Sodium-coupled Taurocholate Transport in the Proximal Convolution of the Rat Kidney In Vivo and In Vitro

FREDERICK A. WILSON, GERHARD BURCKHARDT, HEINI MURER, GERHARD RUMRICH, and KARL J. ULLRICH, *Max Planck Institute for Biophysics, D-6000 Frankfurt am Main, Federal Republic of Germany*

ABSTRACT Using the standing droplet technique in the renal proximal convolution and simultaneous microperfusion of the peritubular capillaries, the zero net flux transtubular concentration difference of taurocholate (ΔC_{TC^-}) at 45 s was determined as a measure of active bile acid reabsorption in vivo. Starting with 0.1 mmol/liter taurocholate in both perfusates the control ΔC_{TC^-} of 0.042 mmol/liter fell to 0.006 mmol/liter ($P < 0.001$) when the Na^+ concentration in the perfusates was reduced to zero. Removal of bicarbonate from the perfusates to alter pH had no influence on ΔC_{TC^-}. When glycocholate was added to the perfusates ΔC_{TC^-} was decreased, while probenecid increased ΔC_{TC^-}.

These observations were extended by studies performed with brush border membrane vesicles derived from renal cortex. The initial (20 s) uptake of 0.01 mmol/liter taurocholate in the presence of a $Na_0^+ > Na_i^+$ gradient was stimulated twofold compared with its uptake in the absence of a Na^+ gradient. Uptake of taurocholate was osmotically and temperature sensitive. Membranes preloaded with unlabeled glycocholate showed accelerated entry of labeled taurocholate (trans-stimulation) only in the presence of Na^+. Replacement of Na^+ in the media with K^+, Li^+, and choline$^+$ decreased initial taurocholate uptake by 49, 53, and 62%, respectively. Stimulation of taurocholate transport by cation gradient diffusion potentials was unlikely inasmuch as the addition of valinomycin under K^+ gradient conditions had no effect. A transmembrane pH gradient ($pH_0 < pH_i$) did not influence initial uptake of taurocholate. Finally, in the presence of Na^+ taurocholate transport showed *cis*-inhibition with unlabeled bile acids and saturation kinetics with respect to increasing taurocholate concentrations. The micropuncture and vesicle data indicate that the net transport of taurocholate in the proximal tubule is the result of an electroneutral Na^+-taurocholate cotransport across the brush border membrane.

INTRODUCTION

Normal total bile acid levels in the peripheral circulation are <8 μmol/liter, as the substances are effectively cleared from the portal blood by the liver. However, in obstructive liver disease bile acid levels may climb to values >0.2 mmol/liter (1). This implies that with obstructive liver disease the remaining excretory pathways for bile acids, e.g., the kidney, are relatively inefficient. Urinary excretion of bile acids is minimized because of tubular reabsorption and binding to serum components which prevents glomerular filtration (2). The reabsorption was first detected in patients with hepatic disease when it was calculated that 24-h urinary excretion of bile acids was less than the amount filtered (3). Subsequent studies in dogs characterized an active reabsorptive process for bile acids in the proximal tubule that is opposed by a tubular secretory flux (4, 5).

Since these studies were conducted, techniques have been devised that directly examine absorption across the renal tubule in vivo (6) and brush border membrane vesicles isolated from renal cortex (7). In the present studies it was possible to discriminate between taurocholate reabsorption and secretion by applying the standing droplet technique in vivo to evaluate local

This work was presented in part on 18 March 1980 at the spring meeting of the German Physiology Society, Kiel, West Germany and presented in abstract form in 1980. *Pflügers Arch. Eur. J. Physiol.* 374(Suppl.): R8.

Dr. Wilson was the recipient of a Senior International Fellowship (F06-TW 00342) of the Fogarty International Center, National Institutes of Health, and a Vanderbilt University Fellowship Award. His present address is AA5206 Medical Center, Vanderbilt University, Nashville, Tenn. 37232.

Received for publication 8 August 1980 and in revised form 25 November 1980.

transport rates in the presence and absence of sodium and probenecid in the perfusates. These studies were extended to observations with brush border vesicles which together suggest that the transport of taurocholate in the proximal tubule is the result of a secondary active, Na^+-dependent reabsorptive process involving coupling of Na^+ and taurocholate flux across the brush border membrane.

METHODS

Standing droplet method and simultaneous microperfusion of the peritubular capillaries. The standing droplet method and simultaneous microperfusion of the peritubular capillaries have been described in detail previously (6). Briefly, 200–240 g male Wistar rats kept on Altromin standard diet were anesthetized with Inactin (Byk Gulden, Constance, West Germany), 120–150 mg/kg body wt intraperitoneally. Each animal was mounted on a 37°C thermostated table, and the left kidney was exposed by flank incision and immobilized in a plastic cup. The kidney was decapsulated and the proximal convoluted tubule, as well as the peritubular blood capillaries, were punctured with 7–8-μm o.d. sharpened glass micropipettes. After 45 s of capillary and tubular perfusion (1–3 μl/min and ~50 nl/min, respectively) with a so-called equilibrium solution containing 0.1 mmol/liter [^{3}H]taurocholate, the luminal perfusate was stopped, enclosed between oil columns and withdrawn 45 s later. To study the time dependence of the development of the transtubular concentration difference of taurocholate, samples were also withdrawn at 2.5, 5, and 10 s. The collection was repeated two to six times without interrupting the capillary perfusion. The samples were pooled to obtain sufficient volume (0.5–1.5 nl) for analysis. To avoid tubular heterogeneity (8) and the influence of perfusion time on the results in some series, the crossed pairs sampling technique (6) was applied. In this case, after the first 45-s luminal samples were taken, the perfusion pipettes were removed and replaced by fresh pipettes containing different test solutions. After 45 s of continuous perfusion with the new solutions, a second series of 45-s luminal samples were obtained from the same tubular loop. In the course of each experiment the sequence, control → test solution, was alternately reversed. When the change (control → test) differed significantly according to the Student's t test (9) from the crossed paired samples (test → control) we concluded that taurocholate transport changed significantly. In pilot experiments taurocholate escaped when samples were placed under paraffin oil to measure sample size. Therefore, 1.75 mmol/liter [^{14}C]urea was added to the perfusates as a volume marker after it was determined that the urea concentration remained constant after the 45-s perfusion. The ^{3}H counts of the labeled taurocholate and the ^{14}C counts of the labeled urea in the capillary perfusate and the sampled luminal perfusates were measured in a scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) with Spectrafluor toluene (Amersham Corp., Arlington Heights, Ill.) as scintillation fluid.

The standard solution used for capillary perfusion contained in mmol/liter: Na^+, 150; Cl^-, 128; HCO_3^-, 25; K^+, 3; Ca^{2+}, 1.5; Mg^{2+}, 1; acetate$^-$, 5; [^{3}H]taurocholate, 0.1; and was gassed with 95% O_2, 5% CO_2. Unless otherwise specified the luminal perfusate composition was Na^+, 134.5; Cl^-, 133.5; HCO_3^-, 4; K^+, 3; Ca^{2+}, 1.5; Mg^{2+}, 1; acetate$^-$, 5; [^{3}H]taurocholate, 0.1; raffinose, 31; and was gassed with 95% O_2, 5% CO_2. With this equilibrium solution, the net flux of water and solutes was zero. When Na^+-free solutions were used, the capillary perfusate was identical to the standard capillary perfusion except all Na^+ was replaced by choline$^+$. In the luminal perfusate the raffinose concentration was lowered to 16 mmol/liter, and the other concentrations in the luminal perfusate were: choline$^+$, 142; Cl^-, 141; HCO_3^-, 4; K^+, 3; Ca^{2+}, 1.5; Mg^{2+}, 1; acetate$^-$, 5; and [^{3}H]taurocholate, 0.1 mmol/liter. When HCO_3^--free solutions were used, all HCO_3^- in the perfusates were replaced by Cl^-. In the luminal perfusate the raffinose concentration was lowered to 10 mmol/liter, and the other concentrations in the luminal perfusate were: Na^+, 145; Cl^-, 149; K^+, 4; Ca^{2+}, 1.5; Mg^{2+}, 1; acetate$^-$, 5; and [^{3}H]taurocholate 0.1 mmol/liter. The capillary perfusate contained in mmol/liter: Na^+, 150; Cl^-, 154; K^+, 4; Ca^{2+}, 1.5; Mg^{2+}, 1; acetate$^-$, 5; and [^{3}H]taurocholate, 0.1.

Brush border membrane vesicles. Brush border membrane vesicles were prepared from renal cortex of 180–220 g, male Wistar rats by a method previously used in our laboratory (7). In essence, renal cortical slices were homogenized in a hypotonic medium. After the addition of $CaCl_2$ (final concentration 10 mM) the brush border membranes were purified by differential centrifugation. Purity of the membranes was assessed by the measurement of alkaline phosphatase (EC 3.1.3.1) and $Na^+ + K^+$ ATPase (EC 3.6.1.3) as described (7). Protein was determined by the method of Lowry et al. (10) after precipitation with ice-cold 10% trichloroacetic acid (TCA) using bovine serum albumin as a standard. The enrichment in specific activity (final pellet per homogenate) of the luminal marker, alkaline phosphatase, was >12 times; whereas, enrichment of the basolateral marker, $Na^+ + K^+$ ATPase, was < 1.0. This purity of the membranes was similar to that reported (7).

Uptake of radiolabeled compounds by isolated brush border membrane vesicles was measured by the rapid filtration technique (11). The basic composition of the incubation medium was 100 mM mannitol and 20 mM Hepes/Tris (pH 7.4). Additions to the incubation media are given in the figure legends. Transport was initiated by adding 5–20 μl of membrane suspension to 25–100 μl of incubation medium kept in a water bath. All experiments were carried out at 37°C with the exception of those presented in Fig. 4 which were also performed at 0° and 24°C. At the desired time interval a 20-μl aliquot was removed from the incubation suspension and diluted in 1 ml of ice-cold stop solution. The stop solution contained 100 mM mannitol, 20 mM Hepes/Tris (pH 7.4), 25 mM $MgSO_4$, 100 mM choline Cl, 0.1 mM taurocholate, and when D-glucose uptake was measured, 0.2 mM phlorizin. The stop solution containing the vesicles was immediately pipetted onto the middle of a filter (cellulose nitrate, 0.65 μm pore size, Sartorius, Göttingen, West Germany) and kept under suction. The filter was then immediately washed with 5 ml of ice-cold stop solution. The radioactivity remaining on the filters was counted with standard liquid scintillation techniques. Rotiszint 33 (Roth, Karlsruhe, West Germany) was used as a scintillator which completely dissolved the radioactivity from the filters. All experiments were performed at least in duplicate with freshly prepared membranes and were repeated at least twice with different membrane preparations for a total of six or more determinations. Absolute solute uptake was expressed as picomole per milligram of protein. Analysis of the data for significant differences was according to the Student's t test (9).

Materials. [G-^{3}H]Taurocholic acid (3.58 Ci/mol sp act), [^{14}C-U]D-glucose (310 μCi/μmol) and [^{14}C]urea (10 mCi/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Taurocholic acid and Hepes were obtained from Serva, Heidelberg, West Germany, and glycocholic and cholic acids from Steraloids, Inc., Pawling, N. Y. Enzymes and substrates for alkaline phosphatase and ($Na^+ + K^+$)-stimulated ATPase assays were obtained from Merck AG, Darmstadt, West Germany and C. F. Boehringer and Sons, Mannheim, West Germany, respectively. Valinomycin was obtained from Boeh-

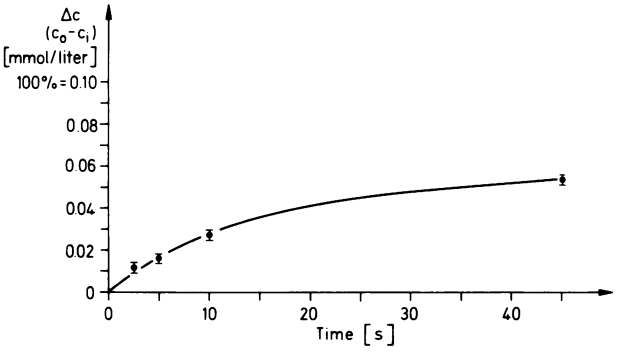

FIGURE 1 Development of the transtubular ΔC of taurocholate with time in the presence of sodium. The taurocholate concentration in the luminal and in the capillary perfusate at time 0 are 0.1 mmol/liter. Hence, the maximal concentration difference $\Delta C = C_{capillary} - C_{lumen}$ ($C_o - C_i$) is 0.1 mmol/liter. Values are obtained with the "equilibrium solution" containing sodium. The development of ΔC in the standing droplet was determined at 2.5, 5, 10, and 45 s. The half-time for the development of ΔC is 9.5 s. The values represent the mean and SE of 9 (2.5, 5, and 10-s values) and 60 (45-s values) determinations, respectively.

ringer. As valinomycin was added in an ethanol solution, equivalent volumes of ethanol were added to control incubations. All other chemicals of the highest purity available were purchased from Merck.

RESULTS

In vivo studies

Development of taurocholate concentration difference. Fig. 1 shows the time dependence for the development of a transtubular concentration difference of taurocholate (ΔC_{TC^-})[1] in the presence of sodium. The half time for reaching the maximal transtubular ΔC_{TC^-} is 9.5 s. A maximal transtubular ΔC_{TC^-} is approached within 45 s. Thus, the ΔC_{TC^-} at 45 s is regarded as the equilibrium transtubular ΔC_{TC^-} at zero net flux conditions. The level flow calculated on the basis of these data is 5.47 pmol/cm²·s (12).

Sodium dependence. Fig. 2 shows the zero net flux ΔC_{TC^-} in the late proximal convolution of the rat kidney in vivo. The mean ΔC_{TC^-} for control animals of 0.042 mmol/liter indicates a 42% drop in luminal concentration and, therefore, active taurocholate reabsorption. Fig. 1 also shows the sodium dependence of the ΔC_{TC^-}. When the Na^+ in the perfusates was replaced by choline$^+$, the ΔC_{TC^-} fell to 0.006 mmol/liter. Thus, in a Na^+-free environment the tubule was practically unable to lower the luminal concentration of taurocholate which suggests that Na^+ is required for active taurocholate reabsorption.

[1] *Abbreviations used in this paper*: ΔC_{TC^-}, concentration difference; Na_i^+, Na_{inside}^+; Na_o^+, $Na_{outside}^+$.

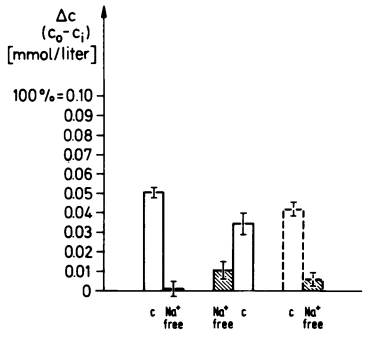

FIGURE 2 The effect of Na^+ on the zero net flux ΔC of taurocholate in the late proximal convolution of the rat kidney in vivo after a 45-s contact time. The taurocholate concentration in the capillary perfusate and hence the maximal possible ΔC was 0.1 mmol/liter. Furthermore, under our zero net flux conditions the transtubular electrical potential difference is +1.9 mV, lumen positive which according to the Nernst equation corresponds at equilibrium to a $(TC^-)_i/(TC^-)_o$ of 1.072. Thus, at the mean transtubular concentration present in our experiment the zero electrochemical potential value would only account for a ΔC_{TC^-} value of −0.007 mmol/liter. Control values (open bars) were obtained with a standard test solution, and test values (shaded bars) were obtained with a Na^+-free solution (see Methods). The sequence, control → test (pair 1), and the reverse sequence, test → control (pair 2), were made. The *P* value refers to the significance of the difference in pair 1 to that in pair 2. The bars with the interrupted outline indicate the mean of nine pairs of control and test measurements; $P < 0.001$.

Effect of pH. As shown in Fig. 3, the transtubular ΔC_{TC^-} always decreased during the second measurement period made at the same tubular location. This decrease was not significantly different ($P > 0.05$) regardless of whether both the lumen and capillaries

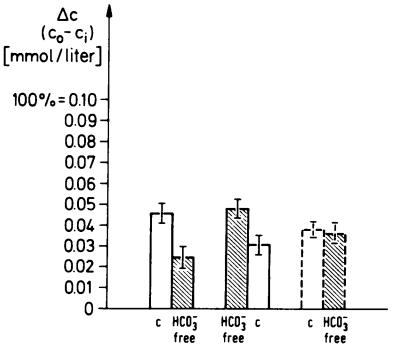

FIGURE 3 Lack of effect of HCO_3^- in capillary and luminal perfusates on taurocholate reabsorption in vivo after a 45-s contact time. Control values (open bars) were obtained with a standard solution containing 25 mmol/Liter HCO_3^- and test values were obtained with a HCO_3^--free solution. The sequence, control → test (pair 1), and the reverse sequence, test → control (pair 2), were made. The *P* value refers to the significance of the difference in pair 1 to that in pair 2. The bars with the interrupted outline indicate the mean of nine pairs of control and test measurements, $P > 0.05$.

were first perfused with 0 and then 25 mmol/liter bicarbonate or vice versa. Thus, changing the luminal and intracellular pH to a value much lower than in the presence of bicarbonate, i.e., ~6.7 and 7.4, respectively (13), had no influence on taurocholate reabsorption.

Effect of glycocholate and probenecid. As shown in Fig. 4, agents that potentially influence the bile acid transport system were added to both perfusates. The addition of a second bile acid, glycocholate, at a concentration sixfold higher than taurocholate decreased the ΔC_{TC^-} by 31% ($P < 0.001$). In contrast, probenecid, added to the perfusates in a concentration of 1 mmol/Liter, increased the ΔC_{TC^-} by 21% ($P < 0.001$). Thus, the structural analogue, glycocholate, inhibited taurocholate reabsorption; whereas, probenecid, an agent known to inhibit renal secretion of organic acids (14), further enhanced net taurocholate reabsorption.

Brush border membrane studies

Sodium dependence. Inasmuch as the above in vivo studies suggested the coupling of Na^+ and taurocholate transport by the proximal tubule, the effect of Na^+ on taurocholate uptake by renal brush border membrane vesicles was first investigated. As shown in Fig. 5, when vesicles were prepared in a Na^+-free medium and incubated in a Na^+-containing buffer, taurocholate showed a rapid initial uptake during the first 2 min. The initial uptake of taurocholate in the presence of the $Na^+_{outside} > Na^+_{inside}$ ($Na^+_0 > Na^+_i$) gradient was stimulated more than two-fold compared with its uptake in the total absence of Na^+, in the absence of a Na^+ gradient across the membrane or when Na^+ initially was present only inside the vesicles. The significant difference ($P < 0.05$) persisted up to 2 min of incubation.

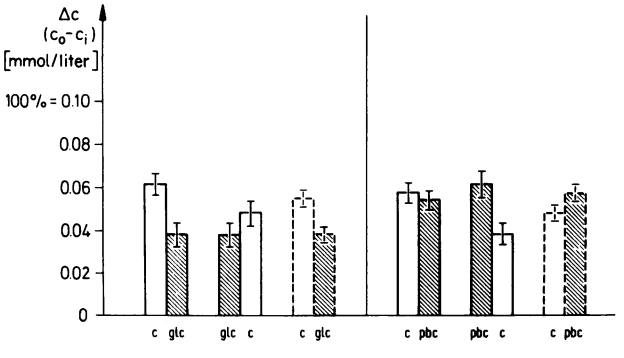

FIGURE 4 Effect of glycocholate (glc) and probenecid (pbc) added in a concentration of 0.6 mmol/liter and 1.0 mmol/Liter, respectively, to the perfusates on proximal taurocholate reabsorption in vivo. Represented are mean ΔC values±SE observed after a 45-s contact time. Open bars indicate control (c) and shaded bars test experiments. Bars with interrupted outline indicate the mean of nine pairs of control and test measurements; $P < 0.001$ in each case.

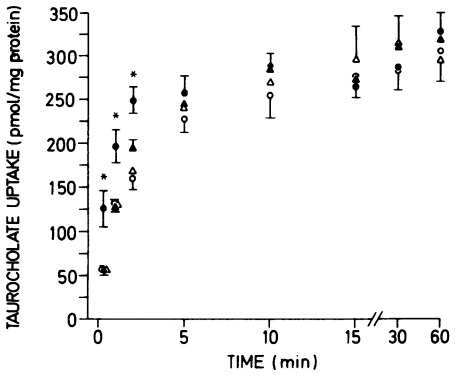

FIGURE 5 Effect of Na^+ on taurocholate uptake by brush border membrane vesicles. ●, 100/0 mmol/liter: Membranes were loaded with 100 mM mannitol, 100 mM choline Cl and 20 mM Hepes/Tris (pH 7.4) and then uptake of 10 μM [^{3}H]-taurocholate was assayed at 37°C in the presence of 100 mM mannitol, 20 mM Hepes/Tris, and 100 mM NaCl. ○, 0/0 mmol/liter: Membranes were loaded and uptake was assayed in the presence of 100 mM mannitol, 100 mM choline Cl, and 20 mM Hepes/Tris. ▲, 100/100 mmol/liter: Membranes were loaded and uptake was assayed in the presence of 100 mM mannitol, 20 mM Hepes/Tris and 100 mM NaCl. △, 0/100 mmol/liter: Membranes were loaded with 100 mM mannitol, 20 mM Hepes/Tris and 100 mM NaCl and uptake was assayed in the presence of 100 mM mannitol, 100 mM choline Cl and 20 mM Hepes/Tris. Each point represents the mean±SE for six determinations. *, for 100/0 is statistically significant from other values ($P < 0.05$).

Metabolism, binding, and diffusion. The next series of experiments were to discriminate membrane transport effects from those associated with metabolism or binding. Membrane vesicles first were incubated up to 60 min with radiolabeled taurocholate. When the vesicular radioactivity was extracted with methanol overnight and submitted to thin-layer chromatography using the solvent system isoamyl alcohol/propionic acid/*n*-propanol/H_2O (60:60:40:30, by vol), >95% of the radioactivity had an R_F value identical to that of taurocholate.

The 60 min uptake of 0.01 mM taurocholate was assayed in media of varying sucrose concentrations. A plot of uptake vs. 1/osmolarity was linear. An estimate of binding was made from extrapolation of the regression line to infinite osmolarity (1/osmolarity = 0) (15). This analysis indicated that under standard conditions >70% of the taurocholate responded to alteration of the intravesicular space by changing osmolarity of the medium. It is assumed that only this part represents free taurocholate kept in the intravesicular space. The remaining taurocholate may be bound to the inner or outer membrane surface of the vesicles or transported into a compartment which is not available for further osmotic shrinkage (16).

Facilitated diffusion of a solute should be preferentially affected by temperature changes. Fig. 6 shows the initial 20-s uptake of taurocholate in the presence of Na^+ and K^+ gradients at 0°, 24°, and 37°C. At 0°C no difference between uptake in the presence of a $Na_o^+ > Na_i^+$ gradient and that in the presence of a $K_o^+ > K_i^+$ gradient was observed. However, at 24° and 37°C taurocholate uptake was stimulated greater than twofold by a Na^+-gradient. At the same time the uptake of taurocholate in the presence of a K^+ gradient increased only slightly.

Finally, an argument for the facilitated diffusion of bile acid is the trans-stimulation of $Na_o^+ > Na_i^+$ gradient taurocholate uptake. As shown in Fig. 7, preloading the vesicles with another unlabeled bile acid, glycocholate, enhanced the subsequent uptake of taurocholate about 1.3-fold compared with non-preloaded vesicles ($P < 0.01$). At the same time taurocholate was not significantly stimulated by preloading with glycocholate in the presence of a $K_o^+ > K_i^+$ gradient ($P > 0.05$). Taken together this series of experiments argues for the presence of Na^+-dependent, facilitated diffusion of nonmetabolized taurocholate across the vesicle membrane.

Cation specificity and diffusion potentials. Fig. 8 shows the effects of inwardly-directed cation gradients of different chloride salts on taurocholate uptake by brush border membrane vesicles. In comparison with K^+, only Na^+ showed a stimulatory effect on initial taurocholate uptake ($P < 0.01$). Li^+ and choline$^+$ were without effect. Thus, replacement of Na^+ in the incubation medium with K^+, Li^+, and choline$^+$ decreased initial uptake of taurocholate.

At pH 7.4, only 1/400,000 of the taurocholate molecules are in the undissociated form. Therefore, it is conceivable that taurocholate flux is coupled via a diffusion potential created by the dissipating Na^+ gradient

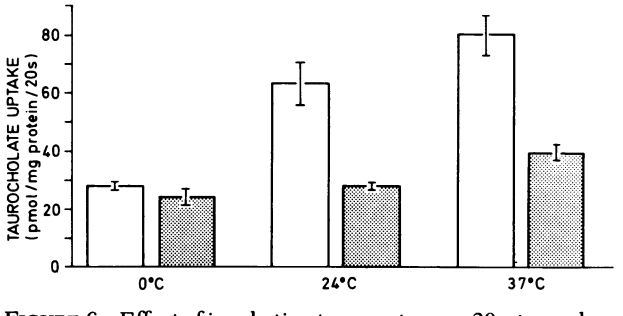

FIGURE 6 Effect of incubation temperature on 20-s taurocholate uptake. Brush border membrane vesicles were loaded with 100 mM mannitol, 100 mM choline Cl, and 20 mM Hepes/Tris (pH 7.4) and incubated at 0°, 24°, and 37°C in the same medium containing 10 μM [^{3}H]taurocholate except that choline Cl was replaced with 100 mM NaCl (open bars) or 100 mM KCl (shaded bars). Each point represents the mean±SE for six determinations.

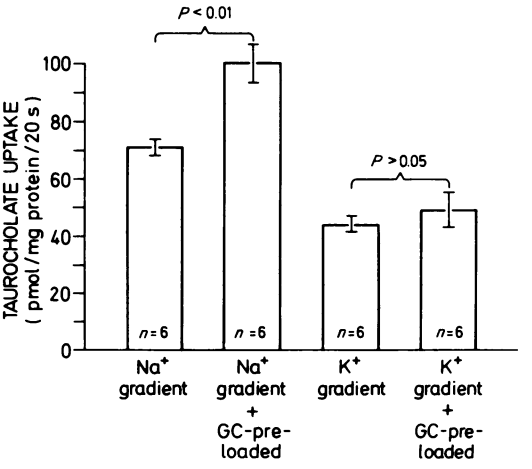

FIGURE 7 Trans-stimulation of Na^+-dependent, 20-s taurocholate uptake. Brush border membrane vesicles, loaded with 100 mM mannitol, 100 mM choline Cl, 20 mM Hepes/Tris (pH 7.4), and ±unlabeled 60 μM glycocholate, were incubated at 37°C in medium containing 10 μM [^{3}H]taurocholate, 100 mM mannitol, 20 mM Hepes/Tris and either 100 mM NaCl (Na^+ gradient) or 100 mM KCl (K^+ gradient). *n* is the number of experimental determinations. GC, glycocholate.

driving the taurocholate anion across the membrane. To test this possibility valinomycin-treated membranes were incubated in a KCl-containing medium. This condition is known to create an inside positive diffusion potential compared to the control without valinomycin, since valinomycin selectively increases the K^+ permeability of the membrane (17, 18). As shown in Fig. 8,

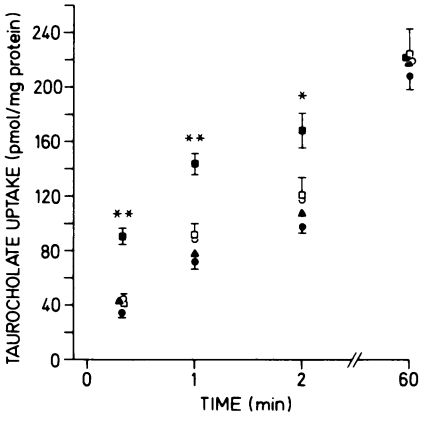

FIGURE 8 Effect of cation replacement and diffusion potential on taurocholate uptake. Brush border membrane vesicles loaded with 300 mM mannitol and 20 mM Hepes/Tris (pH 7.4) were incubated at 37°C in medium containing 10 μM [^{3}H]-taurocholate, 100 mM mannitol, 20 mM Hepes/Tris and either 100 mM NaCl (■), LiCl (□), choline Cl (●), KCl (○), or KCl + valinomycin, 15 μg/mg protein (▲). Each point represents the mean±SE for eight determinations. ** and * are statistical differences of $P < 0.01$ and $P < 0.05$, respectively, between ■ and the other gradients.

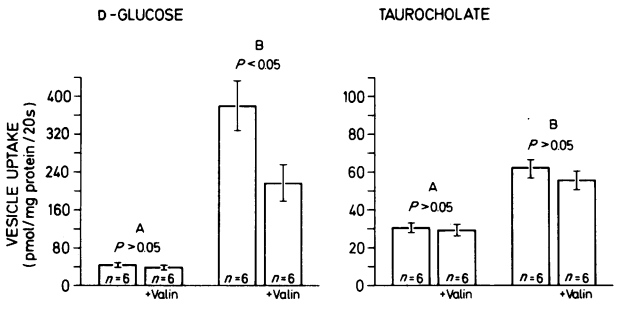

FIGURE 9 Membrane potential and taurocholate uptake. Membranes loaded with 300 mM mannitol and 20 mM Hepes/Tris (pH 7.4) were incubated at 37°C for 20 s in the medium containing 100 mM mannitol, 20 mM Hepes/Tris, 10 μM [^{3}H]-taurocholate, 60 μM [^{14}C]D-glucose and either A: 50 mM choline Cl and 50 mM KCl±valinomycin (valin), 15 μg/mg protein, or B: 50 mM NaCl and 50 mM KCl±valinomycin. n is the number of experimental determinations.

the stimulation of anion diffusion by a cation gradient diffusion potential was unlikely as the addition of valinomycin had no effect on the uptake of taurocholate in the presence of a $K_o^+ > K_i^+$ gradient.

The question, whether the Na^+-taurocholate cotransport is influenced by the membrane potential, is elucidated by experiments described in Fig. 9. An inside positive diffusion potential was created across the brush border membrane by treating vesicles with valinomycin and incubating them in a K^+ medium as described above. In addition, the experiments were carried out in the presence and absence of a Na^+ gradient. To serve as a control, the incubation medium contained [^{14}C]D-glucose which is known to be influenced by the membrane potential (19, 20); e.g., a positive inside diffusion potential inhibits Na^+-dependent glucose transport. Consequently, as shown in Fig. 9, left panel, the valinomycin-induced K^+ diffusion potential inhibited the Na^+-dependent glucose transport. In contrast, as shown in Fig. 9, right panel, no significant effect of the ionophore on taurocholate uptake was noted either in the presence or absence of a $Na_o^+ > Na_i^+$ gradient. Thus, the lack of an effect by valinomycin suggests that the transport of taurocholate occurs via an electroneutral process such as 1:1 Na^+-taurocholate cotransport.

Effect of pH. The binding of bile acid to nonvesiculated intestinal brush border membranes is sensitive to changes in pH (21). Furthermore, secondary active transport of organic acids has been shown recently to be coupled to a pH gradient in renal brush border membrane vesicles (22). Therefore, studies were performed to determine not only the effect of changes in pH on vesicle uptake but also the effect of a proton gradient. As shown in Table I, initial uptake values of taurocholate (0.33 and 1 min) at pH 7.5 (pH_o 7.5/pH_i 7.5) were similar to values obtained at pH 6 (pH_o 6/pH_i 6). Moreover, the imposition of a pH gradient across the vesicle membrane (pH_o 6/pH_i 7.5) resulted in no enhancement of taurocholate uptake either in the presence (Na_o^+ 100/Na_i^+ 0) or absence (Na_o^+ 100/Na_i^+ 100) of a Na^+ gradient. However, at 60 min (equilibrium) taurocholate uptake was higher under conditions of lowest pH on both sides of the vesicle membrane (pH_o 6/pH_i 6) which may represent greater partitioning of bile acid into the vesicle membrane (21).

Substrate specificity. To determine the substrate specificity of the carrier-mediated taurocholate transport process, we tested the effect of unlabeled bile acids and probenecid on the transport of 10 μM [^{3}H] taurocholate and 60 μM [^{14}C]D-glucose under Na_o^+

TABLE I
*Effect of pH on Taurocholate Uptake into Brush Border Membrane Vesicles**

Conditions of study		Taurocholate uptake		
Na_o^+/Na_i^+	pH_o/pH_i	0.33 min	1 min	60 min
		pmol/mg protein		
100/0	7.5/7.5	75.8±9.8	144.0±19.6	275.8±26.4
	6.0/7.5	80.6±5.2	151.0±5.8	326.2±43.4
	6.0/6.0	84.0±12.0	167.0±20.2	501.2±74.81‡
100/100	7.5/7.5	44.6±9.2	113.0±17.6	338.8±32.1
	6.0/7.5	41.0±6.6	132.2±10.0	355.2±44.4
	6.0/6.0	31.8±4.8	131.0±4.8	403.8±45.2

* Brush border membrane vesicles were loaded with 100 mM mannitol, either 100 mM choline Cl (Na_i^+ = 0) or 100 mM NaCl (Na_i^+ = 100) and 20 mM Hepes/Tris either pH 7.5 or 6.0. Vesicles were incubated at 37°C with 100 mM mannitol, 100 mM NaCl (Na_o^+ = 100), 10 μM [^{3}H]taurocholate and 20 mM Hepes/Tris either pH 7.5 or pH 6.0 Data are mean±SEM for six determinations.
‡ Significant difference from pH_o 7.5/pH_i 7.5 value, $P < 0.05$.

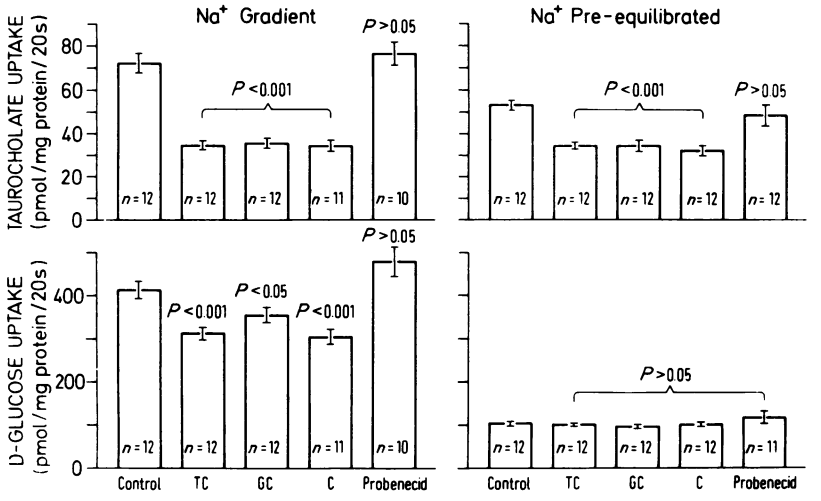

FIGURE 10 Effect of unlabeled bile acids and probenecid on vesicle uptake of taurocholate and D-glucose. Membranes were loaded with 100 mM mannitol, 20 mM Hepes/Tris (pH 7.4), and either 100 mM choline Cl (Na^+ gradient) or 100 mM NaCl (Na^+ pre-equilibrated). Incubations were carried out at 37°C for 20 s in medium containing 100 mM mannitol, 20 mM Hepes/Tris, 100 mM NaCl, 10 μM [^{3}H]taurocholate, 60 μM [^{14}C]D-glucose, and the absence (control) or presence of either 1 mM taurocholate (TC), glycocholate (GC), cholate (C), or probenecid. *n* is the number of experimental determinations.

> Na_i^+ gradient and $Na_o^+ = Na_i^+$ pre-equilibrated conditions. In these experiments the substance in question was offered with the radiolabeled substrates to the vesicle from the outside (*cis* side). As shown in Fig. 10, 1 mM taurocholate, glycocholate, and cholate inhibited the Na^+ gradient stimulated transport of taurocholate and, to a lesser extent, D-glucose. In addition, the bile acids inhibited taurocholate, but not D-glucose, under Na^+ pre-equilibrated conditions. Finally, probenecid showed no effect on the initial, essentially unidirectional, uptake of taurocholate and D-glucose under Na^+ gradient and Na^+ pre-equilibrated conditions. From these observations it appears that bile acids may inhibit the Na^+ gradient-dependent bile acid transport via two mechanisms: (*a*) competition for the same bile acid carrier in the presence of Na^+ (no Na^+ gradient necessary) and (*b*) faster dissipation of the Na^+ gradient required for the rapid uptake of organic solutes such as glucose (23, 24).

Saturability of uptake. The uptake of taurocholate with respect to increasing monomer concentrations of bile acid in the incubation medium was saturable in the presence of a $Na_o^+ > Na_i^+$ gradient (Fig. 11, upper curve). Under $Na_o^+ = Na_i^+$ pre-equilibrated conditions (Fig. 11, middle curve), the velocity of uptake and degree of saturation was less than under conditions of a Na^+ gradient. In the total absence of Na^+ the rate of taurocholate uptake was essentially linear ($r = 0.99$) throughout the bile acid concentration range of 0.01 to 1.0 mM (Fig. 11, lower line). When it was assumed that the carrier-mediated, Na^+ gradient-dependent taurocholate transport represented the difference between uptake in the presence of the Na^+ gradient and in the total absence of sodium, apparent values of V_{max} (2,600

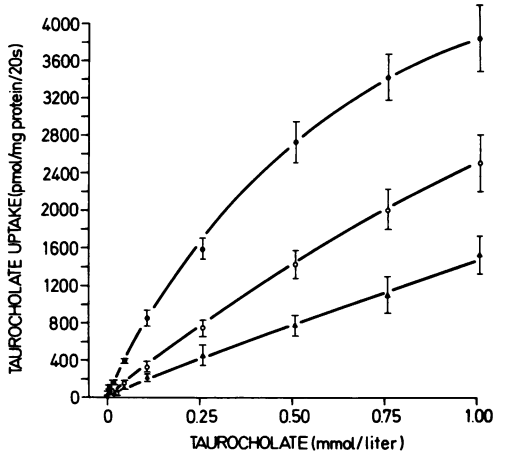

FIGURE 11 Relationship between taurocholate concentration and the Na^+ gradient, Na^+ pre-equilibrated, and Na^+ independent rates for vesicle uptake of the bile acid. ●, 100/0: Membranes were loaded with 100 mM mannitol, 20 mM Hepes/Tris (pH 7.4), 100 mM choline Cl and incubated at 37°C for 20 s in medium containing 100 mM mannitol, 20 mM Hepes/Tris, 100 mM NaCl, and [^{3}H]taurocholate at the indicated concentrations. ○, 100/100: Membranes were loaded and uptake was assayed in the presence of 100 mM mannitol, 20 mM Hepes/Tris, and 100 mM NaCl. ▲, 0/0: Membranes were loaded and uptake was assayed in the presence of 100 mM mannitol, 20 mM Hepes/Tris, and 100 mM choline Cl. Each point represents the mean ± SE for eight determinations.

pmol/mg protein per 20 s) and K_m (0.33 mmol/liter) were derived from data in Fig. 11 based on the Lineweaver Burk plot.

DISCUSSION

These studies in vivo and in brush border membrane vesicles directly examine for the first time bile acid transport across the proximal renal tubule. Our measurements of zero net flux ΔC_{TC^-} indicated that the reabsorption of taurocholate in the proximal convolution occurs against a concentration gradient and that this active transport is sodium-dependent. Furthermore, the reabsorption of taurocholate was unaffected by changes in HCO_3^- concentration, but was inhibited by the structural analogue, glycocholate, and enhanced by probenecid. These findings confirm earlier speculation that bile acids share a common reabsorptive pathway (4, 25). Although the enhancement of taurocholate reabsorption by probenecid suggests that bile acids undergo tubular secretion via the pathway for organic acids (4, 14), the mechanism of enhanced taurocholate reabsorption by probenecid remains to be fully clarified. Measurement of unidirectional fluxes of taurocholate in vivo (data not shown) could not discriminate whether probenecid augmented the reabsorptive or inhibited a secretory component.

The in vivo observations were extended by our studies performed with renal membrane vesicles which showed that sodium dependence and mutual inhibition by other bile acids is located in the brush border membrane. These studies indicated that the stimulation of taurocholate flux by sodium is the consequence of a Na^+-taurocholate cotransport system rather than the result of indirect coupling phenomena such as diffusion potentials or pH effects. Our results also provided limited information on substrate specificity of the taurocholate-Na^+ cotransport system as other bile acids *cis*-inhibited and trans-stimulated the system. Finally, these studies suggested that the enhancement of taurocholate transport by probenecid in vivo does not occur directly at the brush border site.

Several laboratories (24, 26–29) have utilized brush border membrane vesicles isolated from the jejunum and ileum to characterize the intestinal bile acid transport system. Their findings support the existence of a transport system in the brush border membrane that reflects kinetics and characteristics of bile acid transport in intact intestinal preparations (29). Studies with ileal membrane vesicles also allow for comparisons to be made with the present studies. In both vesicles prepared from ileum and proximal tubule, bile acid uptake was Na^+-specific. Uptake values obtained with Li^+, K^+, and choline$^+$ were significantly less than those obtained with Na^+. However, the rapid transport of bile acids across ileal membrane vesicles, resulting in a transient intravesicular accumulation or "overshoot", was not seen here with renal brush border vesicles. In data not presented, an overshoot for D-glucose was observed as described (30) with renal membrane vesicles similar to those used for taurocholate uptake. These observations suggest that the tubular brush border membrane has a lower capacity for active bile acid transport than the ileal brush border membrane. In this situation the Na^+-coupled, taurocholate transport system may be slow with respect to the dissipation of the driving Na^+ gradient, i.e., dissipation by substrate-independent diffusion of Na^+ into the vesicles. The thermodynamic equilibrium between taurocholate and Na^+ is reached at a time when the Na^+ electrochemical potential difference already approaches zero. Thus, no overshoot is expected (31).

Data obtained from ileal vesicles conflict as to whether Na^+-dependent bile acid transport is an electroneutral (28) or electrogenic process (27, 29). The present studies with valinomycin indicate an electroneutral transport of Na^+-taurocholate in renal brush border vesicles. This implies that the chemical potential difference of Na^+, as well as for taurocholate, is the driving force for taurocholate transport across the renal brush border membrane.

In both ileal and renal brush border vesicles competition has been shown between Na^+-dependent bile acid and D-glucose uptake. This inhibitory interaction between bile acid and D-glucose is probably due to faster dissipation of the Na^+ gradient that is required for the rapid uptake of these organic solutes (24). Previous investigators have demonstrated that uptake of D-glucose by brush border membrane vesicles is mediated via a cotransport system that transports both D-glucose and Na^+ from the extravesicular medium to the intravesicular space (19). Furthermore, the cotransport of one organic solute with Na^+ into the vesicles results in accelerated dissipation of the transmembrane electrochemical gradient for Na^+ and thereby inhibits membrane potential-sensitive, Na^+ coupled uptake of the organic solutes (23). As Na^+-dependent taurocholate uptake is a slow process and electroneutral, it is unlikely that this mechanism contributes significantly to the faster breakdown of the Na^+ electrochemical difference across the membrane. The inhibitory effect of taurocholate under Na^+ gradient conditions may be explained by increasing membrane permeability to Na^+. This is supported by the lack of taurocholate inhibition on D-glucose uptake under Na^+ pre-equilibrated conditions (Fig. 10).

In the presence of Na^+, uptake in both ileal and renal membrane vesicles was saturable with respect to increasing substrate concentration and inhibited by other bile acids. Of particular interest in the present studies

was the saturable uptake of taurocholate under Na^+ pre-equilibrated conditions at rates greater than under conditions of no Na^+, where no saturation of uptake was observed. The result is best explained by a facilitated diffusion mechanism working only or at least preferentially with Na^+ present. Evidence for direct coupling of taurocholate and Na^+ fluxes at distinct membrane sites is the saturability of taurocholate only in the presence of Na^+. Previous studies have detailed the interaction between bile acid and the recognition site for transport in the ileum (32, 33). Determinants of this interaction included the steroid moiety of the bile acid molecule, a coulombic attraction between the anionic charge of the bile acid and a cationic site on the transport system and the presence of Na^+ in the medium bathing the mucosal surface of the ileal tissue and a closely positioned anionic site on the transport system. These three factors appear to determine cooperatively bile acid transport.

One final point merits emphasis. Recent studies with organic acids suggest that the energy for the active transport is supplied by a proton gradient across renal brush border membrane vesicles (22). The present studies suggest that such a pH gradient is not an energy source for taurocholate accumulation in the proximal convolution. Furthermore, our studies agree with earlier studies which showed that changes in urinary pH did not have a profound effect on renal clearance of taurocholate in dogs (4). Instead, it appears from the present studies that the transport across the renal brush border membrane and the intracellular accumulation is mediated by the Na^+-taurocholate cotransport. It may be envisaged further that exit from the contraluminal (basolateral membrane) side of the cell proceeds via a Na^+-independent system driven by the chemical gradient for taurocholate and the electrical potential difference, inside negative. The electrochemical potential difference for Na^+ across the luminal membrane is maintained by the action of the $Na^+ + K^+$-stimulated ATPase located in the basolateral membrane. Thus, taurocholate transport may be defined as a secondary active transport.

ACKNOWLEDGMENTS

This research was supported in part by grant AM-21696 from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

REFERENCES

1. Javitt, N. B. 1977. Diagnostic value of serum bile acids. *Clin. Gastroenterol.* **6**: 219–226.
2. Rudman, D., and F. E. Kendall. 1957. Bile acid content of human serum. II. The binding of cholanic acids by human plasma proteins. *J. Clin. Invest.* **36**: 538–542.
3. Rudman, D., and F. E. Kendall. 1957. Bile acid content of human serum. I. Serum bile acids in patients with hepatic disease. *J. Clin. Invest.* **36**: 530–537.
4. Weiner, I. M., J. E. Glasser, and L. Lack. 1964. Renal excretion of bile acids: taurocholate, glycocholate and cholic acids. *Am. J. Physiol.* **207**: 964–970.
5. Zins, G. R., and I. M. Weiner. 1968. Bidirectional transport by the proximal tubule of the dog. *Am. J. Physiol.* **215**: 840–845.
6. Ullrich, K. J., G. Rumrich, and S. Klöss. 1978. Phosphate transport in the proximal convolution of the rat kidney. II. Effect of extracellular Ca^{2+} and application of the Ca^{2+} inophore A 23187 in chronic PTX animals. *Pflügers Arch. Eur. J. Physiol.* **375**: 97–103.
7. Evers, C., W. Haase, H. Murer, and R. Kinne. 1978. Properties of brush border vesicles isolated from rat kidney cortex by calcium precipitation. *Membr. Biochem.* **1**: 203–209.
8. Ullrich, K. J., G. Rumrich, and S. Klöss. 1977. Phosphate transport in the proximal convolution of the rat kidney. I. Tubular heterogeneity, effect of parathyroid hormone in acute and chronic parathyroidectomized animals and effect of phosphate diet. *Pflügers Arch. Eur. J. Physiol.* **372**: 269–274.
9. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. 6th ed. Iowa State University Press, Ames, Iowa. 1-593.
10. Lowry, D. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
11. Berner, W., R. Kinne, and H. Murer. 1976. Phosphate transport into brush border membrane vesicles isolated from rat small intestine. *Biochem. J.* **160**: 467–474.
12. Ullrich, K. J. 1973. Permeability characteristics of the mammalian nephron. *In* Handbook of Physiology. Section 8, J. Orloff and R. W. Berliner, editors. American Physiological Society, Bethesda, Md., **12**: 377–398.
13. Ullrich, K. J., G. Rumrich, and S. Klöss. 1978. Phosphate transport in the proximal convolution of the rat kidney. III. Effect of extracellular and intracellular pH. *Pflügers Arch. Eur. J. Physiol.* **377**: 33–42.
14. Sheikh, M. I., J. V. Moller, C. Jacobsen, and J. Maxid. 1979. Effect of hydrocarbon chain length on renal transport of monosubstituted sulfamyl benzoic acid derivatives and probenecid. *Biochem. Pharmacol.* **28**: 2451–2456.
15. Beck, J. C., and B. Sacktor. 1975. Energetics of the Na^+-dependent transport of D-glucose in renal brush border membrane vesicles. *J. Biol. Chem.* **250**: 8674–8680.
16. Hildman, B., C. Storelli, W. Haase, M. Barac-Nieto, and H. Murer. 1980. Na^+-L-lactate cotransport in rabbit small intestinal brush border membrane vesicles. *Biochem. J.* **186**: 169–176.
17. Henderson, P. J. F., J. D. McGivan, and J. B. Chapell. 1969. The action of certain antibiotics on mitochondrial, erythrocyte and artificial phospholipid membranes. *Biochem. J.* **111**: 521–535.
18. Pressman, B. C. 1968. Ionophorous antibiotics as models for biological transport. *Fed. Proc. Am. Soc. Exp. Biol.* **27**: 1283–1288.
19. Murer, H., and U. Hopfer. 1974. Demonstration of electrogenic Na^+-dependent D-glucose transport in intestinal brush border membranes. *Proc. Natl. Acad. Sci. U. S. A.* **71**: 484–488.
20. Turner, R. J., and M. Silverman. 1977. Sugar uptake into brush border vesicles from normal human kidney. *Proc. Natl. Acad. Sci. U. S. A.* **74**: 2825–2829.

21. Wilson, F. A., and L. L. Treanor. 1977. Characterization of bile acid binding to rat intestinal brush border membranes. *J. Membr. Biol.* **33:** 213–230.
22. Blomstedt, J. W., and P. S. Aronson. 1980. pH gradient-stimulated transport of urate and p-aminohippurate in dog renal microvillus membrane vesicles. *J. Clin. Invest.* **65:** 931–934.
23. Murer, H., K. Sigrist-Nelson, and U. Hopfer. 1975. On the mechanism of sugar and amino acid interaction in intestinal transport. *J. Biol. Chem.* **250:** 7392–7396.
24. Beesley, R. C., and R. G. Faust. 1979. Sodium ion-coupled uptake of taurocholate by intestinal brush border membrane vesicles. *Biochem. J.* **178:** 299–303.
25. Barnes, S., J. A. Summerfield, J. L. Gollan, and B. H. Billing. 1977. Renal mechanisms influencing the bile acid composition of cholestatic urine. *In* Bile Acid Metabolism in Health and Disease. G. Baumgartner, editor. MTP Environment Ltd., Lancaster, England. 89–91.
26. Lack, L., J. T. Walker, and C. H. Hsu. 1977. Taurocholate uptake by membrane vesicles prepared from ileal brush borders. *Life Sci.* **20:** 1607–1612.
27. Lücke, H., G. Stange, R. Kinne, and H. Murer. 1978. Taurocholate sodium co-transport by brush border membrane vesicles isolated from rat ileum. *Biochem. J.* **174:** 951–958.
28. Rouse, D. J., and L. Lack. 1979. Ion requirements for taurocholate transport by ileal brush border membrane vesicles. *Life Sci.* **25:** 45–52.
29. Wilson, F. A., and L. L. Treanor. 1979. Glycodeoxycholate transport in brush border membrane vesicles isolated from rat jejunum and ileum. *Biochim. Biophys. Acta.* **554:** 430–440.
30. Kinne, R., H. Murer, E. Kinne-Saffran, M. Thees, and G. Sachs. 1975. Sugar transport by renal plasma membrane vesicles. *J. Membr. Biol.* **21:** 375–395.
31. Evers, J., H. Murer, and R. Kinne. 1976. Phenylalanine uptake in isolated renal brush border vesicles. *Biochim. Biophys. Acta.* **426:** 598–615.
32. Gallager, K., J. Mauskopf, J. T. Walker, and L. Lack. 1976. Ionic requirements for the active ileal bile salt transport system. *J. Lipid Res.* **17:** 572–577.
33. Bundy, R., J. Mauskopf, J. T. Walker, and L. Lack. 1977. Interaction of uncharged bile salt derivatives with the ileal bile salt transport system. *J. Lipid Res.* **18:** 389–395.