers were measured. Aliquots of 0.1 ml of the medium were pipetted from the basal compartments and the amounts of transported $^{14}$C-sucrose and HRP were determined by measuring the radioactivity and by the colorimetric HRP assay method (12), respectively.

### Cellular Uptake of HRP, $^{14}$C-Sucrose, and HRP-Polylysine Conjugate in MDCK Cell Monolayers

Confluent MDCK cell monolayers grown in six-well cluster plates (Costar) were exposed to the medium containing 0.025% deoxycholate together with HRP (3 mg/ml), $^{14}$C-sucrose (1 μCi/ml), or HRP-polylysine conjugate (3 μg/ml). After incubation at 37°C for 60 min, cells were removed from the plates by trypsin/EDTA, suspended in 2 ml of cold PBS, and transferred to test tubes. After centrifugation, the final cell pellets were washed twice each with 2 ml of cold PBS, then dissolved in 1 ml of 0.1% Triton X-100 in water, and the amount of the intracellular $^{14}$C-sucrose or HRP was determined. Lowry's protein assay (13) was used to normalize the cell number in each sample.

## RESULTS

The effects of various concentrations of deoxycholate on TEER of MDCK-cell monolayers are presented in Fig. 1. In the presence of 0.01% deoxycholate, no change in the TEER of the cell monolayers was found. However, in the presence of 0.05% deoxycholate, a decrease in TEER of the monolayers was observed. The TEER across the cell monolayers fell during the first hour of the treatment to 60% of the control, i.e., from 1356 to 598 Ω · cm$^2$. A rapid recovery of TEER to as high as 120% of the initial values was observed when the deoxycholate-containing medium was replaced by fresh medium without deoxycholate. When the cell monolayers were exposed to 0.1% or higher deoxycholate, a sharp decrease in TEER of the cell monolayers was observed. The decrease in TEER from 1366 to 71 Ω · cm$^2$ in the 1-hr treatment of 0.1% deoxycholate was not recovered during the course of the 6-hr experiment.

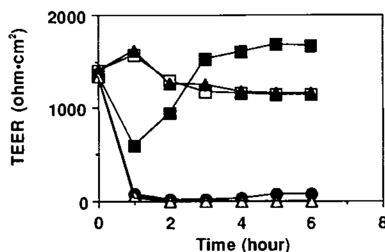


Fig. 1. Effects of various concentrations of deoxycholate on the transepithelial electrical resistance (TEER) of MDCK cell monolayers. The apical surface of the MDCK cells were exposed to medium containing 0.01% (▲), 0.05% (■), 0.1% (●), and 0.5% (△) of deoxycholate during the first hour and then changed to fresh medium for the continuous incubation. TEER in each Transwell was measured at time intervals. Monolayers exposed to medium without deoxycholate were used as controls (□).

To determine if the decrease in TEER in the cell monolayers is due, at least in part, to the alteration of the intercellular gap junctions, the transepithelial transports of $^{14}$C-sucrose and HRP were studied. As shown in Figs. 2A and B, 0.05% deoxycholate induced 2- and 50-fold increases in the transepithelial transport of $^{14}$C-sucrose and HRP, respectively, i.e., an increase from 1.7 to 3.6% of the initial $^{14}$C-sucrose and from 0.3 to 13.6% of the initial HRP transported in 5 hr. The fact that transepithelial transport of HRP in the presence of 0.05% deoxycholate was higher than that of $^{14}$C-sucrose, a fluid phase transport marker, suggests that the transport of HRP was not limited only to the paracellular pathway. Furthermore, when the cell monolayers were exposed to 0.025% deoxycholate, a fivefold increase in HRP transport, i.e., from 0.3 to 1.7% of HRP (Fig. 2A), but not in $^{14}$C-sucrose transport (Fig. 2B), was observed. At this concentration, there was no effect in TEER of the cell monolayers after 5 hr of incubation, i.e., 3075 Ω · cm$^2$ compared to 3066 Ω · cm$^2$ for the control (Fig. 2C).

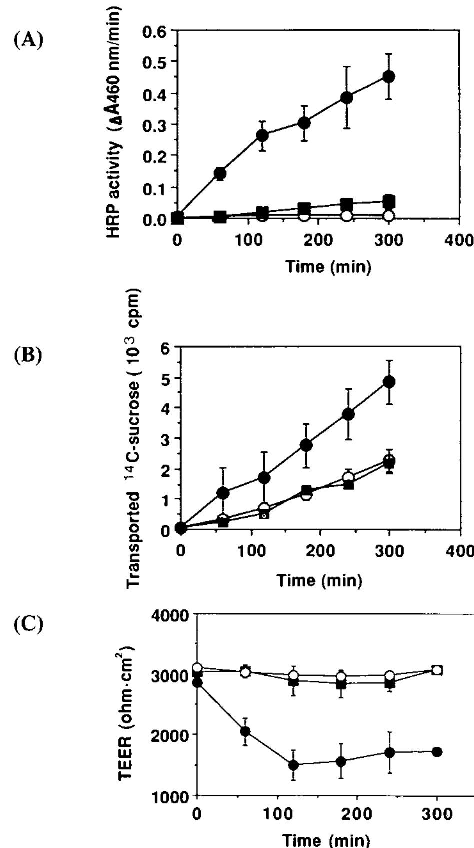


Fig. 2. Effects of deoxycholate on the transport of HRP (A) and $^{14}$C-sucrose (B) and on the TEER (C) in MDCK cell monolayers. Cells were exposed on the apical surface for 5 hr to a medium containing both HRP (1 mg/ml) and $^{14}$C-sucrose (1 μCi/ml) with 0.025% (■), 0.05% (●), or no deoxycholate (○). At various time intervals, 100 μl of the basal medium was pipetted for the measurements of the $^{14}$C-radioactivity and HRP enzymatic activity. Fresh medium was added to the Transwells after each sampling in order to maintain a constant volume of medium in each well. Standard deviations are represented as bars or are smaller than the symbols.

To elucidate further the action of deoxycholate on the transports of $^{14}$C-sucrose and HRP, cellular uptakes of HRP, $^{14}$C-sucrose, and HRP-PLL in MDCK cell monolayers were investigated. As shown in Table I, a 3.3-fold increase in cellular uptake of HRP was observed in the presence of 0.025% deoxycholate. No significant increase was found in the cellular uptake of either $^{14}$C-sucrose or HRP-PLL conjugate in deoxycholate-treated MDCK cells. In fact, in the presence of deoxycholate, the cellular uptakes of $^{14}$C-sucrose and HRP-PLL conjugate were reduced, respectively, to 74 and 33% of that in the controls.

## DISCUSSION

Different mechanisms have been proposed for the enhancement by bile salts on the transepithelial drug transport, including (a) the formation of reverse micelles which, in turn, can form polar channels to allow the diffusion of polar molecules through the plasma membrane (14), (b) the decrease in the viscosity of the mucus layer on the epithelial surface, by which the diffusion of drugs through the plasma membrane can be facilitated (15), (c) the inhibition of the proteinase by bile salts, by which the degradation of protein or peptide can be avoided (16), (d) the interaction of bile salts with $Ca^{2+}$, which may loosen the tight junction between cells and thus may increase the paracellular transport (17). Data presented in this report indicate that deoxycholate may exert its effect on epithelia by at least two different mechanisms. At relatively high concentrations of deoxycholate, e.g., 0.05%, both HRP and sucrose transports were enhanced (Figs. 2A and B). The concentration of deoxycholate used in this transport study, i.e., 0.05% (1.2 m*M*), is lower than the critical micelle concentration (CMC) of this bile salt, i.e., 5 m*M* (18). Therefore, it is unlikely that the increase in transport of HRP and $^{14}$C-sucrose in Figs. 2A and B involved the micelle formation. A decrease in TEER was also observed in the presence of 0.05% of deoxycholate, indicating a loosening of the gap junction in the cell monolayer. This decrease in TEER was reversible (Fig. 1), suggesting that no severe cytotoxicity occurred in the cells. However, the decrease in TEER became irreversible when the concentration of deoxycholate was 0.1% or higher (Fig. 1), possibly because of the damage or detachment of cells in the monolayer under these conditions.

At low concentrations of deoxycholate, i.e., 0.025% or less, no decrease in TEER was observed in MDCK cell monolayers, indicating that the tight junctions were intact (Figs. 1 and 2C). On the other hand, the cross-epithelial transport of the protein marker, HRP, was significantly enhanced (Fig. 2A). Furthermore, a comparable increase in HRP uptake by the deoxycholate-treated MDCK cells was also observed (Table I). Conceivably, such an increase in protein uptake and transport could be due to an increase of the vesiculation of the cell in response to deoxycholate treatment. We show here, however, that sucrose transport was actually reduced under the same conditions that HRP transport was significantly enhanced (Table I). A parallel increase in transcellular transport of the two markers would be expected if there was an increase in vesiculation in the cell. The decrease in the cellular uptake of $^{14}$C-sucrose suggests that deoxycholate may actually suppress endocytosis in the cell. In addition, when HRP was replaced by the polylysine conjugate, HRP-PLL, an even larger decrease in HRP uptake was observed. HRP-PLL is an adsorptive marker for endocytosis, which can be internalized by the cells with a rate 1000-fold higher than that of HRP through the nonspecific binding to the plasma membrane (19). The lack of enhancement on the cellular uptake of HRP-PLL by deoxycholate further confirms that the increase in HRP transport is not due to an increase in the vesiculation in MDCK cells. The striking inhibitory effect of deoxycholate on the cellular uptake of HRP-PLL may be due to an interference of the bile salt with the charge interaction between PLL and plasma membranes. Therefore, it appears that, at low concentrations, deoxycholate has a selective effect on the cellular uptake of HRP. This selectivity could be due to an increase in the diffusion of HRP across the plasma membrane, possibly by the formation of protein–deoxycholate complexes, which were not formed in $^{14}$C-sucrose or HRP-PLL. It can be assumed that the interaction between deoxycholate and HRP, rather than the effect of deoxycholate on the cells, is responsible for the increase of cellular uptake and, subsequently, the transcellular transport of the protein. However, morphological studies of the cells will be required to confirm the lack of increasing vesiculation in the presence of deoxycholate.

In summary, results presented in this report indicate that the mechanism of action of deoxycholate as a penetration enhancer in cultured epithelial monolayers is dependent on the concentration of the bile salt in the medium. At low concentrations, the enhancement of HRP transport is due largely to an increase in the cellular uptake of this protein, most likely by a process other than endocytosis. At higher concentrations, deoxycholate can loosen the tight junction and an increase in the paracellular leakage of fluid in epithelial monolayers can be achieved. Furthermore, results described in this report are in good agreement with those obtained from the effects of deoxycholate on the fresh isolated sheep nasal mucosa in Ussing chambers (20). Because of the simplicity and the reproducibility in cell culture systems, it is conceivable that cultured epithelial cell lines with a good tight junction, such as Strain I MDCK cells, can be useful in elucidating the mechanism and the toxicity of penetration enhancers. This *in vitro* model system may provide impor-

**Table I.** The Effect of 0.025% Deoxycholate on the Cellular Uptake of $^{14}$C-Sucrose, HRP, and HRP–Polylysine Conjugates in MDCK Cell Monolayers[a]

| | Control | 0.025% deoxycholate |
|---|---|---|
| HRP ($\Delta A_{460\ nm}$/min/mg protein) | 1.27 ± 0.08 | 4.17 ± 0.05 |
| $^{14}$C-Sucrose (cpm/mg protein) | 631 ± 39 | 466 ± 34 |
| HRP-PLL ($\Delta A_{460\ nm}$/min/mg protein) | 2.6 ± 0.36 | 0.87 ± 0.29 |

[a] Data represent the means of three measurements ± standard deviations from six-well cluster plates (Costar) with confluent MDCK cells. Concentrations of the markers in the medium were 3 mg/ml HRP, 1 μCi/ml $^{14}$C-sucrose, and 3 μg/ml HRP-PLL. No endogenous HRP activity was detectable in the untreated MDCK cells.

tant information regarding the design of effective and safe penetration enhancers for noninvasive drug delivery.

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